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Modulation pré et post-récepteur de l'exposition aux glucocorticoïdes :
Rôles du diabète de type 1 et de la vitamine A

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RÉSUMÉ

L'action physiologique des glucocorticoïdes (GC) est de mobiliser certaines ressources de l'organisme pour s'adapter à des changements d'origine endogène ou exogène susceptibles de perturber l'homéostasie de l'organisme. Des facteurs métaboliques/nutritionnels modifient l'intensité d'action des GC. Ils agissent au niveau i) de l'activation de l'axe corticotrope, ii) de leur biodisponibilité des GC (régulation « pré-récepteur »), iii) de l'activation transcriptionnelle des récepteurs des GC (régulation « post-récepteur »).
L'objectif général de ce travail repose sur l'exploration du rôle de certains facteurs métaboliques/nutritionnels dans la modulation pré- et post-récepteur de l'action des GC sur l'organisme.

Dans une approche clinique, notre attention s'est tout d'abord focalisée sur le rôle de l'équilibre diabétique et/ou l'état inflammatoire des patients atteints de diabète de type I et le métabolisme pré-récepteur du cortisol. Par l'étude en spectrométrie de masse des métabolites du cortisol, nous montrons une augmentation significative de l'activité de la hydroxystéroïde-déshydrogenase 1, principale enzyme de régénération intracellulaire du cortisol. Cette augmentation est corrélée à des marqueurs de l'inflammation chez les enfants diabétiques. Ces résultats suggèrent un lien entre le diabète et l'existence d'une inflammation à bas bruit et l'augmentation de l'exposition cellulaire aux GC.

Dans une approche expérimentale, nous nous sommes ensuite intéressés à l'action de l'acide rétinoïque all trans (atAR), métabolite actif de la vitamine A, sur l'activité transcriptionnelle des GC. Nous avons choisi un modèle *in vitro* de cellules hippocampiques en raison d'effets contrastés de l'atAR et des GC sur les fonctions mnésiques *in vivo*. Nous observons une interaction entre les voies de signalisation transcriptionnelle de l'atAR et des GC sur leurs propres récepteurs et sur des protéines de la plasticité synaptique. Par ailleurs, l'atAR est responsable de modifications de la phosphorylation du récepteur aux GC altérant ainsi ses fonctions transcriptionnelles. Enfin atAR et GC modifient différemment l'organisation du cytosquelette d'actine sans modification transcriptionnelle ou traductionnelle.

La compréhension du rôle de certains facteurs environnementaux dans la signalisation des GC pourrait permettre de réduire certains des effets délétères du stress. L'utilisation de certains nutriments, vitamine A par exemple, pourrait atténuer certaines conséquences d'un tonus glucocorticoïde excessivement prolongé. Des travaux cliniques ont débuté dans cette direction.

ABSTRACT

Physiological actions of glucocorticoids (GC) are to mobilize body resources and to adapt to endogenous or exogenous changes that might disrupt the homeostasis of the body. Nutritional & metabolic factors may modify the intensity of GC action in: i) the activation of the corticotrope axis and their secretion by the adrenals, ii) their bioavailability ("pre-receptor" regulation), iii) the transcriptional activation of their receptors ("post-receptor" regulation).

The main target of this work is to explore the role of some metabolic/nutritional endogenous or exogenous factors in modulating pre- and post-receptor action of GC.

Our attention first focused on the role of diabetes and the related inflammation in patients with type I diabetes, and pre-receptor metabolism of cortisol. We showed that a significant increase in the activity of hydroxysteroid dehydrogenase 1, the main enzyme of intracellular cortisol regeneration, is correlated with markers of inflammation in diabetic children. This suggests a link between diabetes and the low-level chronic inflammation and increased cellular exposure to GC

Then, we focused on the action of *all-trans* retinoic acid (atRA), the active metabolite of vitamin A on the transcriptional activity of GC. We used an *in vitro* model of hippocampal cells as GC and atRA have contrasted effects on mnesic processes *in vivo*. We observed a transcriptional interaction between the GC and retinoic pathways targeting their receptors and genes involved in neuronal plasticity. atRA also affects the phosphorylation of the GC receptor and modifies its transcriptional activity. Lastly, both atRA and GC affect cellular organisation of actin cytoskeleton.

The knowledge acquired by studying the action of nutritional molecules on GC action could be used to easily reduce the deleterious effects of GC in chronic stress. Clinical studies have started in this direction.

SOMMAIRE

AVANT-PROPOS

Liste des publications et prix

En cours

Brossaud J, Ducint D, *et al.* Urinary glucocorticoid metabolites in the subclinical hypercortisolism diagnosis of adrenal tumour

Brossaud J, Redonnet A *et al.* Retinoic acid modified phosphorylated status of the glucocorticoid receptor

Soumis

Brossaud J, Corcuff J.B. Rebaudioside A and cortisol metabolism: sweet news for consumers**.**

Brossaud J, Pallet, V., *et al.* Vitamin A, endocrine tissues and hormones: interplay & interactions.

Brossaud J, Gatta B, *et al.* Could different ways to estimate serum free cortisol modify the evaluation of adrenal function?

2013

Brossaud J, Gosse P, *et al.* Phasing-in plasma metanephrines determination
European journal of Endocrinology 2013 Jun 29.

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2012 Prix ELAS forum (octobre 2012)

LISTE DES ABRÉVATIONS

11β-HSD1	11 beta-hydroxystéroïde-déshydrogénase de type 1
11β-HSD2	11 beta-hydroxystéroïde-déshydrogénase de type 1
ACTH	*adrenocorticotropic hormone*
ADN	acide désoxyribonucléique
AF-1	*activation function-1*
AF-2	*activation function-2*
allo-THF	allo-tétrahydrocortisol
AP-1	*activator protein-1*
atAR	acide rétinoïque *all-trans*
AVP	arginine vasopressine
axe HPA	*hypothalamic pituitary adenal axis*
BDNF	brain-derived neurotrophic factor
CBG	*corticosteroid binding globulin*
CBP	*cAMP-responsive element-binding protein (CREB)-binding protein*
CDK5	cyclin dependent kinase 5
COUP-TFI	*chicken ovalbumin upstream promoter-transcription factor* I
CRABP	*cellular retinoic acid-binding proteins*
CRBP	*cellular retinol binding protein*
CRH	*corticotropin releasing hormone*
CRP	protéine C-réactive
DBD	*DNA-binding domain*
Dex	dexaméthasone
DLT	dépression à long terme
DR	direct repeat
ERK	*extracellular signal-regulated protein kinase*
GABA	acide gamma-aminobutyrique
GAP43	*neuromodulin*
GC	glucocorticoïde
GR	*glucocorticoid receptor*
GRE	*glucocorticoid-responsive element*
GSK-3	*Glycogen synthase 3*
HAT	histone acétyltransferase
HDAC	histone désacétyltransférase
HDL	*high density lipoprotein*
HSP	h*eat shock protein*
JNK	*c-Jun NH2-terminal protein kinase*
LBD	*ligand binding domain*
LDL	*low density lipoprotein*
LRAT	*lecithin:retinol acetyltransferase*
MAP Kinase	*mitogen-activated protein kinase*
MR	*mineralocorticoid receptor*
MSK1	*mitogen and stress-activated kinase 1*
N-CoR	*nuclear co-repressor*
NF-κB	*nuclear factor-kappa B*
nGRE	*negative glucocorticoid-responsive element*
NL1	*nuclear location sequence* 1

NL2	*nuclear location sequence 2*
NTD	*N-terminal domain*
p/CAF	facteur associé au p300/CBP
PI3K	*phosphatidylinositol 3-kinase*
PI-PLC	*phosphoinositide-specific phospholipase C*
PKCδ	p*rotein kinase C delta type*
PLT	potentialisation à long terme
PVN	noyau paraventriculaire
POMC	propiomélanocortine
STRA6	*stimulated by retinoic acid gene 6*
PPARs	*peroxisome proliferator–activated receptors*
PRAME	*preferentially expresed antigen in melanoma*
RALDH	*retinaldehyde dehydrogenase*
RAR	*retinoic acid receptor*
RARE	*retinoic acid-responsive element*
RBP	*retinol-binding protein*
RC3	*neurogranin*
RDH	*retinol deshydrogenase*
REH	*retinyl ester hydrolase*
RIP140	*receptor interacting protein of 140 kDa*
ROR	*retinoid-related orphan receptor*
RXR	*retinoic X receptor*
SMRT	*silencing mediator for retinoid and thyroid hormone receptors*
SNC	système nerveux central
SR-BI	*scavenger receptor class B, type I*
SRC	*steroid receptor coactivators*
STRA6	*stimulated by retinoic acid gene 6*
THE	tétrahydrocortisone
THF	tétrahydrocortisol
TIF1α/Trim24	*transcription intermediary factor-1α*
TNFα	tumor necrosis factor α
TPR	*tetratricopeptide repeat protein*
TTNPB	(E)-4[2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphtalenyl)-1-propenyl]benzoic acid
TTR	transthyrétine
VLDL	*very low density lipoprotein*

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INTRODUCTION

En 1950, Edward Kendall reçoit le Prix Nobel de Physiologie ou Médecine pour la découverte de la structure des glucocorticoïdes et de leurs propriétés thérapeutiques (Kendall 1953). Ces travaux font suite à ceux de Thomas Addison décrivant en 1855 le décès de patients présentant une destruction du cortex surrénalien. Grâce aux traitements par glucocorticoïdes, Edward Kendall révolutionne la prise en charge de la pathologie décrite par Addison mais également celle des pathologies inflammatoires. L'usage massif des glucocorticoïdes met simultanément en évidence les effets délétères potentiels de tels traitements.

Le terme de glucocorticoïde (GC) désigne à la fois les hormones stéroïdiennes naturelles (comme le cortisol chez l'Homme et la corticostérone chez les rongeurs) et les corticoïdes de synthèse (comme la prednisone ou la dexaméthasone). Il fait référence aux propriétés hyperglycémiantes de ces hormones. En effet, l'action physiologique des GC est de mobiliser certaines ressources de l'organisme pour surmonter et/ou s'adapter à des changements internes ou externes susceptibles de perturber l'équilibre homéostatique de l'organisme. La libération de GC par les glandes surrénales s'inscrit donc dans un contexte de réponse à un changement endogène ou exogène, désigné le plus souvent sous le terme de stress. Des milliers de publications scientifiques, de documents de vulgarisation et de fictions médiatiques ont fait suite aux travaux de Hans Selye explicitant une définition du stress (Szabo et al. 2012). Notons ici que la notion de stress n'est pas superposable à l'interprétation commune de « tension » psychologique.

L'activation et le rétrocontrôle au niveau du système nerveux central de l'axe hypothalamo-hypophyso-surrénalien (ou axe HPA pour *hypothalamic pituitary adenal axis*) constituent le pivot de la régulation de la sécrétion des GC. Néanmoins, la régulation de l'activité des GC ne se limite pas à la régulation de leur sécrétion. Ainsi, à la suite de la sécrétion des GC, un système modulateur du transport du cortisol aux tissus cibles et de disponibilité aux cellules régule l'accès de l'hormone à ses cibles, et cela, en amont du récepteur du cortisol (régulation « pré-récepteur »). En aval de la fixation du cortisol sur son récepteur (régulation « post-récepteur »), l'effet glucocorticoïde dépend principalement de l'activité transcriptionnelle du récepteur aux GC.

Les évènements perçus par l'organisme comme des stress ne sont pas les seuls à moduler l'imprégnation en GC. En effet, l'exploration de la variabilité de la réponse au stress dans notre

laboratoire[1] nous a amenés à explorer d'autres niveaux de régulation de l'exposition des tissus aux GC par d'autres facteurs endogènes ou exogènes. L'action des GC et la réponse de l'organisme sont fonctions de caractéristiques génétiques (Mormede et al. 2011). Mais des facteurs métaboliques endogènes et exogènes tels que l'alimentation sont également impliqués dans la modification de l'intensité d'action des GC. On retrouve par exemple une influence de l'obésité, du diabète (Bujalska et al. 1997; MacLullich and Seckl 2008), de diverses hormones (Miranda et al. 2013), des perturbateurs enzymatiques (réglisse) (Frey and Ferrari 2000), des vitamines (A, D) (Obradovic et al. 2006; Marissal-Arvy et al. 2013). Ces facteurs modulent l'action des GC au niveau i) de l'activation de l'axe HPA, ii) de leur sécrétion par les surrénales, iii) de leur biodisponibilité aux tissus, iv) de leur biodisponibilité à leur récepteurs intracellulaires, v) de l'activation transcriptionnelle de leur récepteurs (Seckl 2004; Perogamvros et al. 2012; Moisan 2013; Stimson et al. 2013).

Etudier la régulation pré- et post-récepteur est crucial pour comprendre l'action des GC. Néanmoins, l'exploration de l'imprégnation glucocorticoïde est souvent focalisée sur les caractéristiques de la réponse sécrétoire des GC. Nous avons choisi de nous intéresser à la modulation des effets des GC après qu'ils aient été sécrétés et transportés jusqu'aux cellules cibles. Notre travail porte sur l'étude de modulations nutritionnelle et métabolique pré et post-récepteur des GC.

[1] Le laboratoire de Nutrition et Neurobiologie Intégrée, NutriNeurO, étudie l'impact de la nutrition sur les fonctions cérébrales et le développement de troubles de l'humeur et de la cognition. Le laboratoire est composé de deux équipes de recherche: (1) « Psychoneuroimmunologie et Nutrition : approches expérimentales et cliniques (2) « Nutrition, récepteurs nucléaires et vieillissement cérébral »

OBJECTIFS

Objectifs

L'objectif général de ce travail repose sur l'exploration du rôle de facteurs métaboliques/nutritionnels endogènes et exogènes dans la modulation de l'action des GC sur l'organisme. Le domaine d'étude ne comprend ni la modulation de la sécrétion des GC ni leur transport.

Nous nous intéresserons d'une part à la modulation de l'exposition cortisolique des cellules par l'étude des hydroxysteroïde-déshydrogenases 1 et 2, principales enzymes du métabolisme cellulaire inactivateur réversible des GC. Ce métabolisme intracellulaire permet la conversion régulée d'un GC actif en un métabolite inactif et inversement. Cette action en amont du récepteur du cortisol a pour conséquence de moduler les effets des GC postérieurement à leur sécrétion.

Nous nous intéresserons d'autre part à la modulation de l'activité principale des récepteurs des GC, c'est-à-dire leur activité transcriptionnelle. Il s'agit là de la modulation des effets des GC, en aval des récepteurs des GC, par des évènements intracellulaires interagissant avec la voie de signalisation transcriptionnelle des GC.

Les deux hypothèses qui ont guidé ce travail sont les suivantes :

- Hypothèse I : Il existe une altération du métabolisme pré-récepteur des GC chez des sujets présentant des anomalies du métabolisme glucidique. Les cibles de notre attention seront les hydroxysteroïde-déshydrogenases 1 et 2, deux enzymes responsables de ce métabolisme pré-récepteur. Notre modèle sera celui de sujets diabétiques insulino-dépendants. Si une altération du métabolisme des GC existe bien, elle pourrait être la conséquence du désordre métabolique, de l'inflammation à bas bruit ou du traitement par l'insulinothérapie.

- Hypothèse II : Des nutriments indispensables à la survie d'un individu sont capables de moduler les effets cellulaires des GC. Les cibles de notre attention seront la vitamine A et son métabolite actif, l'acide rétinoïque all-trans (atAR). Ils sont capables de moduler l'activité transcriptionnelle des glucocorticoïdes. Notre modèle sera celui de cellules neuronales hippocampiques traitées par de l'atAR et par un agoniste des récepteurs des GC, la dexaméthasone. Si une altération des effets du GC existe, elle pourrait alors être la conséquence de synergie ou d'antagonisme de l'activité transcriptionnelle des GC par une modification de la phosphorylation du récepteur aux GC *via* des effets transcriptionnels ou des effets non génomiques des rétinoïques.

Les objectifs de ce travail sont donc de tester ces deux hypothèses.

Objectifs testant l'hypothèse I : objectifs n°1 et 2

- Mettre au point une technique permettant l'exploration *in vivo* chez l'homme du métabolisme du cortisol. Ceci revient à mettre au point le dosage par spectrométrie de masse du cortisol urinaire et de ses huit principaux métabolites (PUBLICATION N°1).

- Explorer le métabolisme du cortisol dans les urines de sujets diabétiques traités par insulinothérapie pour évaluer l'activité des deux enzymes responsables du métabolisme du cortisol les hydroxysteroïde-déshydrogenases 1 et 2. Le but sera d'établir s'il existe un lien entre l'équilibre diabétique ou l'état inflammatoire des patients diabétiques et le métabolisme du cortisol (PUBLICATION N°2).

Objectifs testant l'hypothèse II : objectifs n°3 et 4

- Evaluer, après une revue bibliographique exhaustive, le rôle de la vitamine A dans le fonctionnement des systèmes endocriniens (PUBLICATION N°4).

  Evaluer l'existence d'une éventuelle modulation par l'atAR de l'effet des GC sur l'expression de protéines impliquées dans la différentiation et la plasticité neuronale au regard de l'abondante littérature à propos des effets des GC sur les neurones (PUBLICATION N°5 et N°7).

- Préciser les effets du traitement concomitant de l'atAR et de la dexaméthasone sur des neurones hippocampiques et explorer différents mécanismes intracellulaires potentiellement impliqués dans la modification de la phosphorylation du récepteur aux GC et responsables d'une modulation des effets GC par l'atAR (PUBLICATION N°6).

La connaissance de ces facteurs environnementaux et métaboliques dans la signalisation des glucocorticoïdes et les mécanismes impliqués devrait permettre d'atténuer voire de normaliser certaines dysrégulations de la réponse corticotrope vis-à-vis d'un stress comme c'est le cas de l'hyperactivité cortisolique observée chez le patient diabétique ou chez la personne âgée. Dans le premier objectif les travaux présentés ici constituent le départ de la recherche de ces facteurs. Dans le deuxième objectif, la vitamine A a déjà fait l'objet de travaux préliminaires de notre équipe. Elle a été identifiée comme un bon candidat dans la modulation de l'activité cortisolique.

CHAPITRE I

Modulation pré-récepteur de l'exposition aux glucocorticoïdes : rôle du diabète de type I

I. Glucocorticoïdes : données bibliographiques

A. La sécrétion de glucocorticoïdes et l'axe hypothalamo-hypophyso-surrénalien

a) Fonctionnement de l'axe hypothalamo-hypophyso-surrénalien

L'axe HPA constitue le principal système neuroendocrinien responsable de la réponse au stress, c'est-à-dire d'une perturbation de l'homéostasie, physique ou émotionnelle, de l'organisme.

L'activation de l'axe HPA commence par la libération de la corticolibérine ou *corticotropin releasing hormone* (CRH) (Figure 1). Ce peptide de 41 acides aminés est synthétisé par les neurones du noyau paraventriculaire (PVN) de l'hypothalamus. Ces neurones reçoivent des projections neuronales de multiples régions cérébrales notamment de l'amygdale et de l'hippocampe capables d'intégrer des stimulations internes ou externes à l'organisme. Ainsi, la libération de CRH est stimulée *via* différents systèmes : le système sympathique avec la sécrétion de catécholamines et un réseau de neurones GABAergiques. La sécrétion de CRH s'accompagne de la libération de l'arginine vasopressine (AVP) qui potentialise l'action du CRH (Herman and Cullinan 1997; Aguilera et al. 2008). Les projections des neurones hypothalamiques vers l'antéhypophyse libèrent le CRH au niveau des cellules corticotropes. Le CRH y active la transcription du gène de la pro-opiomélanocortine (POMC) qui sera clivée en hormone adrénocorticotrope ou ACTH (*adrenocorticotropic hormone*). L'ACTH est un peptide de 39 acides aminés qui, sécrété dans la circulation générale, agit sur les corticosurrénales (Roberts and Herbert 1977; Voigt et al. 1990). L'ACTH active la biosynthèse des glucocorticoïdes en stimulant la stéroïdogenèse (Manna et al. 2009). Enfin, les glucocorticoïdes exercent un rétrocontrôle négatif sur la sécrétion de CRH et d'ACTH contrôlant ainsi leur propre sécrétion par la présence de leurs récepteurs au niveau hippocampique, hypothalamique et hypophysaire (Dallman et al. 1987).

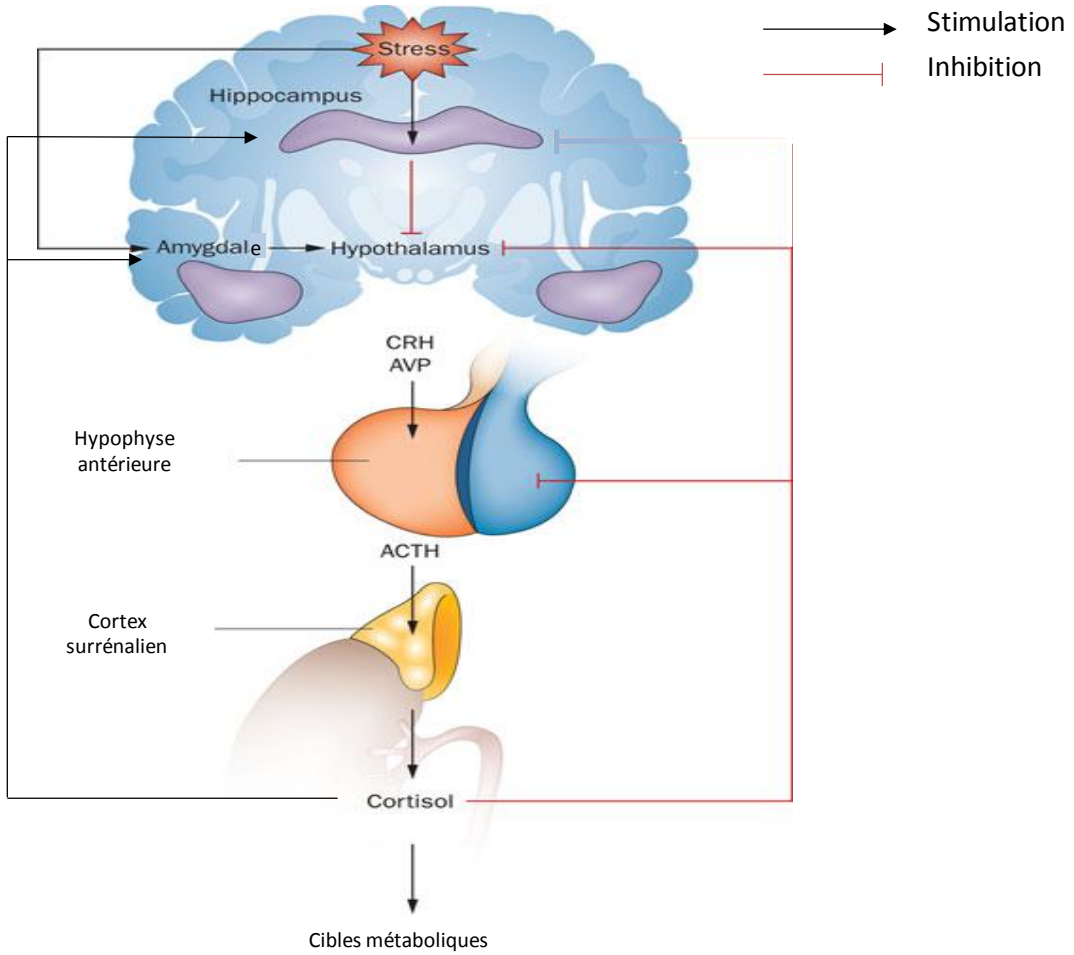


Figure 1 : Représentation schématique de l'axe hypothalamo-hypophyso-surrénalien. Adaptée de Sandi 2004.

ACTH : adrenocorticotropic hormone; AVP : arginine vasopressin; CRH : corticotropin-releasing hormone.

b) Biosynthèse des glucocorticoïdes par les glandes surrénales

Les hormones glucocorticoïdes sont des hormones stéroïdiennes (Payne and Hales 2004). Chez l'homme, le cortisol constitue le glucocorticoïde physiologiquement majoritaire (Kendall 1953). Chez les rongeurs, il s'agit en revanche de la corticostérone. Le terme de glucocorticoïdes (GC) est utilisé ici pour désigner l'hormone active dans l'une ou l'autre espèce. Ces hormones sont synthétisées par la partie corticale de la glande surrénalienne en réponse à une situation de stress de l'organisme. Ce sont des hormones lipophiles issues du métabolisme du cholestérol (Figure 2). La stéroïdogenèse consiste en une cascade enzymatique et conduit, depuis le cholestérol, à différente catégorie de stéroïdes : les glucocorticoïdes (zone fasciculée de la surrénale), les minéralocorticoïdes (zone glomérulée) et les androgènes (zone réticulée). Une fois libérés dans la

circulation sanguine, les glucocorticoïdes se lient à la transcortine ou CBG (*Corticosteroid Binding Globulin*) de faible capacité mais avec une forte affinité et à l'albumine avec grande capacité mais une faible affinité (Moisan 2013).

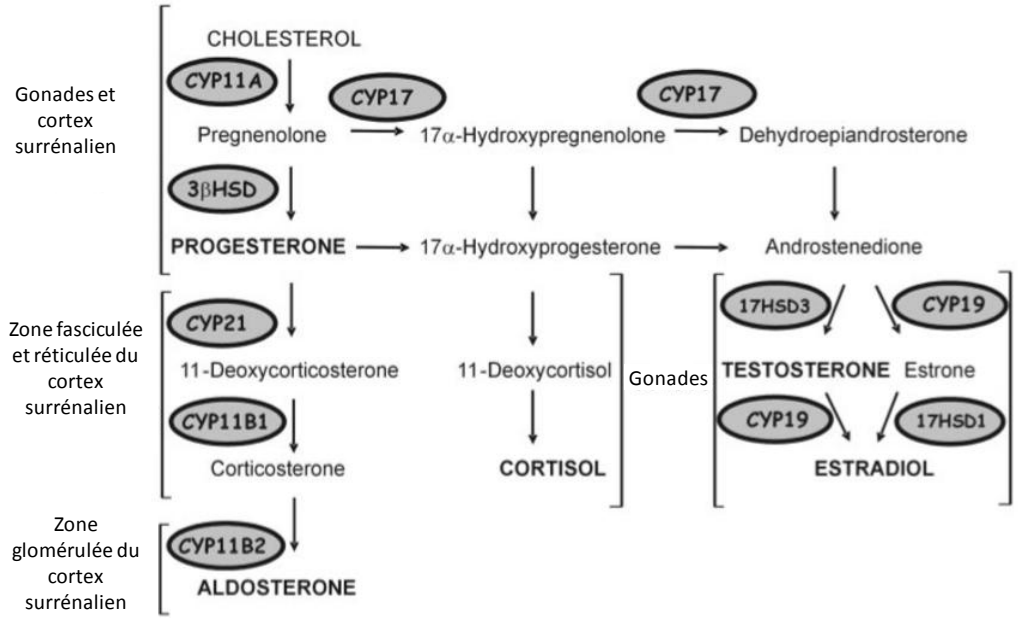


Figure 2 : Biosynthèse des hormones stéroïdiennes par les surrénales et les gonades. Adapté de Payne and Hales 2004

3βHSD : 3β-hydroxystéroïde-déshydrogenase; 17βHSD : 17β-hydroxystéroïde-déshydrogénase ; CYP11A1 : Cytochrome P450scc ou cholestérol desmolase ; CYP11B1 : Cytochrome P450-11β ou 11β-hydroxylase ; CYP17 : Cytochrome P450c17 ou 17α-hydroxylase ; CYP19 : Cytochrome P450arom ou aromatase ; CYP21 : Cytochrome P450c21 ou 21-hydroxylase.

c) Cycle nycthéméral de la sécrétion

La sécrétion basale d'ACTH n'est pas constante au cours de la journée mais est soumise à un rythme circadien ou cycle nycthéméral. En conséquence, la sécrétion de GC suit le même rythme. La concentration sérique d'ACTH et de GC est maximale au début de la phase active c'est-à-dire le matin chez l'Homme et le soir chez les rongeurs (animal nocturne) en lien avec une augmentation des pics de sécrétion de CRH et d'AVP. Ces variations ne sont pas simplement liées à l'alternance jour-nuit mais impliquent une « horloge biologique » de contrôle de l'expression de gènes. Elle permet à l'organisme une anticipation des besoins en GC en fonctions des activités d'éveil et de veille (Chung et al. 2011; Spiga et al. 2011).

d) Stress et activation de l'axe corticotrope

Lors de la perception d'une perturbation interne ou externe, le cerveau active de nombreux circuits neuronaux pour permettre des réponses physique, émotionnelle ou métabolique adaptées à la situation de stress. Cette perception passe par la coopération de plusieurs structures cérébrales et du système sympathique (sécrétion d'adrénaline et de noradrénaline). Il s'en suit une sécrétion de CRH et d'AVP par l'hypothalamus, une activation de l'axe HPA et une libération de GC par les surrénales. Les concentrations sanguines de GC sont maximales 15-30 min après la perception du stimulus, puis diminuent lentement pour atteindre un niveau basal 60-90 min plus tard (Joels et al. 2006).

Le système est rapidement régulé par le rétrocontrôle négatif qu'exercent les GC sur leur propre sécrétion (Herman and Cullinan ; de Kloet et al. 2005). Ce système est indispensable à l'adaptation de l'individu face à un environnement en perpétuel changement. Néanmoins, lors de situations de stress chronique, le retour à l'état basal n'est plus observé. Une réponse inadaptée, c'est-à-dire excessive au regard de l'élément déclencheur, survient alors. Apparaissent ainsi les conséquences néfastes des GC aussi bien en périphérie (diabète, hypertension, ostéoporose, obésité centrale) qu'au niveau du SNC (dépression, déficience apprentissage et de mémoire).

La sécrétion basale selon un rythme nycthéméral et l'augmentation de cette sécrétion par la perception d'un stress constituent les éléments pivots de la régulation de la sécrétion de GC. Il existe néanmoins d'autres systèmes de régulation de l'imprégnation en GC intervenant durant le transport des GC puis plus en aval à des niveaux intracellulaires pré-récepteur et post-récepteur.

B. La biodisponibilité des glucocorticoïdes : régulation pré-récepteur

a) La Corticosteroid Binding Globulin

La CBG est une glycoprotéine monomérique sécrétée par le foie et capable de lier 80% du cortisol (ou de la corticostérone) circulant(e). La liaison non spécifique des GC avec l'albumine concerne 15% du total circulant. La part de GC sous la forme libre, c'est-a-dire non liés ni à la CBG ni à l'albumine, représente seulement 5% du cortisol total. Or, seule cette forme libre peut diffuser au niveau intracellulaire et exercer son action sur les récepteurs aux glucocorticoïdes. La forme libre constitue la forme active de l'hormone (Lin et al. 2010; Moisan 2013). Longtemps considérée comme une simple protéine de transport du cortisol ou de la corticostérone, la CGB participe en réalité à la régulation de la biodisponibilité du GC aux tissus (Qian et al. 2011; Minni et al. 2012; Moisan 2013). D'une part, dans une situation de stress où la sécrétion de GC par les surrénales est

très importante, les capacités de liaison des GC à la CBG sont rapidement « dépassées » entraînant de ce fait une augmentation des GC libres. D'autre part, un mécanisme plus actif intervient pour une régulation locale selon les besoins en GC (Henley and Lightman 2011). La CBG est en effet sensible à l'action de protéase notamment de l'élastase des polynucléaires neutrophiles. Ainsi, sur un site inflammatoire, la CBG peut être clivée et libérer massivement des GC. Enfin, il existe plusieurs situations métaboliques endogènes ou exogènes dans lesquelles le niveau d'expression de la CBG varie comme par exemple les estrogènes, l'insuline, l'interleukine 6, la perte de poids, … (Manco et al. 2007; Henley and Lightman 2011).

b) La 11 beta hydroxystéroïde-déshydrogénase de type 1 et de type 2

Un autre niveau de régulation de l'action des GC se situe au niveau cellulaire et consiste en une inactivation du GC actif (cortisol ou corticostérone) en GC inactif (cortisone ou 11-déhydro-corticostérone) (Gathercole et al. 2013).

La réaction enzymatique responsable n'est pas obligatoirement un prélude au catabolisme du GC mais peut constituer une désactivation temporaire de la signalisation par les GC. En effet la déshydrogénation du carbone 11 du stéroïde survient grâce à l'action de la 11 beta hydroxystéroïde-déshydrogénase de type 2 (11β-HSD2). Elle est réversible par l'action 11 beta hydroxystéroïde-déshydrogénase de type 1 (11β-HSD1). La 11β-HSD2 est NAD-dépendante et elle est présente dans les tissus cibles de l'effet minéralocorticoïde des GC tels que le néphron distal, le colon, les glandes salivaires, la peau et le placenta. La 11β-HSD1 possède une activité NADPH-dépendante. Dans la cellule, elle est co-localisée avec l'hexose-6-phosphate déshydrogénase qui lui fournit le NADPH (Chapman et al. 1997). Elle est ubiquitaire dans l'organisme et présente en forte concentration au niveau du foie, du tissu adipeux, du système nerveux central, des cellules du système immunitaire (Gathercole et al. 2013).

Le rôle de la 11β-HSD2 est de prévenir l'action des GC au niveau des récepteurs aux minéralocorticoïdes. En effet, dans les organes exprimant la 11β-HSD2, l'inactivation des GC empêche leur action sur les récepteurs minéralocorticoïdes. Ceci permet donc une action exclusive de l'aldostérone sur les récepteurs aux minéralocorticoïdes. Au contraire, la 11β-HSD1 permet une action des GC à la fois sur les récepteurs minéralocorticoïdes et sur les récepteurs glucocorticoïdes (Gathercole et al. 2013). L'action de ces enzymes est loin d'être négligeable dans l'effet GC puisque la production de cortisol ou corticostérone est équivalente à la production de ces hormones par les glandes surrénaliennes (40 nmol/min) (Basu et al. 2004; Stimson et al. 2013).

L'importance de ce niveau cellulaire de régulation n'a été comprise que récemment avec la description d'une augmentation de l'activité 11β-HSD1 dans le syndrome métabolique (Bujalska et al. 1997; Stewart et al. 1999). En effet, les signes cliniques caractéristiques de ce syndrome (obésité, diabète de type 2 ou insulino-résistance, hypertension) sont très proches de ceux de l'hypercorticisme (syndrome de Cushing) à la différence – notable – qu'il n'existe pas d'augmentation du cortisol sérique chez les patients avec un simple syndrome métabolique. C'est l'augmentation locale, en particulier au niveau des tissus adipeux, de l'exposition aux GC qui est responsable du tableau clinique (Seckl 2004; Staab and Maser 2010). C'est dans ce cadre qu'ont été décrits les deux principaux facteurs régulateurs de l'activité de la 11β-HSD1 : l'inflammation et la nutrition (Walker and Andrew 2006). Ils permettent d'expliquer le lien entre syndrome métabolique et activité de l'enzyme. L'interleukine-1 et le *tumor necrosis factor α* (TNFα) augmentent cette activité (Chapman et al. 2013b). L'ingestion d'un repas est responsable de l'augmentation de la production de cortisol par la 11β-HSD1, augmentation variable selon le type de macronutriments composant le repas (lipides, glucides ou protides) (Stimson et al. 2013). Selon les études, l'insuline diminuerait ou augmenterait l'activité de la 11β-HSD1 (Voice et al. 1996; Staab and Maser 2010; Chapman et al. 2013a) alors que la glycémie l'augmente (Livingstone et al. 2000; Fan et al. 2011). La perte de poids est associé à une diminution de cette activité (Morton 2010; Rask et al. 2013).

Très peu d'auteurs ont étudié l'activité 11β-HSD1 chez les sujets présentant un diabète de type 1 alors qu'il existe évidemment chez ces patients une dérégulation de la glycémie et de l'insulinémie mais également une inflammation à bas bruit (Targher et al. 2001). Les études sont contradictoires. Certaines ne montrent aucune modification de l'activité de cette enzyme (Kerstens et al. 2003), d'autres une augmentation (Simunkova et al. 2011). Enfin, l'exemple de la régulation de l'exposition du système nerveux central aux GC illustre bien l'importance et le rôle crucial de 11β-HSD1 et 11β-HSD2 dans l'action MR et/ou GR dépendante des GC sur les tissus cérébraux. (voir paragraphe : *Glucocorticoïdes et plasticité neuronale : hypothèse mécanistique*) (Wyrwoll et al. 2011).

L'évaluation de l'activité de ces enzymes peut être faite soit par des méthodes isotopiques *in vivo* (Andrew et al. 2002), soit *in vitro* par le dosage des métabolites du cortisol par chromatographies gazeuse ou liquide couplée à la spectrométrie de masse (voir paragraphe suivant).

c) Élimination

Lié à la CBG et à l'albumine, le cortisol est relativement protégé de la dégradation. Sa demi-vie plasmatique est comprise entre 60 et 100 minutes alors qu'elle est beaucoup plus courte pour les autres stéroïdes. Son catabolisme vise à diminuer l'activité biologique de l'hormone et à augmenter sa solubilité dans l'eau pour permettre une élimination urinaire (Figure 3).

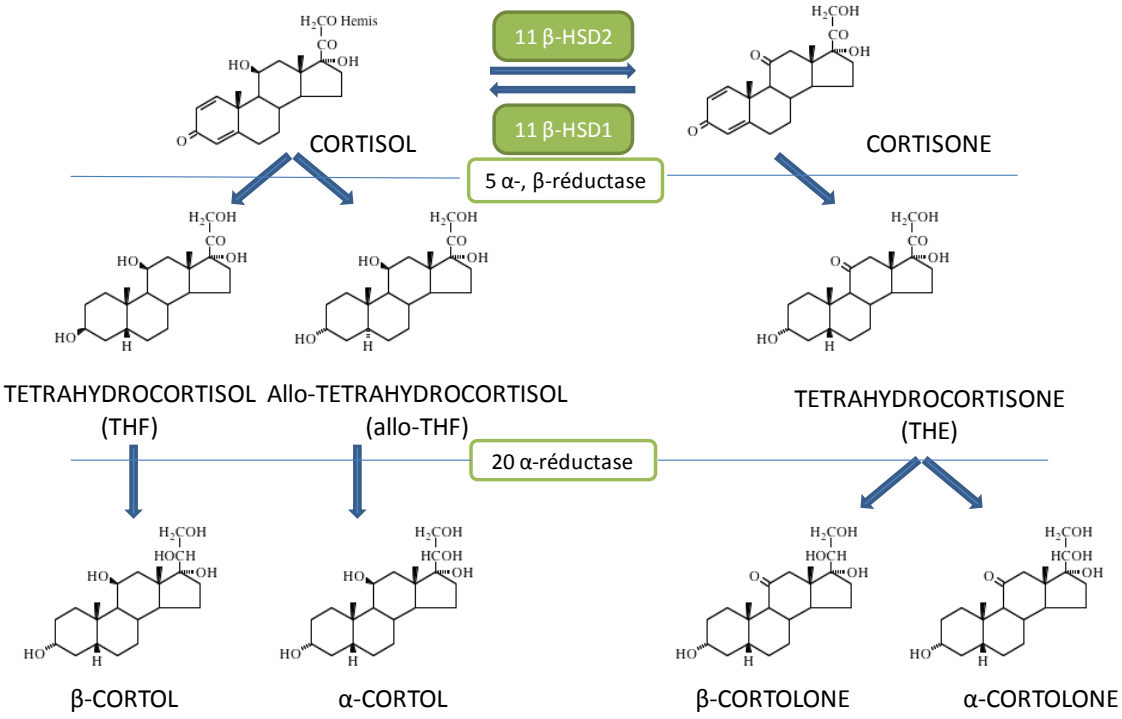


Figure 3 : Catabolisme du cortisol et de la cortisone

Ainsi le cortisol sera réduit au niveau de la double liaison en $C_{4\text{-}5}$. Cette réaction irréversible conduit à la formation d'hydrocortisol très rapidement réduit par la 5 α-réductase ou la 5 β-réductase en position C_3 (fonction cétone) pour donner le tétrahydrocortisol (THF) et son isomère l'allo-tétrahydrocortisol (allo-THF). THF et allo-THF sont conjugués avec l'acide glucuronique en position 3-hydroxyl et éliminés dans les urines. Ils peuvent également être réduits (en position C_{20}) en α et β cortols par la 20 α-réductase. La cortisone subit le même schéma de dégradation conduisant au tétrahydrocortisone (THE), α et β cortolones (Peterson 1960; Rittmaster 1995).

Le foie est le principal site du métabolisme du cortisol. Les stéroïdes non-conjugués sont filtrés au niveau du rein et largement réabsorbés. Ainsi l'activité de ces différentes enzymes participe à la concentration en GC circulant par leur implication dans la clairance des GC.

Comme cela a pu être expliqué précédemment, la 11 βHSD1 est essentiellement présente au niveau

du foie et de la graisse viscérale. Son activité rénale étant négligeable, le rapport (THF+allo-THF)/THE reflète essentiellement la conversion de la cortisone en cortisol. A l'inverse, la 11-βHSD2 étant principalement exprimé au niveau rénal, le ratio des concentrations urinaires de cortisol/cortisone est le reflet chez l'Homme de la conversion locale du cortisol en cortisone. C'est sur cette base théorique qu'a été mise au point l'évaluation de l'activité *in vitro* des deux enzymes par dosage en spectrométrie de masse des différents composés (initialement par chromatographie gazeuse (Andrew et al. 1998) puis par chromatographie liquide (Raffaelli et al. 2006).

La CBG, les 11β-HSD1 et 2, l'élimination des GC constituent une régulation de l'accessibilité des GC à leurs récepteurs. Une fois que les GC se sont liés à leurs récepteurs, interviennent des mécanismes moléculaires de régulation de l'activité transcriptionnelle des GC.

C. Le mécanisme d'action des glucocorticoïdes et la régulation post-récepteur

a) Les récepteurs aux glucocorticoïdes

L'effet des GC résulte de leur liaison à deux types de récepteurs nucléaires : ceux spécifiques des GC (*Glucocorticoid receptor* ou GR ou GR de type I ou NR3C1) ou ceux communs avec les minéralocorticoïdes (*Mineralocorticoid receptor* ou MR ou GR de type II ou NR3C2). Ils appartiennent à la superfamille des récepteurs nucléaires et à la famille des récepteurs aux stéroïdes (Heitzer et al. 2007). Le récepteur MR se caractérise par une affinité beaucoup plus importante pour les GC que le récepteur GR. Les récepteurs MR seront donc activés dès les basses concentrations de GC alors que l'effet des GC *via* les GR ne s'observera que pour de fortes concentrations de GC (situation de stress par exemple) (Reul and de Kloet 1985). Notre propos se limitera ici à la description du GR.

L'équipe d'Allan Munck est la première à identifier et caractériser le récepteur nucléaire des GC (Wira and Munck 1970). Le gène du récepteur aux GC chez l'Homme est localisé au niveau du chromosome 5 (souris, chromosome 18). L'épissage alternatif du gène codant pour le GR conduit à deux types de récepteurs de structure très proche, GRα et GRβ. GRα correspond à la forme la plus étudiée responsable de l'activité classiquement décrite pour ce récepteur nucléaire. Beaucoup moins étudié, l'activité et la fonction de GRβ ne sont encore partiellement comprises. Cette isoforme agirait comme un dominant négatif de l'activité transcriptionnelle de GR (Zhou and Cidlowski 2005; Duma et al. 2006).

b) Structure des GR

Le GR est une protéine monomérique de 776 acides aminés (90 kDa). Il présente une structure classique des récepteurs de cette famille comprenant six domaines structurels et fonctionnels (Figure 4) (Evans 1988; Kumar and Thompson 1999).

- Le domaine A/B en partie N-terminale de la protéine (appelé aussi NTD pour *N-terminal domain*) a un rôle dans l'initiation et la régulation de la transcription puisqu'il contient un domaine AF-1 (*activation function-1*). AF-1 interagit directement avec les facteurs de transcription. Son activation est indépendante de la liaison du récepteur avec le ligand (Dahlman-Wright et al. 1995).
- Le domaine C, configuré en doigts de zinc, est la région de fixation à l'ADN (acide désoxyribonucléique) appelée DBD pour *DNA-binding domain*. Ce domaine, très conservé, est considéré comme caractéristique de la super famille des récepteurs nucléaires. La structure en doigt de zinc permet une conformation tridimensionnelle nécessaire à la reconnaissance de l'élément de réponse de l'ADN. Ce domaine est également impliqué dans la dimérisation du récepteur.
- Le domaine D (*nuclear location sequence* ou NL1) correspond à une région charnière impliquée dans la fixation à l'ADN et dans l'adressage du récepteur dans le noyau.
- Les domaines E et F correspondent à la région de liaison du ligand nommée LBD pour *ligand binding domain*. Cette région contient une deuxième fonction activatrice de la transcription (AF-2 pour *activation function 2*), une interface de dimérisation et une région d'interaction pour les protéines de choc thermique HSPs (*Heat shock protein*). L'AF-2 présente une activation ligand-dépendant. En effet, la fixation du ligand au niveau du domaine LBD entraîne un changement de conformation du récepteur (rapprochement de l'hélice 12 du domaine LBD). Ce changement permet l'interaction de facteurs activateurs de la transcription (coactivateurs) avec le domaine AF-2. Au contraire, la fixation d'antagonistes s'accompagne d'un éloignement de l'hélice 12 et donc du recrutement de facteurs répresseurs de la transcription (corépresseurs) (Kauppi et al. 2003). Dans ce domaine se situe également une seconde séquence impliquée dans l'adressage au noyau, NL2.

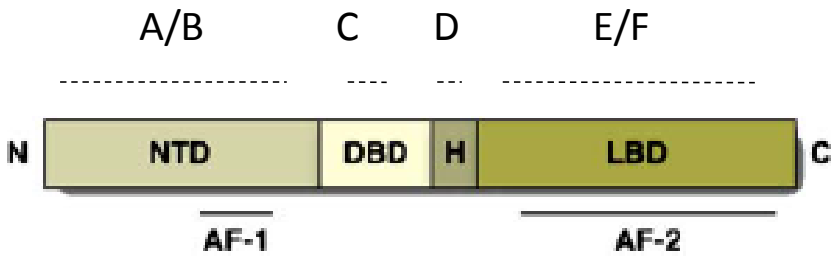


Figure 4 : Représentation schématique des différentes régions du récepteur aux glucocorticoïdes GR. Adapté de Heitzer et al. 2007.

NTD : *N-terminal domain* ; DBD : *DNA-binding domain* ; LBD : *ligand binding domain*

Le récepteur GR est omniprésent dans l'organisme. De fortes concentrations sont retrouvées au niveau du système immunitaire, des os, des poumons, du foie, du tissu adipocytaire et du cerveau (Morimoto et al. 1996; van der Laan and Meijer 2008). L'inactivation complète du gène du récepteur aux GC dans les modèles d'animaux transgéniques est létale dès les premières heures de vie (Cole et al. 1995).

c) Voie génomique d'activation des GR

En l'absence de ligand, le GR est principalement situé dans le cytoplasme cellulaire, sous la forme d'un complexe hétéro-oligomérique comprenant une protéine chaperonne de type HSP90, un peptide p23 et une *tetratricopeptide repeat protein*, TPR (**Figure 5**). Ce complexe permet l'exposition du domaine LBD du récepteur et sa séquestration dans le cytoplasme en masquant les séquences NL1 et NL2 (Pratt 1993).

Les GC libres sont liposolubles ; cette propriété leur permet de diffuser à travers la membrane cellulaire. Au niveau du cytoplasme, la fixation du ligand entraîne un changement de conformation du récepteur et une dissociation du complexe. Les séquences NL1 et NL2 étant démasquées, elles sont reconnues par des protéines de translocation nucléaire. Le récepteur peut migrer vers le noyau (Heitzer et al. 2007).

Dans le noyau, le GR forme alors un homodimère au niveau d'une séquence d'ADN du promoteur de gène cible appelée *glucocorticoid-responsive elements* (GREs) et spécifique du GR (Drouin et al. 1992). Classiquement, cette séquence est composée de deux séquences palindromique de six nucléotides séparées par trois nucléotides : AGAACAnnnTGTTCT. Mais de nombreuses variations de ce site sont possibles (Cato et al. 1984; Nicolaides et al. 2010). Il peut s'agir, selon le promoteur, d'un GRE activant la transcription du gène cible c'est-à-dire transactivateur, ou réduisant la transcription du gène cible, c'est-à-dire transrépresseur (désigné alors comme nGREs)(Nicolaides et al. 2010).

La fixation du dimère sur un GRE entraîne le recrutement i) de coactivateurs de la famille p160 des récepteurs aux stéroïdes (*steroid receptor coactivators*, SRCs) tel que SRC-1 (ou NcoA1), SRC-2 (ou TIF-2 ou GRIP1) et SRC-3 (ou p/CIP, RAC3, ACTR ou AIB1) ii) du p300 et de son homologue le *cAMP-responsive element-binding protein (CREB)-binding protein* (CBP) iii) du facteur associé au p300/CBP (p/CAF) (van der Laan and Meijer 2008). Ces coactivateurs servent de pont entre le GR et le complexe d'initiation de la transcription. De plus, l'activité histone acétyltransferase

(HAT) de ces protéines initie la décondensation de la chromatine facilitant ainsi l'initiation de l'activité transcriptionnelle de l'ARN polymérase par le GR (Trousson et al. 2007; Nicolaides et al. 2010). Dans le cas d'une fixation du dimère sur un nGRE, des corépresseurs (*Nuclear corepressor*, N-CoR et *silencing mediator for retinoid and thyroid hormone receptors*, SMRT) seront préférentiellement recrutés (van der Laan and Meijer 2008; Ramamoorthy and Cidlowski 2013). Leur activité histone désacétyltransférase (HDAC) inhibe alors la transcription du gène cible par le GR. Néanmoins, le mécanisme précis permettant d'expliquer ces différences de recrutement n'est pas encore totalement élucidé (Surjit et al. 2011). Un second mécanisme de transrépression des GC opère par l'intermédiaire de l'interaction des monomères GR avec des facteurs de transcription, sans liaison à l'ADN (comme par exemple le *nuclear factor-kappa B*, NF-κB et le *Activator Protein-1*, AP-1). Le GR vient simplement empêcher la fixation des facteurs de transcription sur leur promoteur et donc leur activité transcriptionnelle (Scheinman et al. 1995; McKay and Cidlowski 1998).

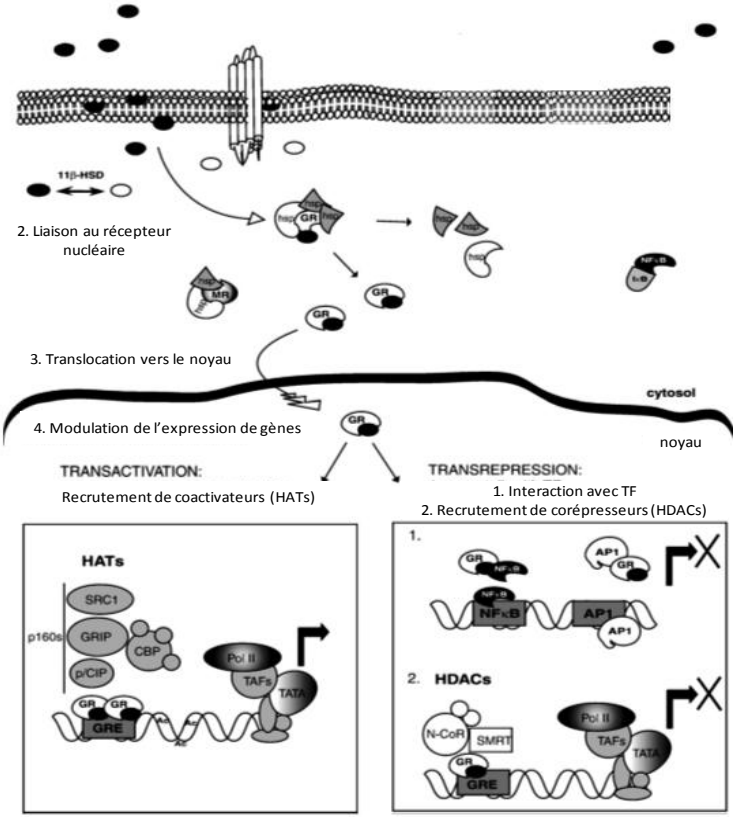


Figure 5 : Voie d'activation génomique de l'activité transcriptionnelle du récepteur aux glucocorticoïdes GR. Adapté de (van der Laan and Meijer 2008).

GR : *glucocorticoïdes receptor* ; HSP : *Heat shock protein* ; NF-κB : *nuclear factor-kappa B* ; AP-1 : *Activator Protein-1* ; HATs : histone acétyltransferase ; p160s : famille p160 des récepteurs aux stéroïdes ; SRC1 : *steroid receptor coactivators 1* ; GRIP : *Glutamate receptor-interacting protein* ; CBP : *cAMP-responsive element-binding protein (CREB)-binding protein* ; Pol II : ARN Polymérase II ; TAFs : *TBP-associated factors* ; HDACs: histone désacétyltransférase ; N-CoR : *Nuclear co-repressor*, SMRT : *silencing mediator for retinoid and thyroid hormone receptor* ; GRE : *glucocorticoid-responsive elements*

d) Voie non génomique d'activation des GR

A elle seule, l'activation transcriptionnelle GR-dépendante n'est pas suffisante pour expliquer l'ensemble des effets des GC notamment ceux survenant quelques minutes après l'exposition aux traitements (ce délai ne laisse pas le temps à l'expression de protéines de gènes cibles) (Haller et al. 2008; Groeneweg et al. 2012). Effectivement, il existe un certain nombre de mécanismes résultant de l'action spécifique des GC sur les cellules sans l'intervention du GR et/ou la modification de la transcription génique. Ils peuvent être regroupés en :

- effets directs des GC sur les lipides membranaires,
- effets directs des GC sur les protéines membranaires (pompes ioniques, récepteurs de neurotransmetteurs),
- effets indirects par modifications des voies de signalisation de protéines cytoplasmiques comme les protéines de la famille des Mitogen-activated protein kinases ou MAP Kinases (*extracellular signal-regulated protein kinases* (ERK), *c-Jun NH2-terminal protein kinase* (JNK), et MAPK p38) (Gutierrez-Mecinas et al. 2011; Ayroldi et al. 2012), la *phosphoinositide-specific phospholipase C* (PI-PLC) ou la *phosphatidylinositol 3-kinase* (PI3K) (Haller et al. 2008). Ces effets sont qualifiés par certains auteurs d'indirects plutôt que non-génomiques car ils s'accompagnent en général également de modifications transcriptionnelles. Ils pourraient être la conséquence de l'action des GC sur un GR membranaire (Haller et al. 2008).

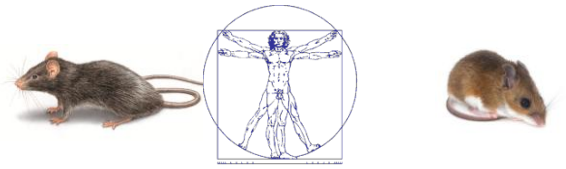
Les effets génomiques et non-génomiques des GC sont néanmoins intimement liés puisque les modifications des voies de signalisation des kinases par les GC peuvent être impliquées dans les modifications post-traductionnelles de l'état de phosphorylation GR lié à la présence du ligand (Galliher-Beckley and Cidlowski 2009).

e) Modifications post-traductionnelles : importance de la phosphorylation

Bien que l'activité transcriptionnelle du récepteur aux GC dépende essentiellement de la liaison à son ligand, les modifications post-traductionnelles du récepteur modulent cette activité. L'ubiquitination, la sumomylation et la dégradation par le protéasome affectent la stabilité du récepteur et diminuent l'activité transcriptionnelle en augmentant son *turn-over* (Ismaili and Garabedian 2004).

Au-delà de sa simple expression, c'est avant tout le statut phosphorylé du GR qui joue un rôle critique dans l'effet des GC. La phosphorylation du GR contrôle à la fois sa stabilité et son *turn-over*, ses déplacements entre les compartiments cellulaires, sa fixation au promoteur, son interaction avec les cofacteurs, la durée et l'intensité de son activité transcriptionnelle.

La première preuve de phosphorylation du GR est apportée en 1983 par l'équipe de Housley et Pratt. Les auteurs montrent l'incorporation de ^{32}P-orthophosphate au niveau du GR (Housley and Pratt 1983). Rapidement, une cartographie des différents sites de phosphorylation du GR humaine et murine a pu être établie (Figure 6). Elle montre que ces sites sont très conservés et une correspondance entre les différentes espèces a été proposée (Bodwell et al. 1991). Sept phosphorylations ont été caractérisées chez l'homme : serines 122, 150, 212, 220, 234 et 315 et thréonine 159.



S134	S113	S122
S162	S141	S150
T171	S135	T159
S224	S203	S212
S232	S211	S220
S246	S226	S234
S329	S308	S315

Figure 6 : Sites de phosphorylations du récepteur aux glucocorticoïdes GR chez le rat, l'homme et la souris. Adapté de Ismaili and Garabedian 2004.

Ces phosphorylations sont principalement situées dans la région N-terminale et dans le domaine de liaison à l'ADN du récepteur. Ceci suggère leur rôle dans la liaison du récepteur à l'ADN ou avec les facteurs de transcription et donc dans la modification de l'activité transcriptionnelle du GR (Galliher-Beckley and Cidlowski 2009). Afin de clarifier l'impact de ces phosphorylations, l'activité transcriptionnelle de GR présentant des mutations dirigées vers des sérines et thréonines phosphorylées a été comparée au GR naturel. Ces premières études ont d'abord montré qu'il n'existait pas de différence d'activité entre GR muté et GR naturel (Almlof et al. 1995). Puis, Webster et ses collaborateurs ont montré d'une part l'importance de ces phosphorylations dans la

stabilité du récepteur et d'autre part que leur implication dans l'activité transcriptionnelle du GR était réelle mais très variable d'un promoteur à un autre (Webster et al. 1997). Cette variabilité de l'activité transcriptionnelle en fonction du promoteur a été confirmée par d'autres études (Kino et al. 2007; Trousson et al. 2007). La modification de l'activité transcriptionnelle par la phosphorylation de GR repose à la fois sur une stabilisation du récepteur sous une conformation particulière entraînant la modification de l'interaction du récepteur avec les co-régulateurs (Garza et al. 2010) et sur une translocation nucléaire accrue ou inhibée (Webster et al. 1997; Dobrovolna et al. 2012).

Ces phosphorylations sont pour certaines constitutives (Ser141, Ser150). D'autres sont dépendantes de la fixation de l'agoniste. Des études récentes ont pu préciser qu'en l'absence de ligand, une phosphatase située à l'extrémité N-terminale du GR, maintenait certains récepteurs à l'état déphosphorylé. La liaison du GC au GR décroche le complexe HSP/p23/phosphatase. Le GR devient alors la cible de différentes kinases : p38 MAPK ou CDK5 (*cyclin-dependent kinase 5*) pour Ser211, JNK ou CDK5 pour Ser226, CDK2 ou ERK pour Ser203, GSK-3 (*Glycogen synthase 3*) pour Ser404 (Ismaili and Garabedian 2004).

L'impact de ces phosphorylations sur l'activité transcriptionnelle de GR n'est pas parfaitement connu. Les données concernant leurs rôles sont souvent contradictoires selon les auteurs du fait de l'importance du contexte cellulaire dans l'effet observé. Les phosphorylations sérine 211, 226 et 203 sont les plus étudiées. La présence d'un phosphate en position Ser211 est en général citée comme activatrice (Webster et al. 1997; Miller et al. 2005; Chen et al. 2008). Néanmoins, dans les cellules neuronales, elle a été décrite comme inactivatrice de l'activité GR (Kino et al. 2007). La majorité des publications suggère le rôle atténuateur de la phosphorylation Ser226 sur l'activité transcriptionnelle de GR par augmentation de l'affinité du récepteur pour ces corépresseurs (Rogatsky et al. 1997). Enfin, le GR phosphorylé en position Ser203 ne migre pas vers le noyau et ne semble pas capable de se lier à l'ADN. D'autres études sont nécessaires pour préciser ces fonctions au niveau cellulaire.

Les effets génomiques et non génomiques du GR au niveau cellulaire expliquent les actions des GC à l'échelle d'un organisme entier.

D. Actions des glucocorticoïdes dans l'organisme

Les glucocorticoïdes sont indispensables à la survie. Ils participent à la réponse adaptative de l'organisme face un déséquilibre de l'état d'homéostasie. Les GC mobilisent les ressources énergétiques nécessaires à cette réponse et atténuent les réactions immunitaires et inflammatoires générées par le stress. Le terme de glucocorticoïde fait référence aux propriétés de régulation du glucose de ces hormones mais ils ont également un rôle essentiel dans les métabolismes glucidique, protéique et lipidique. Ils interviennent aussi dans la régulation de l'immunité et la fonction rénale. Enfin, ils ont une influence importante sur le développement général, la croissance, le métabolisme osseux et l'activité cérébrale (Figure 7) (Rittmaster 1995; Sapolsky et al. 2000).

Effets sur le métabolisme
Augmentation de la glycémie, de la néoglucogenèse,de la glycogenèse, de la résistance à l'insuline
Augmentation de la lipolyse
Augmentation de la protéolyse
Effets immunologiques (à concentration pharmacologique)
Stabilisation des membranes lysosomales
Bloquage des bradykinine, histamine et interleukine-1 et -2
Diminution de la perméabilité vasculaire
Augmentation des polynucléaires neutrophiles
Bloquage de la diapédèse, de la chemotaxis et de la phagocytose des polynucléaires neutrophiles
Diminution des lymphocytes circulants (et plus particulièrement les lymphocytes T)
Diminution de la synthèse d'anticorps par les lymphocytes B
Diminution de la circulation des monocytes et éosinophiles
Effets sur les tissus confonctifs et sur la peau
Diminution de la formation de collagène
Atrophie cutanée par inhibition des fibroblastes
Effets sur le squelette et le métabolisme calcique
Diminution de la calcémie
Accélération de l'ostéoporose
Effets cardiaques
Augmentation de la fréquence et de la contractibilité cardiaque par augmentation de la sensibilité aux catécholamines
Effets rénaux
Augmentation de la filtration glomérulaire
Inhibition de la vasopressine
Effets sur la croissance et le développement
Inhibition de la croissance squelettique
Augmentation de la maturation du surfactant pulmonaire et des systèmes hépatiques et gastrointestinaux

Figure 7 : Principales actions des glucocorticoïdes dans l'organisme. Adapté de Rittmaster 1995.

Leurs actions immunologiques, en particulier pour les GC synthétiques, en font le traitement de choix des pathologies inflammatoires.

a) Effets sur le système nerveux central

Les effets des glucocorticoïdes sur le système nerveux central sont nombreux (Fietta and Delsante 2009). Les MR sont localisés dans le cortex préfontal, l'amygdale et en forte concentration dans l'hippocampe. Les GR sont exprimés de manière plus ubiquitaire dans le cerveau, à la fois dans les cellules gliales et les neurones, avec de fortes concentrations au niveau de l'hippocampe et du PVN de l'hypothalamus.

D'importants effets comportementaux sont décrits. Les GC sont impliqués dans la régulation du sommeil puisqu'ils sont responsables d'une diminution du sommeil paradoxal et d'une augmentation du sommeil lent (Friess et al. 2004). Plusieurs études montrent aussi l'implication des GC dans la prise alimentaire. Les GC augmentent l'appétit et la prise alimentaire même si leurs effets peuvent être inverses lors d'une situation de stress (Bazhan and Zelena 2013). Ils influencent également l'activité locomotrice (Stone and Lin 2008). Concernant les effets des GC sur l'humeur, ils peuvent être associés soit à des sentiments d'euphorie (Born et al. 1989; Het and Wolf 2007), soit à des sentiments négatifs (Schmidt et al. 1999; Swaab et al. 2005; Wolkowitz et al. 2009) dans le cas d'exposition à de forte doses ou chronique à des GC. Ces deux derniers effets (sur la locomotion et l'humeur) expliquent en partie que les GC puissent induire un état dépressif (Murray et al. 2008). Cet effet est particulièrement bien décrit chez les personnes présentant une dérégulation de l'axe corticotrope comme par exemple la dépression secondaire à un syndrome de Cushing ou à une prise de GC exogènes (Wolkowitz et al. 2009). Dépression et activité de l'axe HPA sont d'autant plus imbriqués qu'il a été montré plus récemment que la dépression était également responsable d'une hypercortisolisme.(Swaab et al. 2005; Carroll et al. 2012).
A l'inverse, une hypoactivité de l'axe HPA (en particulier celle survenant dans la maladie d'Addison) est également responsable de troubles dépressifs (Wolkowitz et al. 2009).

b) Effets sur la cognition et la mémoire.

Les effets des GC sur la cognition et la mémoire sont complexes. McEwen et ses collaborateurs sont les premiers à décrire l'importante densité des récepteurs aux corticostéroïdes au niveau de l'hippocampe, région clé de la mémoire, de l'apprentissage et de la cognition (McEwen et al. 1987; Lupien and Lepage 2001). Suivront alors d'importantes recherches chez l'animal et chez l'Homme montrant l'effet paradoxal des GC impliquant l'hippocampe mais aussi d'autres structures cérébrales comme l'amygdale et cortex pré-frontal. L'exposition aigüe et à faible dose des GC améliorent les performances mnésiques permettant une adaptation vis-à-vis de la perception d'un stress. En revanche, une exposition chronique et/ou à fortes doses diminuent ces mêmes

performances (Lupien and Lepage 2001; McEwen 2007). Ainsi une administration de fortes doses de prednisone (80mg par jour) pendant 5 jours altère chez l'Homme les capacités de mémorisation alors qu'une administration unique de 1mg de dexaméthasone n'a pas d'effet délétère (Wolkowitz et al. 2009). Les patients traités par GC présentent une diminution du volume hippocampique et des troubles de la mémoire déclarative Ces observations s'inscrivent dans une théorie selon laquelle le niveau de performances mnésiques suit une courbe en U inversé en fonction de la concentration en GC (de Kloet et al. 2005; Joels et al. 2006).

c) Glucocorticoïdes et plasticité neuronale : hypothèse mécanistique

Comme décrit plus haut, l'augmentation à court terme des niveaux GC est normalement adaptative et bénéfique, mais l'exposition prolongée à des niveaux élevés de GC comme lors d'un stress chronique conduit à des effets délétères. Cette courbe en U inversé est à rattacher directement aux mécanismes moléculaires survenant au niveau des cellules du SNC. En effet, lorsque l'exposition aux GC survient à de faibles concentrations ou en aigu, l'occupation des récepteurs MR (forte affinité pour les GC) sera importante alors que l'occupation des GR (faible affinité pour les GC) sera moindre. Dans cette configuration, les effets des GC seront optimaux en termes de réponse comportementale et de mémorisation de l'information. En cas de réponse des GC excessive et/ou prolongée, les GC occuperont à la fois les MR mais aussi les GR. Ce ratio d'occupation MR/GR augmenté se traduit par une apparition des effets délétères des GC au niveau cellulaire (Sousa et al. 2008; Dorey et al. 2012; Yau and Seckl 2012).

Les effets délétères du stress et des GC sont expliqués par la survenue de dommages, principalement au niveau hippocampique. De nombreux auteurs rapportent une diminution de la neurogenèse et de la prolifération neuronale ((Murray et al. 2008; Anacker et al. 2013), une atrophie des dendrites associée à une diminution de la densité et de la complexité des épines dendritiques (Conrad et al. 2007; Alfarez et al. 2009), à une perturbation de la potentialisation à long terme (Pavlides et al. 1996) et une diminution de la plasticité neuronale (Krugers et al. 2006) lors de l'exposition prolongée à un stress ou aux GC. L'implication des GC dans la mort neuronale reste controversée : pour certains auteurs les GC auraient une toxicité neuronale propre, pour d'autres ils augmenteraient la vulnérabilité aux agents neurotoxiques (Conrad 2008). Ces effets seraient en partie liés à la diminution de la sécrétion de neurotrophines comme le *brain-derived neurotrophic factor* (BDNF) (Schaaf et al. 1997; Suri and Vaidya 2013).

Au vu de l'importance des effets des GC et surtout de la balance de leur action sur les récepteurs MR et GR, on comprend le rôle fondamental des déshydrogénases dans les fonctions du système

nerveux central. Les 11β-HSD1 et 11β-HSD2 modulent en permanence l'exposition cellulaire aux GC. L'expression cérébrale de la 11β-HSD1 prédomine par rapport à celle de la 11β-HSD2. Alors que la 11β-HSD2 y est très peu exprimée, la 11β-HSD1 est fortement exprimée au niveau de l'hippocampe, du cervelet et du cortex dans les neurones et les cellules gliales (Sandeep et al. 2004). La preuve de l'implication de la 11β-HSD1 dans les effets délétères cérébraux et cognitifs des GC est apportée par des modèles de souris où la surexpression de l'enzyme est associée à une détérioration des performances mnésiques de l'animal. A l'inverse, l'inactivation du gène de la 11β-HSD1 ou l'utilisation d'inhibiteurs de son activité améliore significativement ces mêmes performances (Holmes et al. 2010; Yau et al. 2011; Yau and Seckl 2012).

II. Modifications pré-récepteurs de l'exposition aux glucocorticoïdes : résultats

La moitié de la production du cortisol dans l'organisme résulte de l'action de la 11 β -HSD1 qui convertit la cortisone en cortisol. Il s'agit d'une étape importante de la régulation de la biodisponibilité en GC dans l'organisme et en particulier au niveau du SNC. Plusieurs facteurs métaboliques endogènes ou exogènes comme l'insuline, la glycémie et l'inflammation modulent cette exposition pré-récepteur aux GC.

Il existe chez les patients atteints de diabète de type I (ou insulino-dépendant) à la fois une dérégulation de la glycémie, de l'insulinémie et une inflammation à bas bruit. En effet, la survenue du diabète correspond à une insuffisance de sécrétion d'insuline par le pancréas en lien avec une destruction des ilots beta de Langerhans souvent d'origine auto-immune. Le traitement consiste en l'injection sous-cutanée d'inuline recombinante. Sans que les causes ne soient réellement connues, cette pathologie se déclare de plus en plus tôt dans l'enfance. Son incidence est donc croissante chez le jeune enfant : elle augmente de 7% par an chez l'enfant de moins de 4 ans contre 3% chez l'enfant et l'adolescent tout âge confondu.

Le premier objectif de ce travail est de mettre en évidence, chez l'enfant, le lien entre les modifications métaboliques dues au diabète de type I et une modification de l'activité de l'axe corticotrope. Cette étude, menée par le pédiatre Pascal Barat, ciblera une modification pré-récepteur de l'exposition aux glucocorticoïdes.

Pour évaluer l'exposition pré-récepteur et en particulier l'activité de la 11β-HSD1, nous avons mis au point un dosage du cortisol et de huit de ses métabolites par une méthode de chromatographie liquide couplée à la spectrométrie de masse. Un travail préliminaire (PUBLICATION N°1) a été mené pour valider notre dosage dans une situation physiologique (patients contrôle), une situation d'augmentation de la sécrétion de GC (patients atteints de la maladie de Cushing et patients présentant un adénome cortisolique), d'augmentation subnormale (patients présentant un syndrome de Cushing sub-clinique), d'inflammation à bas bruit (patients obèses).

Nous avons ensuite (PUBLICATION N°2) comparé deux populations d'enfants : ceux atteints depuis plus d'un an d'un diabète de type I (n=45) à leur fratrie non atteinte (n=23). Une évaluation de i) l'équilibre glycémique (glycémie et dosage de l'hémoglobine glyquée), ii) de l'inflammation (la protéine C-réactive de haute sensibilité CRP, interleukine 6) et iii) de la concentration du cortisol et de ses métabolites dans les premières urines du matin (reflet de la sécrétion nocturne) a été faite dans ces deux groupes.

Enfin, d'autres facteurs métaboliques modulent la biodisponibilité pré-récepteur en GC. L'influence de l'alimentation, en particulier la composition en protides, glucides et lipides des repas, sur l'activité de la 11β-HSD1 est maintenant bien décrite. Il existe également des principes actifs de plantes capables de modifier cette activité. L'exemple le plus connu est l'acide glycyrrhizzique du réglisse qui inhibe la conversion cortisol en cortisone par la 11β-HSD2. Récemment, une équipe a rapporté un cas d'une hypertension artérielle avec œdème et hypokaliémie en lien possible avec l'inhibition de la 11β-HSD2 par les rébaudiosides du stévia (Esmail and Kabadi 2012). Des extraits de cette plante sont commercialisés pour leur important pouvoir sucrant et donc largement utilisée par les patients atteints de diabète de type II afin de limiter les apports caloriques. Il nous est apparu intéressant de d'étudier ce métabolisme après la prise de rébaudioside A, commercialisé pur en France (et non d'un simple extrait de stevia). Nous présentons les résultats dans une population de personnes saines, d'une prise modérée de rébaudioside A sur douze heures (PUBLICATION N°3).

A. PUBLICATION N° 1 : validation d'une méthode de dosage des métabolites urinaires du cortisol chez l'homme

Urinary glucocorticoid metabolites in the subclinical hypercortisolism diagnosis of adrenal tumour

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En préparation

Urinary glucocorticoid metabolites in the subclinical hypercortisolism diagnosis of adrenal tumour

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No reprints are necessary

Introduction

The increasing prevalence of adrenal incidentalomas (≈ 4%), is related to the simultaneous increases of the age of the population and of the quality as well as quantity of medical imaging procedures (1-3). To discover an incidentaloma elicits questions about its nature as one may propose a follow-up or a surgical treatment depending on its secretion and on the clinical background (age and other risk factors). Thus, to carefully clinically as well as biologically determine the secretion disturbance is critical when the patient does not present a clear ACTH-independent Cushing syndrome. Indeed, the necessity of operating so-called subclinical hypercortisolism with minor or non-specific clinical signs (obesity, hypertension, type 2 diabetes, osteoporosis,...) is still debated (1, 4, 5). The first obvious difficulties are the definition, frequency and diagnosis of subclinical hypercortisolism (4). Further debates are about the risk of evolution towards clear oversecretion and subsequent metabolic consequences *vs* no evolution, the cost of surgery *vs* the cost and duration of follow-up, etc, *i.e.* the ratio benefits/risks of surgery *vs* follow-up (6-10).

The more common tests for assessing incidentaloma secretion used are dexamethasone suppression test (DST), urinary free cortisol (UFC), ACTH at 08h00, serum or salivary cortisol at 00h00. However, none is perfect or consensual although all perform better when combined and repeated (6) (11, 12). More specifically, UFC concentration determined by immunoassays (irUFC) shows poor sensibility and specificity particularly in obese population (13, 14). This may partly be because obesity, hypertension or type 2 diabetes tend to increase hypothalamo-pituitary adrenal axis activity with moderately increased secretion of cortisol and its metabolites (15). As cortisol and metabolites related structures share common antigenic epitopes cortisol immunoassays suffers of cross-reactivity and subsequently overestimate UFC (16-18). For these both reasons irUFC determination is criticized for the frequency of the false positive results. The high-performance liquid chromatography instruments coupled to tandem mass spectrometers (UPLC-MSMS) can overcome these specificity problems and several studies demonstrate some advantages of

spectrometric techniques for the diagnosis of Cushing's syndromes (19, 20). Initially, a cortisone/cortisol ratio was propose to improve the diagnosis of Cushing's syndrome, later an enlarged metabolic analysis was envisioned to identify benign AT or adrenocortical carcinoma (21, 22).

Here, we hypothesised that to specifically assay urinary cortisol (msUFC) and eight cortisol metabolites by mass spectrometry could be more efficient than to assay cortisol by immunoassay to diagnose sub clinical adrenal tumours. Patients with adrenal tumours were compared with normal or obese and with patients with Cushing's disease to investigate the potential benefit of a multiplex assay in the assessment of incidentalomas.

Subjects, material and methods

Subjects

Samples of 24h urines analysed in our routine laboratory activity were provided by patients (n=260) hospitalised in the endocrine department. Clinical and biological characteristics are summarised in the Table 1. Patients with hydrocortisone or anticortisolic treatments or with adrenocortical carcinoma, hyperaldosteronism or pheochromocytoma were excluded of the analysis.

Firstly, we compared cortisol and metabolites values of 48 healthy subjects (Nl, patients without any hormonal anomalies) to 143 obese subjects (Ob), 26 patients with confirmed active Cushing's disease (CD, initial diagnosis or relapsing diagnosed on clinic, biologic and imagery criteria) and 43 patients with an AT. Clinical and biological data concerning patients are presented in the Table 1.

	Ctl			AT	
	N	**Ob**	**CD**	**AT-**	**AT+**
age (yrs)	45 [25-72]	46 [19-74]	46 [20-72]	58 [39-72] ***	56 [26-71]
sex ratio	0.3 [37/11]	0.28 [112/31]	0.4 [18/8]	0.35 [20/7]	0.33 [12/4]
BMI (kg/m²)	23 [18-30]	39 [31-55] ***	29 [23-42] **	27 [21-40]	25 [18-38]
Cortisol 8h (nmol/L)	544 [186-767]	485 [264-784]	605 [296-997]	477 [264-852]	477 [295-864]
ACTH 8h (pmol/L)	8 [1.7-12.1]	5.3 [1.2-18.0]	13.6 [2.8-42.0] *	4.3 [1.4-11.2]	1.8 [1.2-3.5] ***
irUFC (µg/24h)	34 [19-79]	28 [9-91]	211 [102-1696] ***	41 [15-78]	93 [18-530] ***
1-mg DST(nmol/L)	43 [27-63]	42 [27-119]	307 [67-705] ***	51 [27-117]	108 [63-847] ***
Cortisol 0h (nmol/L)	145 [62-281]	137 [44-290]	449 [191-912] ***	155 [40-419]	224 [136-820] *
ACTH 0h (pmol/L)	2.5 [1.3-5.9]	2.4 [1.2-9.2]	13 [2.7-37.7] ***	1.5 [1.2-7.9] *	1.2 [1.2-2.4] ***

Table 1

Biological data of the investigated subjects: median [2.5^{th} – 97.5^{th}] centile. Nl & Ob normal and obese subjects respectively, CD & AT patients with Cushing's disease & adrenal tumour (including AT+ secreting and AT non secreting adrenal tumour). * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$ compared to Nl subjects.

Secondly, we classified this latter population in AT patients without oversecretion ("silent" tumours, AT-, n=27) or with oversecretion (AT+, n=16) to compare them to a control population (Ctl, *i.e.* Nl + Ob subjects). The discovery of the tumour was fortuitous for all AT- (incidentaloma) and for 13 patients AT+. For 2 AT+ patients the tumours were discovered on clinical signs and 1 when investigating a recent hypertension. This classification used the following criteria (11): serum cortisol levels after 1 mg overnight dexamethasone-suppression test (1 mg DST) > 3.0 µg/dL (83 nmol/L), elevated irUFC levels (over normal values i.e. 90 ng/mL), 8h ACTH levels < 10pg/mL (<2 pmol/L). Patients with at least 2 of these 3 positive criteria were considered as AT+ (9 with 2 criteria and 5 with 3 criteria). Two patients also included in this category had only 1 positive criterion but underwent surgery. The first one displayed 2 positive criteria were obtained shortly after the initial investigation. The second one had an increase uptake nor-iodocholesterol scintigraphy that prompted surgery. In the group AT-, 10 patients presented 1 criterion and 17

patients 0 criterion. Finally, eleven AT+ and 2 AT- patients were operated of their adenoma.

All patients were tested for irUFC. 1 mg-DST was performed in 40, 89, 42, 93 and 88% of Nl, Ob, CD, AT- and AT+ subjects, respectively. ACTH 00h00 was performed in 21, 19, 100, 85 and 100% of Nl, Ob, CD, AT- and AT+ subjects, respectively.

Cortisol and ACTH immunoassay

ACTH serum concentrations were assayed by IRMA (ACTH RIA, B.R.A.H.M.S., Germany) according to the manufacturer instructions. Inter-assay coefficients of variation (CV) were 4.0 and 4.2 % at 35 and 332 ng/L, respectively; Functional sensitivity was 5.3 ng/L (23). Serum cortisol was assayed by an automated analyser (DXi800, Beckman Coulter). Inter-assay CV were 10.4, 6.5 and 6.1 % at 85, 456 and 830 nmol/l, respectively. irUFC was assayed by RIA (Spectria CORTISOL RIA, IDS France). Inter-assay CV were 12.0 and 7.0 % at 14 and 127 µg/24h, respectively; the manufacturer's normal range was [20-90] µg/24h.

Urinary steroid profiling: high performance liquid chromatography-tandem mass spectrometry

Steroids standards (cortisol, cortisone, tétrahydrocortisol (THF), allo-tétrahydrocortisol (allo-THF), tétrahydro-cortisone (THE), alpha-cortol, beta-cortol, alpha-cortolone, beta-cortolone) were purchased from Steraloids Inc Newport and the internal standard (IS) (6-alpha-methylprednisolone) from Sigma-Aldrich (St Louis, USA). Dichloromethane and sodium acetate, methanol and ammonium acetate were provided by VWR (VWR International, LLC, West Chester, PA, USA). The beta-glucoronidase/arylsulfatase was provided by Roche Applied Science (Penzberg, Germany).

Stock solutions were prepared in methanol for each standard and the IS at the concentration of 1 mg/mL. An eight-point calibration curve was prepared by mixing each steroid solutions and by serial dilutions to obtain 10000, 5000, 1000, 500, 100, 50, 10, 5 ng/mL concentrations. After

addition of 150 µL of sodium acetate buffer and 50 µL of IS (10 µg/mL), the calibration curve was extracted by DCM in the same conditions as urine samples.

A 1.2 mL aliquot of urine was centrifuged at 3000 rpm and 125 µL of sodium acetate buffer (0.5 M pH=5.1) and 25 µL of beta-glucoronidase/arylsulfatase were added. After a 3 h incubation at 55°C, 50µL of IS were added and samples were extracted with 5 mL of dichloromethane. Finally, 4 mL of the latter were evapored and reconstituted with 300 µL of mobile phase.

Two µL were injected into Acquity UPLC separative module (Waters, Millport, USA) controlled by the Masslynx Software. Separation was performed at 50°C on Acquity UPLC® BEH C8 1.7µm 2.1x100 mm column and precolumn 3/PK BEH C18 1.7µm VanGuard^{TM} 2.1x5 mm in the following conditions: flow rate of 0.4mL/min with a mobile phase consisted of a combination of phase A (ammonium acetate 10mM) and phase B (methanol) for a total run time of 10 min (flow rate %A %B: [0 min] 65/35; [4.5 min] 40/60; [6 min] 5/95; [7.5 min] 65/35). An Acquity TQD detector (Waters) with electrospray ionization (ESI) in positive ion mode was used for detection in multiple reaction monitoring (MRM) mode. Operative conditions were: temperature ESI source, 120°C; desolvation temperature, 400°C; cone gas flow 50L/h; desolvation gas flow, 900 L/h; capillary voltage 1.5kV; pression of MS collision gas (argon), 3 10^{-3} mBar. Mass transition, limit of sensitivity, linearity and CV intra inter are shown in the Table 2.

	Molecular weight	Retention time	Parent ion	Quantification transition	Cone voltage	Collision energy	Calibration range	Limit of quantification
		(min)	(m/z)	ion (m/z)	(V)	(eV)	(ng/mL)	(ng/mL)
cortisol	362.5	4.4	363.5	120.9	32	27	10-10000	10
cortisone	360.5	4.1	361.5	162.9	38	25	10-10000	10
THF	366.5	5.4	331.0	301.0	30	20	10-10000	250
allo THF	366.5	5.3	384.3	313.2	10	15	10-10000	100
THE	364.5	5.6	365.5	329.0	20	15	10-10000	100
alpha cortol	368.5	5.2	386.2	297.0	15	15	10-10000	50
beta cortol	368.5	5.4	386.2	297.0	15	15	10-10000	100
alpha cortolone	366.3	5.4	349.2	331.1	20	10	10-10000	100
beta cortolone	366.3	5.6	349.2	331.1	20	10	10-10000	50
6 Alpha methylprednisolone (IS)	375.2	5.2	375.2	339.0	22	12		

Table 2

Technical characteristics of UPLC-MSMS for msUFC and metabolites determination: calibration, limits of quantification and quality control.

Statistics

All data were presented as median [2.5^{th}-97.5^{th}] centiles. Calculations and statistics were done using the Excel 98, MedCalc and Statistica softwares. We compared each group using Kruskal-Wallis non-parametric test and Dunn's *post doc* test. Significant differences were considered for $p < 0.05$.

Results

There was no significant difference between irUFC, msUFC or metabolites excretion in Nl *vs* Ob subjects (Table 3).

	irUFC	msUFC	cortisone	THF	alloTHF	THE	α-cortol	β-cortol	α-cortolone	β-cortolone
N										
median (µg/24h)	34	73	115	2193	227	2706	154	243	1036	247
[2,5th-97,5th percentile]	[13-79]	[25-135]	[38-193]	[809-4111]	[92-1056]	[1020-6124]	[48-313]	[75-650]	[439-1956]	[81-570]
Ob										
median (µg/24h)	28	65	85	2501	274	3475	165	237	819	194
[2,5th-97,5th percentile]	[9-91]	[15-214]	[16-310]	[538-8408]	[66-1219]	[573-9055]	[37-464]	[44-697]	[125-1956]	[31-503]
CD										
median (µg/24h)	211 ***	362 ***	301 ***	8637 ***	490 **	8561 ***	609 ***	965 ***	2712 ***	907 ***
[2,5th-97,5th percentile]	[102-1696]	[180-2155]	[135-619]	[4561-37710]	[120-2053]	[1742-23472]	[226-3431]	[328-6900]	[1222-5392]	[385-3260]
AT										
median (µg/24h)	48	100 *	124	3064 ***	240	3947 **	167	288	717	390
[2,5th-97,5th percentile]	[15-212]	[43-533]	[28-429]	[1305-11839]	[111-623]	[781-9924]	[65-519]	[125-946]	[207-2191]	[207-1188]

Table 3

Concentrations (median [2.5^{th} centile – 97.5^{th} centile]) of irUFC, msUFC and metabolites of normal (Nl) and obese (Ob) subjects and of patients with Cushing's disease (CD) and adrenal tumours (AT). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to normal subjects.

Conversely, irUFC, msUFC and all metabolites were significantly over-excreted in the patients with active CD compared to Nl subjects. When Nl subjects were compared to AT subjects, there was no significant difference of irUFC whereas msUFC, THF and THE excretions were significantly increased in AT.

The population of Nl and Ob subjects was pooled in a control population (Ctl) because all UFC

and metabolites excretions were similar. AT patients were sub classified in AT+ and AT- patients (see M&M). There was no significant difference between Ctl *vs* AT- subjects for irUFC, msUFC or metabolites excretions (Table 4).

	irUFC	msUFC	cortisone	THF	alloTHF	THE	α-cortol	β-cortol	α-cortolone	β-cortolone
Ctl										
median (µg/24h)	29	68	100	2400	245	3261	163	239	909	202
[2,5th-97,5th percentile]	[10-89]	[17-208]	[19-256]	[727-8048]	[70-1208]	[823-8938]	[41-462]	[48-692]	[176-1974]	[39-553]
AT-										
median (µg/24h)	41	82	106	2752	315	3568	147	258	645	296
[2,5th-97,5th percentile]	[15-78]	[43-213]	[39-311]	[1440-6871]	[129-629]	[1226-7741]	[61-408]	[128-635]	[281-1101]	[96-747]
AT+										
median (µg/24h)	93 ***	185 *** ¤	156 **	4054 **	200	4601 **	252 ¤	471 *	1079	512 **
[2,5th-97,5th percentile]	[18-530]	[63-720]	[39-405]	[1129-12066]	[114-477]	[1521-11269]	[80-829]	[124-1114]	[269-2199]	[153-1288]

Table 4

Concentrations (median [2.5th centile – 97.5th centile]) of irUFC, msUFC and metabolites of control patients (Ctl: pooled normal and obese subjects) and of patients with adrenal tumours with oversecretion (AT+) or without oversecretion (AT-) (see material and methods). * p< 0.05, ** p< 0.01, *** p< 0.001 compared to normal subjects; ¤ p< 0.05 compared to AT- subjects.

Conversely, irUFC, msUFC and some metabolites excretions (cortisone, THF, THE, β-cortol, β-cortolone) were significantly higher in AT+ subjects. Unexpectedly, irUFC was similar in AT+ *vs* AT- patients whereas msUFC and α-cortol excretions were significantly different between these groups.

Receiver-operating characteristics (ROC) analyse were performed to determine the most discriminating analytes in Ctl *vs* AT+ populations and in AT+ vs AT- populations. The 3 best metabolites differentiating AT+ patients from Ctl subjects were: msUFC (area under the ROC curve (AUC): 0.698, 95 CI % 0.635-0.756), β-cortolone (0.729, 95% CI 0.652-0.797) and THF (0.66, 95 % CI 0.596-0.721). The 3 best metabolites differentiating AT+ from AT- patients were: msUFC (0.817, CI 95% 0.670-0.918), α-cortolone (0.773, CI 95% 0.552-0.919) and THF (0.734, CI 95% 0.577-0.857).

Discussion

We compared urinary cortisol metabolites in patients with secreting (AT+) or non-secreting (AT-) adrenocortical tumours. We hypothesised that one analyte assayed by mass spectrometry could perform better than irUFC to distinguish AT+ from AT-.

Firstly, we studied metabolites excretion in a control population to define reference values. The population studied included a large proportion of obese subjects. Indeed obesity is one non-specific clinical signs of Cushing's syndrome. Furthermore, the prevalence of obesity is higher in patients with clinically silent adrenal adenomas, with or without subclinical hypercortisolism (1). So screening for CS is frequent in obese populations. Some studies reported an elevation of cortisol metabolites excretion in obese subjects (13, 24)(25). Thus, we investigated whether to be overweight could change irUFC, msUFC or metabolites. We found neither significant modification of excretion of these analytes nor correlation between any analyte and BMI (data not shown). This is in agreement with some authors that also found no significant correlation between UFC and BMI. More precise parameters of fat distribution not available here could be linked to modifications of cortisol metabolism such as THFs/THE ratio (26-29). The reference values we found here in our control population are similar with values obtained previous studies (26). msUFC values was found lower in the few studies when enzymatic hydrolysis was not used before analysis (30). In our hands, msUFC was also slightly lower when pre-analytical metabolite hydrolysis was not performed (data not shown).

Secondly, as expected the population of patients with CD had clearly elevated msUFC and metabolites. No patient presented here had ambiguous or intermittent CD hence these results. Reports of cortisol metabolites excretion in CD is lacking although mass spectrometry assay of cortisol is not rare.

Thirdly, we classified the population of subjects with AT according to published criteria to biologically distinguish AT with secreting from apparently non-secreting AT (6). It must be noted that these criteria do not fully overlap with the recommendations of the Endocrinology Society (1, 12, 31) but they represent the routine practice this study. Indeed the French Society of Endocrinology published in 2008 a consensus recommending the screening of hypercortisolism with 1 mg dexamethasone test, urinary cortisol serum or salivary cortisol and ACTH (32). It must be noted that here salivary cortisol was performed in only 37% of the patients with AT. We thus focused our classification on the more frequently used tests: dexamethasone test, UFC and plasma ACTH. In this study, msUFC and metabolites were significantly increased in whole AT population while irUFC was not significantly increased. This was also true in AT+ *vs.* AT- populations. In agreement with Eller-Vainicher *et al.* did not find any difference between the patients that were 'improved' after chirurgical treatment *vs* those that were 'not improved' (33). These results could relate to the lower specificity of irUFC.

msUFC, α-cortolone and THF seem to be the best parameters to distinguish AT+ from AT- patients. These results confirm other publications that found significant differences between subjects with "silent" incidentaloma *vs* normal subjects for THF, α-cortol and allo-THF using a GC-MSMS assay (21). However, Alk *et al.* found a difference between adrenal benign tumours and control population using the sum of all cortisol metabolites concentrations (22) as we found that the ROC of the sum of msUFC, α-cortolone and THF URL presented the largest area.

Our simple and inexpensive multiplex LC-MSMS assay of cortisol and metabolites appears helpful for the identification of SH. Some of these new biomarkers can overcome the lack of specificity of irUFC (34, 35). This retrospective study must be confirmed by a prospective study with a larger population of AT as well as a long lasting follow-up of patients whether operated or

not.

This study cannot identify the earliest analyte to be increased in SH. msUFC seems a better biomarker than irUFC. Whether α-cortolone or THF increase are synchronous or more precocious than msUCF increase in subclinical hypercortisolism is an open question.

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B. PUBLICATION N°2 : Activité de la 11β-HSD1 chez les enfants diabétiques de type I

Nocturnal activity of 11 β-hydroxy steroid dehydrogenase type 1 is increased in type 1 diabetic children

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Original article

Nocturnal activity of 11β-hydroxy steroid dehydrogenase type 1 is increased in type 1 diabetic children

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Abstract

Aim. – The objective of this study was to investigate low-grade inflammation in children with type 1 diabetes (T1D) and its association with cortisol levels as well as its bioavailability through 11β-hydroxy steroid dehydrogenase type 1 (11β-HSD1) activity.

Methods. – Children with T1D ($n = 45$) and their non-diabetic siblings ($n = 28$) participated in the study. Interleukin-6 (IL-6) and high-sensitivity C-reactive protein (CRPhs) were measured between 1400 and 1800 h. Glucocorticoid metabolites were measured in the first morning urine on clinic day and 11β-HSD1 activity was estimated by tetrahydrocortisol/tetrahydrocortisone (THF/THE) ratio.

Results. – Diabetic patients presented with an increased THF/THE ratio compared with controls (median: 0.68 [range: 0.45–1.18] *vs* 0.45 [0.27–0.98], respectively; $P < 10^{-3}$). There was no difference between diabetic patients and controls for IL-6 (0.6 ng/mL [0.6–6.8] *vs* 0.6 [0.6–2.2], respectively; $P = 0.43$) and CRPhs (0.4 mg/L [0–7.4] *vs* 0.3 [0–8.2]; $P = 0.26$, respectively). When adjusted for age, gender and BMI, the THF/THE ratio was significantly associated with CRPhs ($\beta = 0.32$, $P = 0.02$) in diabetic patients, but not in controls.

Conclusion. – Low-grade inflammation assessed by plasma CRPhs and IL-6 concentrations was not detectable in our cohort of T1D children. Nocturnal 11β-HSD1 activity was increased and associated with plasma CRPhs concentration in diabetic patients. These results may be explained by either a direct or inflammation-mediated effect of the relative hepatic lack of insulin due to subcutaneous insulin therapy.

Keywords: Type 1 diabetes mellitus; Children; Inflammation; Pituitary–adrenal axis; 11β-hydroxy steroid dehydrogenase type 1; High-sensitivity C-reactive protein; Interleukin-6

Résumé

Augmentation de l'activité 11β-hydroxysteroïde déshydrogénase de type 1 chez les enfants diabétiques de type 1.

Objectif. – Mesurer l'inflammation à bas bruit chez des enfants présentant un diabète de type 1 (DT1) et chercher des associations potentielles entre cette inflammation et l'activité de la 11β-hydroxysteroïdes deshydrogénase de type 1 (11βHSD1) qui contribue à la biodisponibilité tissulaire du cortisol.

Méthodes. – Quarante-cinq enfants DT1 et 28 frères et sœurs non diabétiques ont participé à cette étude. L'interleukine-6 (IL-6) et la protéine C-réactive de haute sensibilité (CRPhs) ont été mesurées entre 14 h et 18 h. Les métabolites urinaires des glucocorticoïdes ont été dosés sur les premières urines du matin le jour de la consultation. L'activité 11βHSD1 a été estimée par le rapport tetrahydrocortisols/tertahydrocortisone (THF/THE).

Résultats. – Les enfants diabétiques présentaient une augmentation du rapport THF/THE par rapport aux témoins (médiane 0,68, extrêmes [0,45–1,18] vs 0,45 [0,27–0,98], $P < 10^{-3}$, respectivement). Il n'y avait pas de différence entre les deux groupes pour les dosages d'IL-6 (0,6 ng/ml [0,6–6,8] vs 0,6 [0,6–2,2], $P = 0,43$, respectivement) et de CRPhs (0,4 mg/l [0–7,4] vs 0,3 [0–8,2], $P = 0,26$, respectivement). Après ajustement pour

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l'âge, le genre et l'IMC, le rapport THF/THE était associé à la CRPhs (β = 0,32, P = 0,02) chez les enfants diabétiques mais non chez le témoins.

Conclusions. – Nous ne mettons pas en évidence d'inflammation à bas bruit par les mesures de CRPhs et d'IL-6 dans notre cohorte d'enfants atteints de DT1. L'activité nocturne de la 11βHSD1 est augmentée et associée aux concentrations plasmatiques de CRPhs chez les enfants DT1. Ces résultats pourraient s'expliquer par la carence relative en insuline au niveau hépatique engendrée par l'insulinothérapie sous-cutanée.

Mots clés : Diabète de type 1 ; Enfants ; 11β-hydroxysteroïdes deshydrogénase de type 1 ; Inflammation ; Axe corticotrope ; Interleukine-6 ; Protéine C-réactive

1. Introduction

The incidence of type 1 diabetes (T1D) is increasing worldwide in children and teenagers by an average of 3% per annum, but is up to 7% in children younger than 4 years [1–3]. This alarming increase calls for the development of new strategies for preventing chronic complications associated with the disease in this population. One strategy would be to identify new markers associated with the developing pathophysiology of these complications.

Markers of inflammation have been shown to be associated with microvascular complications and cardiovascular disease in T1D [4,5]. Furthermore, levels of interleukin-6 (IL-6) could be elevated in young adults with T1D, but no clinical evidence of microvascular or macrovascular complications, suggesting that a low-level chronic inflammatory state may play a key role in the early stages of atherogenesis and in the development of microvascular disorders [6].

Cortisol is the main hormone involved in the endogenous anti-inflammatory process. Local cortisol bioavailability is highly dependent on the activity of 11β-hydroxy steroid dehydrogenase type 1 (11β-HSD1) enzyme. Indeed, 11β-HSD1 is the essential agent in peripheral cortisol metabolism, converting inactive cortisone to the active hormone cortisol mainly in the liver, adipose tissue and brain. This enzyme, extensively studied in subjects with obesity and the metabolic syndrome, has been implicated in the regulation of their low-grade inflammatory states [7,8]. However, little is known of the peripheral activity of 11β-HSD1 in T1D.

Early recognition of changes in the balance between inflammatory and anti-inflammatory processes could be of interest in detecting the susceptibility for developing future complications of diabetes. Thus, the objective of the present study was to investigate low-grade inflammation in children with T1D and its association with cortisol levels as well as its bioavailability through 11β-HSD1 activity.

2. Patients and methods

2.1. Patients' recruitment

Children with T1D of at least 1 year's duration (n = 45) attending the diabetes clinic at the Children's Hospital, CHU Bordeaux, France, were invited to participate, along with 28 non-diabetic siblings serving as controls. Informed consent was obtained from the participating children and their parents (clinical trial registration: NCT 01099956).

2.2. Data collection

Data regarding gender, age, duration of diabetes, body mass index (BMI), waist circumference (WC) and blood pressure were collected. The night before coming to the clinic, the patients were asked to collect their first morning urine. Patients with known urine emission or known hypoglycaemia during the night were not included. A blood sample taken by micropuncture was obtained for HbA_{1c} (DCA Vantage HbA_{1c} Reagent Kit, Siemens Healthcare Diagnostics, Suffolk, UK), capillary blood glucose (blood analyzer, Olympus Company, Center Valley, PA, USA), IL-6 (human IL-6 immunoassay, Bio-Rad Laboratories, Hercules, CA, USA; intra- and interassay coefficients of variation were 7.4% and 7.2%, respectively) and high-sensitivity C-reactive protein (CRPhs) (latex assay, Olympus; intra- and interassay coefficients of variation were 2.8% and 3.4%, respectively) measurement between 1400 and 1800 h.

Chronic hyperglycaemia was defined as a mean HbA_{1c} value > 7.5%. Chronic exposure to hyperglycaemia was calculated by collecting all available HbA_{1c} test results from the patients' medical records. The time course surveyed by the HbA_{1c} tests was calculated by multiplying the number of tests by 3 months – the periodicity of each test – and then dividing by the duration of the patient's diabetes (in months). To minimize the effect of incomplete data, patients with an HbA_{1c} survey of < 50% of the duration of their diabetes (n = 7) were excluded from the analysis of chronic exposure to hyperglycaemia.

2.3. Urine glucocorticoid metabolite measurement

Glucocorticoid metabolites were measured by liquid chromatography and mass spectrometry (ACQUITY UPLC System and TQD detector with electrospray ionization, Waters Ltd, Elstree, Hertfordshire, UK). Briefly, 6-alpha-methylprednisolone was used as the internal standard, and hydrolysis with β-glucuronidase was performed before dichloromethane extraction. Analyte concentration was related to creatinine concentration (analyte/cr). Total cortisol metabolite excretion was calculated as α and β tetrahydrocortisol (THF) + tetrahydrocortisone (THE) + cortols + cortolones. Whole-body equilibrium between cortisol and cortisone, as determined by the balance between tissue-specific activities of 11β-reductase and 11β-dehydrogenase activities, was inferred from the ratio of THF/THE. Renal 11β-dehydrogenase activity was inferred from the urinary cortisol/cortisone ratio.

Table 1
Type 1 diabetic children and siblings characteristics.

	Diabetic patients ($n=45$)	Sibling control subjects ($n=23$)	P
Age (years)	11.0 [5.0–17.0]	10.0 [6.0–17.0]	0.9
Sex ratio	1.25	0.91	0.6
BMI (Z score)	0.7 [−1.0–2.8]	0.4 [−1.2–2.8]	0.4
Waist circumference (cm)	68.0 [48.5–91.0]	65.0 [52.0–103.0]	0.7
Systolic blood pressure (mmHg)	104 [85–136]	104 [86–128]	0.9
Diastolic blood pressure (mmHg)	58 [43–72]	56 [47–72]	0.2
Last HbA_{1c} (%)	8.0 [6.5–10.5]	5.3 [4.4–5.8]	<0.001
Last glycaemia (mmol/l)	8.2 [0.9–27.5]	4.8 [3.7–6.6]	<0.001
Duration of diabetes (months)	72 [13–136]		
Mean HbA_{1c} since diabetes onset (%)[a]	7.9 [6.5–11.0]		
Number of HbA_{1c} >7.5% since diabetes onset[a]	9.5 [1–33]		
Number of HbA_{1c} <7.5% since diabetes onset[a]	4 [0–27]		
Insulin doses (U/kg/d)	0.9 [0.3–1.8]		

Values are expressed as medians [range]. BMI is expressed as Z score for sex and age. Comparisons were made using the Mann-Whitney test, with a P value <0.05 indicating a statistically significant difference.

[a] $n=36$. All available HbA_{1c} test results were collected from medical records since diabetes discovery. The time length surveyed by the HbA_{1c} tests was calculated by multiplying the number of tests by 3 months, the periodicity of each test, and dividing by duration of the individual's diabetes in months. To minimize the effect of incomplete data, participants with HbA_{1c} "coverage" less than 50% of their duration of diabetes ($n=9$) were excluded.

2.4. Statistical analysis

Continuous data are presented as medians [range]. BMI values are given as the Z score for gender and age, according to published data for the French population [9]. Comparisons were made using the Mann-Whitney test with a P value <0.05 indicating a statistically significant difference. Factors potentially associated with IL-6, CRPhs and THF/THE were studied using a bivariate linear-regression model, with IL-6, CRPhs and THF/THE as dependent variables. The multivariate analysis included adjustments for age, gender and BMI (with standard deviations for gender and age). Because of their non-normal distribution, CRPhs and IL-6 results were log-transformed prior to the association studies.

3. Results

3.1. Patients' characteristics

The study included 45 children with T1D and 23 siblings without the disease. Their clinical and biological characteristics are shown in Table 1. Children with T1D and their sibling controls were matched in terms of age, BMI, WC and blood pressure. Predictably, parameters associated with diabetes, such as the last HbA_{1c} and last glycaemia values, were higher in patients with T1D than in the siblings.

3.2. Urine glucocorticoid metabolites and low-grade inflammation markers

Comparisons between diabetic patients and controls for urine glucocorticoid metabolites and low-grade inflammation markers are shown in Table 2. Diabetic patients presented with an increased THF/THE ratio ($P<10^{-3}$) compared with the controls. There was also a trend towards higher THF/cr and lower THE/cr ratios in patients with diabetes, but no significant difference was evident between the groups for IL-6 and CRPhs concentrations.

3.3. Clinical factors associated with IL-6 and CRPhs

In diabetic patients, IL-6 concentrations ($\beta=0.34$, 95% CI: 0.04–0.64; $P=0.03$), but not CRPhs levels, were associated with age. When corrected for age, no association was found between either IL-6 or CRPhs and BMI, WC, blood pressure, last glycaemia and HbA_{1c} values, doses of insulin, diabetes duration or chronic exposure to hyperglycaemia.

In the controls, IL-6 and CRPhs levels were only associated with BMI ($\beta=0.61$, 95% CI: 0.14–1.05; $P=0.01$ and $\beta=0.68$, 95% CI: 0.30–1.06; $P=0.01$, respectively) when corrected for age.

3.4. Factors associated with urine glucocorticoid metabolites

In diabetic patients, the THF/THE ratio was associated with age (Table 3), and this association remained even after parameters were adjusted for duration of diabetes. When adjusted for age, gender and BMI, the THF/THE ratio was significantly associated with CRPhs levels in diabetic patients, but not with IL-6 levels (not significant; Table 3). After adjusting for age, no association was shown for the THF/THE ratio with BMI, WC, blood pressure, last glycaemia and HbA_{1c} values, doses of insulin, duration of diabetes or chronic exposure to hyperglycaemia (data not shown). Neither THF nor THE was associated with BMI, WC, blood pressure, last glycaemia and HbA_{1c} values, doses of insulin, diabetes duration, or chronic exposure to hyperglycaemia, IL-6 or CRPhs levels.

In controls, no association was found between the THF/THE ratio or THF or THE concentrations and age, BMI, WC, blood pressure, IL-6 or CRPhs levels.

Table 2
Urine glucocorticoids metabolites and low-grade inflammation markers.

	Diabetic patients ($n=45$)	Sibling control subjects ($n=23$)	P
Creatininuria (nmol/l)	9.5 [2.0–19.7]	12.0 [3.3–25.9]	0.10
F/cr (µg/nmol)	4,6 [1.0–30.9]	3.6 [2.6–13.3]	0.21
E/cr (µg/nmol)	6.5 [2.5–38.0]	5.6 [2.9–17.1]	0.15
THFs (5α + 5β THF)/cr (µg/nmol)	110 [39–396]	85 [45–188]	0.07
THE/cr (µg/nmol)	139 [55–441]	178 [67–508]	0.09
Total metab/cr (µg/nmol)	337 [164–1037]	344 [171–902]	0.97
F/E	0.68 [0.40–1.53]	0.72 [0.44–1.18]	0.68
5 α TFH/5β THF	0,12 [0,02–0,42]	0,12 [0,03–1,18]	0.07
THFs/THE	0.68 [0.45–1.18]	0.45 [0.27–0.98]	<0.0001
IL-6 (ng/ml)	0.6 [0.6–6.8]	0.6 [0.6–2.2]	0.43
CRP (mg/l)	0.4 [0–7.4]	0.3 [0–8.2]	0.26

Values are expressed as medians [range]. Comparisons were made using the Mann-Whitney test, with a P value < 0.05 indicating a statistically significant difference.

4. Discussion

The objective of our present study was to investigate whether low-grade inflammation and changes in cortisol or peripheral cortisol metabolism were present in children with T1D. It was found that the THF/THE ratio, suggesting nocturnal whole-body 11β-HSD1 activity, was increased in diabetic patients and that this increase was associated with CRPhs levels. de Lacerda et al. [10] attributed the diurnal variation in metabolic clearance rate of cortisol to variations of HSD activity. However, different studies have found that 11β-HSD1 activity undergoes minimal variations throughout the day [11,12]. On the other hand, the expression of 11β-HSD1 genes has been found to have a circadian rhythm in human adipose explants [13]. Our present study shows that diabetes has an impact on 11β-HSD1 activity.

IL-6 is a powerful inducer of the inflammatory hepatic acute-phase response and also stimulates hepatic production of CRPhs. IL-6 plasma concentration is doubled in young patients with a 12-year duration of diabetes, but no clinical evidence of micro- or macrovascular complications compared with controls [6]. In our present population of children with shorter durations of diabetes (median of about 6 years), there was no difference in CRPhs or IL-6 levels. This suggests that the concentrations of these parameters may not be the earliest to be modified in T1D in children. However, strong associations were observed in both markers with BMI in controls that were lost in the diabetic children. This suggests that other associated factors could be precociously influencing CRPhs and IL-6 concentrations in diabetic children, and hyperglycaemia could be one of those factors [14,15]. However, neither recent nor chronic metabolic control criteria were associated with CRPhs and IL-6 levels in our study. In fact, the only factor associated with CRPhs (and marginally with IL-6) was the nocturnal THF/THE ratio.

Table 3
Associations with THFs/THE ratio on multivariate analyses.

	Coefficient (95%CI)	P
Diabetic patients		
Age	0.57 (0.32 to 0.82)	<0.0001
Age[a]	0.51 (0.23 to 0.78)	<0.001
IL-6[b]	0.30 (–0.004 to 0.56)	0.053
CRP[b]	0.32 (0.12 to 0.58)	0.02
Controls		
Age	0.33 (–0.09 to 0.76)	0.11
IL-6[b]	0.49 (–0.14 to 1.1)	0.12
CRP[b]	0.30 (–0.31 to 0.90)	0.32

CI: confidence interval.
[a] Adjusted for duration of diabetes.
[b] Adjusted for age, gender and BMI.

Endogenous glucocorticoid activity depends upon glucocorticoid output from the adrenal gland under the control of the hypothalamic–pituitary–adrenal (HPA) axis. However, intracellular glucocorticoid concentrations can differ greatly from blood levels due to the action of the 11β-HSD1 enzyme, which converts inactive cortisone to active cortisol [16]. In the liver, cortisol and cortisone are metabolized by irreversible inactivation by A-ring reductases, leading to urinary excretion of cortisone and cortisol metabolites (THE and THF) [17]. Thus, whole-body 11β-HSD1 enzyme activity may be assessed by the THF/THE ratio. However, in healthy people, whole-body regeneration of cortisol through 11β-HSD1 activity is mainly provided by visceral adipose tissue and the liver [16]. 11β-HSD1 has been found in other tissues, including the kidneys [18], but the influence of these forms on whole-body regeneration of cortisol is unknown. As our diabetic patients and controls presented with identical BMI and WC, it may be hypothesized that the difference between them in terms of THF/THE ratio were more likely the result of variations in hepatic 11β-HSD1 activity. Also, it may be assumed that variations in the THF/THE ratio were not due to changes in renal 11β-HSD type 2 activity, which converts cortisol to cortisone, as the E/F ratio was identical between groups. Previously, 11β-HSD1 enzyme activity was found to be normal in 24-h urine collections analyzed in adults [19] and children [20] with T1D. Interestingly, an increased ratio was found when glucocorticoid metabolites were analyzed in nocturnal urine samples. The circadian rhythm of cortisol secretion leads to low plasma cortisol concentrations and therefore low cortisol metabolite production at night. This

basal condition allows the exclusion of stimuli such as food intake, exercise and stress, which can all greatly affect cortisol levels. For this reason, this nocturnal measure should help in detecting subtle changes in HPA-axis activity. Indeed, it was found that an increase in 11β-HSD1 activity was accompanied by a trend towards greater excretion of cortisol metabolites and less excretion of cortisone metabolites in diabetic patients. This result may be explained by nocturnal hypoglycaemia stimulating cortisol secretion. Although the absence of nocturnal hypoglycaemia was among our inclusion criteria, it was not possible to completely exclude the occurrence of unknown nocturnal hypoglycaemia.

Another explanation may stem from the stimulating effect of inflammation on 11β-HSD1 expression. It has been suggested that proinflammatory mediators such as IL-1β and tumour necrosis factor (TNF)-α increase 11β-HSD1 expression, thereby promoting local glucocorticoid availability and, thus, local anti-inflammatory action [7]. In our present study, the THF/THE ratio was associated with serum CRPhs concentrations in the diabetic children, but not in the controls, suggesting that a proinflammatory state could stimulate 11β-HSD1 expression in diabetes. However, the association was weak with IL-6 concentrations, and plasma CRPhs concentrations were not increased in the T1D children. This apparent discrepancy may result from the fact that CRPhs is mainly produced by hepatocytes whereas IL-6 is provided by T cells and macrophages. For this reason, it may be speculated that an hepatic proinflammatory state might stimulate hepatic 11β-HSD1 expression, thereby explaining the increase in THF/THE ratio. The latter would also not lead to elevated plasma CRPhs concentrations because of the increased intracellular production of cortisol by the liver.

As 11β-HSD1 gene transcription is increased by glucose concentration in hepatocytes [21], elevated 11β-HSD1 activity may be explained by an enhanced hepatic glucose output due to a global lack of insulin. However, as no association was found between THF/THE ratio and recent or chronic metabolic control criteria reflecting such a lack of insulin, this hypothesis is not considered likely. Alternatively, with a conventional insulin regimen, insulin is not delivered into the portal circulation, but through a non-physiological subcutaneous route, leading to a relative hepatic lack of insulin [22]. This could contribute to enhanced hepatic 11β-HSD1 activity either directly, as 11β-HSD1 gene transcription is decreased by insulin [23], or indirectly, by promoting an hepatic proinflammatory state [24]. Thus, the increase in THF/THE ratio in nocturnal urine could be explained by the relative hepatic lack of insulin at night due to subcutaneous insulin therapy.

In conclusion, low-grade inflammation, as assessed by plasma CRPhs and IL-6 concentrations, was not detectable in our cohort of children with T1D. However, nocturnal 11β-HSD1 activity was increased in diabetic patients and associated with plasma CRPhs concentrations. These results may be explained by either a direct or an inflammation-mediated effect of the relative insulin deficiency due to conventional insulin therapy. Thus, 11β-HSD1 activity may be an early marker of an inflammatory state associated with T1D.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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C. PUBLICATION N° 3 : Le rébaudioside A, extrait du Stévia, module -t-il l'activité de la 11β-HSD2 ?

Rebaudioside A and cortisol metabolism:

sweet news for consumers

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Abstract

Background

Stevia extracts, a traditional sweetener was reported to cause of reversible oedema, hypokalemia and elevated blood pressure presumably through modification of the activity of 11β-hydroxysteroid-dehydrogenase type 2 (HSD2), an enzyme involved in the cortisol/cortisone reversible shuttle.

Aims

We investigated the effects, on cortisol metabolites, of an acute ingestion of rebaudioside A an active compound of stevia extracts. Indeed, considering the prevalence of diabetes as well as usage of sweeteners, such an enzyme inhibition by rebaudioside would have dire consequences.

Results

No modification of HSD2 activity was seen in healthy subjects after ingestion of 12 pellets of Canderel "green" in a day. No effect on HSD1 activity, the enzyme reversing the action of HSD2 was recorded either.

Conclusion

In terms of cortisol metabolism, it appears safe to use rebaudioside. Most importantly, as rebaudioside does not affect HSD2/HSD1 cortisol metabolism, it should not promote metabolic syndrome via abdominal fat and local cortisol regeneration.

Key words

Food sweetener, rebaudioside, 11β-hydroxysteroid-dehydrogenase

Introduction

Extracts of Stevia Rebaudiana Bertoni are traditional food sweeteners in South America. Stevioside and other sweet-tasting compounds such as steviobioside and rebaudiosides were then isolated from Stevia leaves. Subsequently, rebaudioside A has been authorised in many countries as a sweetener [1].

Reversible oedema, hypokalemia and elevated blood pressure were reported in a patient that ingested large amounts of Stevia extracts [2]. This was synchronous to a modification of the cortisol/cortisone ratio in urine. Stevia extract was presumed guilty through modification of the activity of 11β-hydroxysteroid-dehydrogenase type 2 (HSD2), the enzyme implicated with HSD1 in the inactivation/reactivation shuttle of cortisol in various tissues including abdominal fat. Considering the worldwide prevalence of diabetes and the great number of diabetics using sweeteners, such an enzyme inhibition by rebaudioside would have dire consequences. We thus investigated the specific effects of rebaudioside on the excretion of metabolites cortisol.

Subjects and methods

Healthy subjects (n=23) ingested 4 pellets/meal of a commercially available sweetener containing rebaudioside A (Canderel "green", total 12 pellets in a day). Nocturnal urine was collected the mornings before and after ingestion of the sweetener. The subjects did not ingest any sweetener at least a month before the study. No adverse events were reported. The metabolic consequences of this sweetener ingestion on HSD2 and HSD1 activities was investigated through the excretions of cortisol, cortisone, tetrahydrocortisol and allo-tetrahydrocortisol (THFs), and tetrahydrocortisone (THE) using cortisol/cortisone and THFs/THE ratios, respectively [3, 4]. Intraassay variabilities were <8%. Age and BMI of the subjects (10M/14F) were 34 [23;50] yr and 22 [17;27] kg/m² (median [range]).

Results

Cortisol/cortisone and THFs/THE basal ratios were: 0.60 [0.33;1.26] and 0.62 [0.49;0.99] (Figure 1). Inter-individual coefficients of variation of these ratios were 32 and 21%, respectively. We found no significant modification of the ratios after rebaudioside ingestion (paired t-test, Figure 1). Intraindividual variations of the ratios were: 8 [3;49] and 9 [0;26]%.

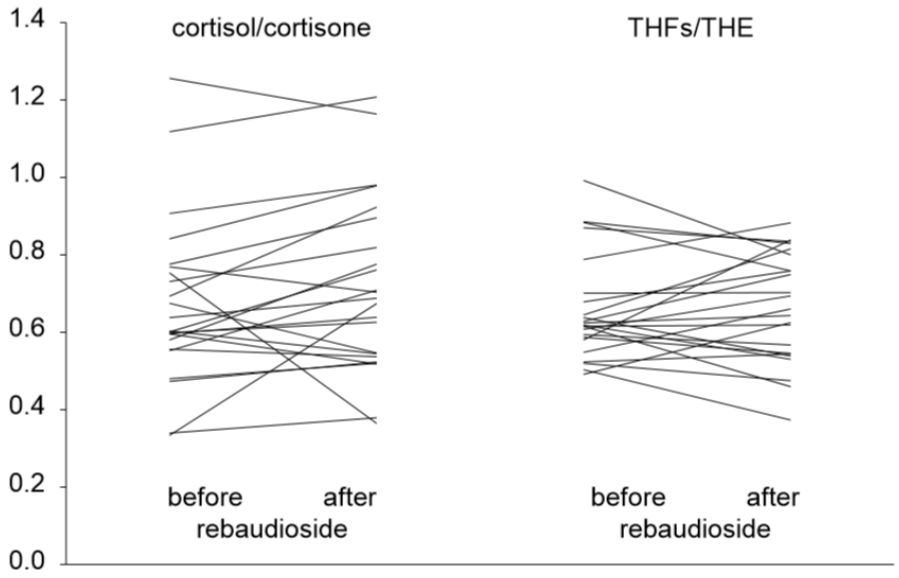

Figure 1:
Individual cortisol/cortisone and (tetrahydrocortisol + allo-tetrahydrocortisol)/tetrahydrocortisone ratios reflecting the activities of 11β-hydroxysteroid dehydrogenase type 2 & 1, respectively before and after rebaudioside A ingestion.

Discussion

This absence of significant effect of rebaudioside on HSD1 or HSD2 activities is reassuring although it is not definite proof of the absence of consequences for a long-term intake. However, prior publications about rebaudioside reported no modification of blood pressure or ion urinary excretion [5]. Our study has limits: the absence of diabetics in this population. Hypothetically, enzymes activities may be modified by diabetes and superimposed rebaudioside could act

differently in such a context.

Finally, what could explain the results of Esmail *et al*. [2]? Stevia extracts powder is a mixture of molecules that can be extremely variable according for instance of the origin of the plant, time of harvest, no error of the plant genus... Important consumption of such an uncharacterized mixture could have caused side effects. Cortisol metabolite ratios have noticeable inter-individual variations suggesting variable enzyme activity. Combinations of uncharacterized stevia extracts and uncommon enzyme activity could have resulted in unusual side effects. In this respect, it appears safer to use well-defined sweeteners. Most importantly, as rebaudioside appears neutral in terms of HSD2 and HSD1 cortisol metabolism, rebaudioside should not promote metabolic syndrome *via* abdominal fat and local cortisol regeneration [6].

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The authors wish to thank our colleagues that, sometimes against their preferences, sugared tea, coffee, milk …

Conflict of interest

No potential conflicts of interest relevant to this article were reported.

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Authors' contribution

JBC researched data and wrote, reviewed, and edited the manuscript. JB performed the assays, and reviewed and edited the manuscript.

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III. Conclusion

La première partie de ce travail de thèse a permis de mettre au point le dosage du cortisol urinaire et de ses métabolites par chromatographie liquide couplée à la spectrométrie de masse. Il s'agit maintenant d'une technique cotée au Référentiel Hors Nomenclature des actes de Biologie Médicale (BHN500). La validation de cette technique a été faite sur une population présentant une sécrétion normale, élevée ou sub-normale de cortisol. Au vu de ces résultats, nous confirmons la possibilité de discriminer à la fois d'importantes ou de faibles différences de sécrétion et de métabolisation du cortisol.

Grâce à cette technique, nous avons pu évaluer les activités enzymatiques de la 11β-HSD1 et de la 11β-HSD2 lors d'une situation de perturbation supra-physiologique de l'axe HPA : le diabète de type I. En effet, plusieurs équipes ont montré que les patients atteints de diabète de type I ou de type II présentent une augmentation basale de l'activité de l'axe HPA. Cette hyperactivité pourrait être en rapport avec une insuffisance de l'effet répresseur des GC sur l'axe HPA après la survenue d'hypoglycémie (Chan et al. 2005). Chez le sujet diabétique de type II, la dérégulation de l'insulinémie, la glycémie et l'inflammation à bas bruit sont responsables de l'augmentation de l'activité de la 11β-HSD1. Nous montrons ici que les mêmes anomalies présentent chez le patient diabétique de type I sont responsables d'une augmentation de l'activité nocturne de la 11β-HSD1. Si nous ne trouvons pas d'inflammation à bas bruit chez les enfants diabétiques par les mesures de la CRP et d'IL6, nous observons néanmoins une corrélation entre l'activité de cette enzyme et la CRP chez les enfants atteints de diabète de type I contrairement aux enfants témoins.

Enfin le dosage du cortisol et de ses métabolites a permis d'évaluer les effets du rébaudioside A sur l'activité de la 11β-HSD2. Contrairement au cas rapporté dans la littérature, nous ne retrouvons pas l'augmentation de la biodisponibilité pré-récepteur des GC par inhibition de l'activité de la 11β-HSD2 par le rébaudioside A dans nos conditions expérimentales (sujets sains, prise ponctuelle du rébaudioside). L'effet rapporté par Esmail et Kabadi était probablement celui d'un composé non identifié présent dans l'extrait de stévia (Esmail and Kabadi 2012).

Ces premiers résultats présentent nos principaux travaux sur la régulation du métabolisme des GC après leur sécrétion et en amont de leur fixation à leurs récepteurs. Le chapitre suivant présentera à travers l'exemple de la vitamine A l'importance de cette régulation par un facteur exogène d'origine alimentaire en aval de la fixation des GC sur leurs récepteurs.

Chapitre II

Modulation post-récepteur de l'exposition aux glucocorticoïdes : rôle de la vitamine A

I. Vitamine A : données bibliographiques

Le terme « vitamine A » regroupe un ensemble de composés dont l'activité chez l'Homme est similaire. Le métabolite actif physiologique est l'acide rétinoïque *all-trans* (atAR). La forme circulante et stockée dans l'organisme est le rétinol.

A. Sources de vitamine A et absorption

Le rétinol est une vitamine liposoluble essentielle à la formation et à l'homéostasie d'un grand nombre de tissus dans l'organisme. Il provient chez les mammifères exclusivement de l'alimentation car il ne peut être synthétisé *de novo* par l'organisme. Les produits d'origine animale comme le foie ou les œufs constituent la principale source de rétinol. Les aliments d'origine végétale contiennent de la pro-vitamine A ou beta-carotène, précurseur du rétinol (Blomhoff and Blomhoff 2006). Néanmoins, seule une petite partie sera transformé en rétinol (12 µg de beta-carotène est converti en 1µg de rétinol) (Theodosiou et al. 2010). Dans les tissus animaux, l'apport de rétinol se fait sous la forme de carotènes ou de retinyl ester (Figure 8). Ces derniers nécessitent d'être hydrolysés en rétinol dans la lumière intestinale par la *retinyl ester hydrolase* (REH) (van Bennekum et al. 2000). Au niveau des entérocytes de l'intestin, le rétinol est pris en charge par la *cellular retinol binding protein II* (CRBP-II) (Zhang et al. 2002). Les carotènes sont absorbés grâce à la *scavenger receptor class B, type I* (SR-BI) (van Bennekum et al. 2005) puis sont également complexés à la CRBP-II pour enfin être réduits en rétinol par la *retinal reductase*. Ainsi complexé le rétinol peut être estérifié par la *lecithin:retinol acetyltransferase* (LRAT) en rétinyl ester. Il est alors excrété au niveau de la lymphe et pris en charge par les chylomicrons. En passant dans la circulation générale, la vitamine A des chylomicrons est transportée jusqu'aux tissus cibles - pour 30% environ de la totalité absorbée - ou jusqu'au foie pour être stockée (D'Ambrosio et al. 2011).

B. Métabolisme hépatique

Dans les hépatocytes, les rétinyl esters sont hydrolysés en rétinol pour être ensuite transférés dans les cellules stellaires hépatiques où ils seront stockés sous forme estérifiée (Figure 8)(Blomhoff and Blomhoff 2006). Lors de besoin en rétinol, celui-ci est retransféré au niveau de l'hépatocyte. Il est alors lié à la *retinol-binding protein* (RBP) (Quadro et al. 1999) pour être libéré dans la circulation générale sous forme d'un complexe ternaire retinol-RBP-transthyrétine (transthyrétine ou TTR

protéine de transport également des hormones thyroïdiennes) selon un ratio de 1:1. Plus de 95% du rétinol circule sous forme complexée, le reste correspond à des retinyl esters associés à des lipoprotéines (*very low density lipoprotein* (VLDL), *low density lipoprotein* (LDL) et *high density lipoprotein* HDL) ou de l'atAR fixés à l'albumine (D'Ambrosio et al. 2011).

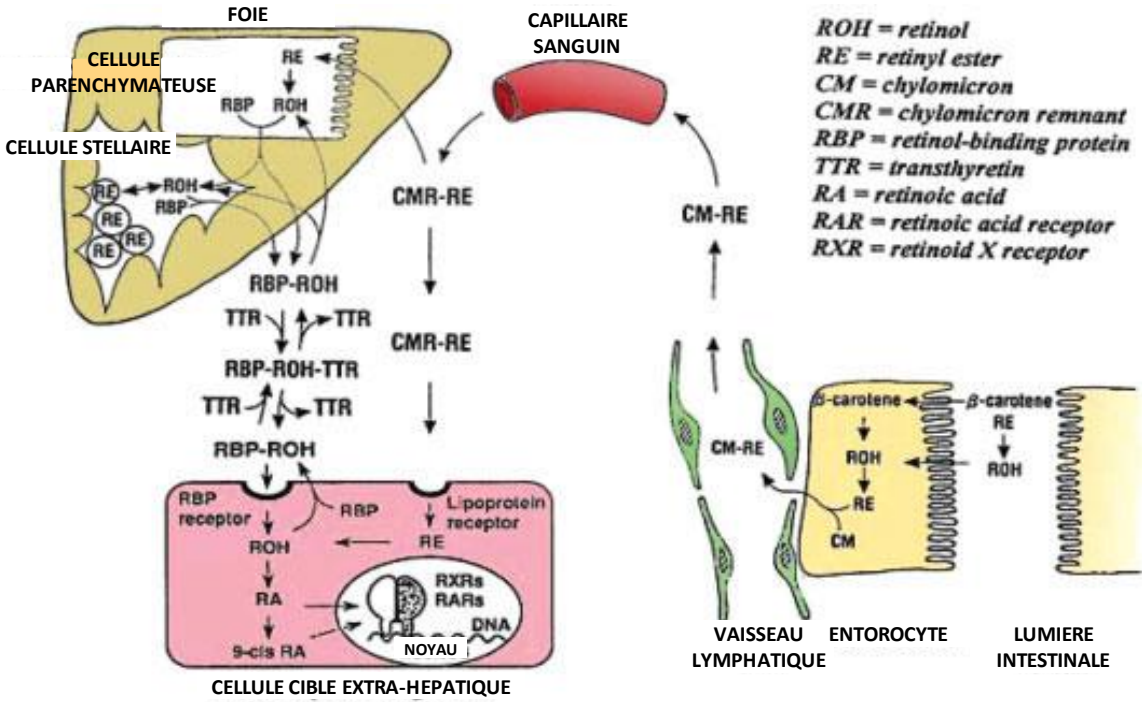

Figure 8 : Transport et Métabolisme hépatique du rétinol. Adapté de Blomhoff and Blomhoff 2006.

C. Métabolisme cellulaire

Bien qu'il existe des preuves du libre passage de l'atAR au travers de la membrane cytoplasmique cellulaire, l'essentiel du rétinol est transporté dans la cellule grâce à un transporteur de la RBP, STRA6 pour *stimulated by retinoic acid gene 6* (Kawaguchi et al. 2007) (Figure 9). Ce récepteur membranaire lie spécifiquement la RBP et participe à l'incorporation du rétinol dans le cytoplasme.

Au niveau cytosolique, le rétinol est pris en charge par les protéines porteuses cellulaires CRBP-I et CRBP-II. Il peut également servir de substrats à différentes enzymes cytosoliques ou microsomales appelées *retinol deshydrogenase* (RDH) qui oxydent le *all-trans* rétinol en all-*trans* rétinaldehyde (ou rétinal). Enfin une oxydation irréversible transforme l'*all-trans* rétinal en atAR sous l'action de la *retinaldehyde dehydrogenase 2* (RALDH2) (Duester et al. 2003).

L'atAR se lie à des RBPs cytosoliques, les *cellular retinoic acid-binding proteins* (CRABP-I et CRABP-II). Impliquées dans le transport de l'atAR vers le noyau, CRABP-I et CRABP-II modulent

la capacité de l'atAR à se fixer à ses récepteurs spécifiques (Dong et al. 1999).

La dégradation des dérivés rétinoïques en 4-hydroxy AR et 4-oxo AR est assurée par le cytochrome CYP26A1 (Thatcher and Isoherranen 2009).

Des isomères, bien que représentant des formes mineures, peuvent être retrouvés dans la cellule. Il s'agit du 13-*cis* AR, 11-*cis* rétinal et du 9-*cis* AR. Le 11-*cis* rétinal ne se retrouve qu'au niveau des cellules rétiniennes ; il est spécifiquement impliqué dans la photo-transduction (Parker and Crouch 2010). Enfin, la pertinence physiologique du 9-*cis* AR reste encore à prouver et son activité naturelle est controversée. C'est un agoniste des RXR (*retinoic X receptors*, voir paragraphe suivant) mais avec une concentration inférieure au pico-molaire, il n'a pu être détecté que dans le pancréas (Kane 2012). L'équilibre entre les isomères pourrait être sous le contrôle de protéines chaperonnes d'isomérisation comme la *glutathione S-transferase*.

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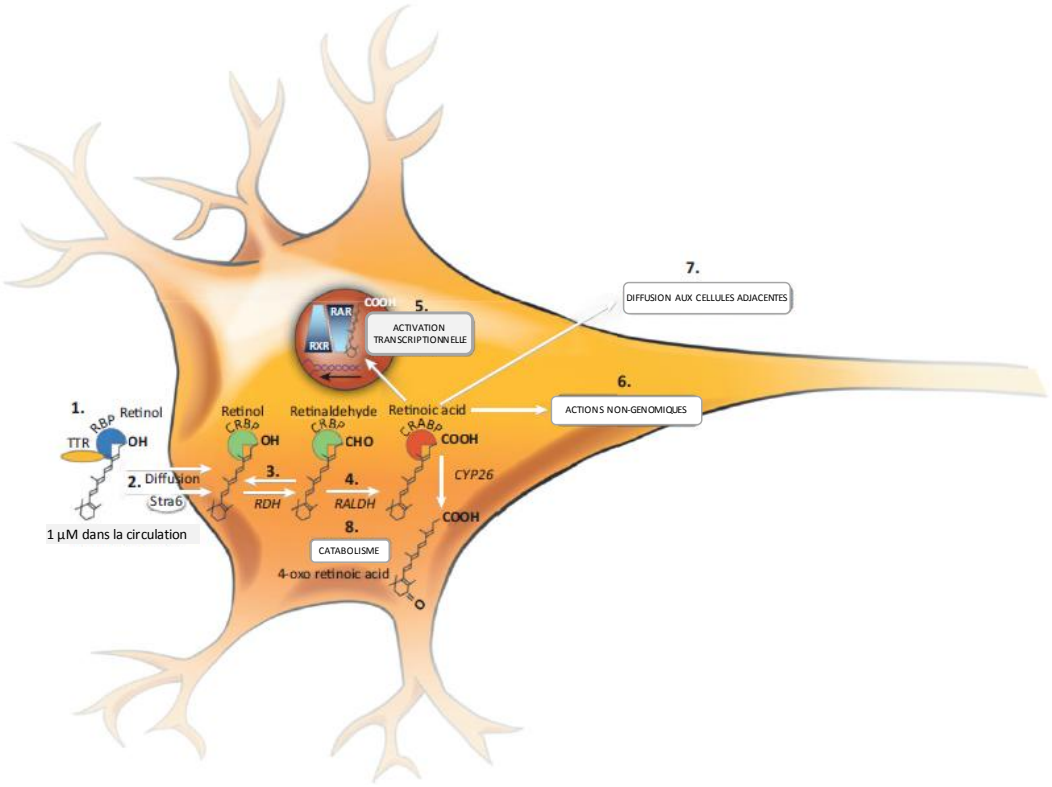

Figure 9 : Métabolisme cellulaire du rétinol : exemple de la cellule neuronale.
Adapté de Shearer et al. 2012.

TTR : transthyrétine, RBP : *retinol-binding protein*, Stra6 : *stimulated by retinoic acid gene 6*, CRBP : *cellular retinol binding protein*, RDH : *retinol deshydrogenase*, RALDH : *retinaldehyde dehydrogenase*, CRABP : *cellular retinoic acid-binding proteins*, CYP26A1 : cytochrome p61A1, RAR : *retinoic acid receptor*, RXR : *retinoic X receptor.*

D. Mécanisme d'action

a) Voie génomique

Les effets physiologiques de l'atAR sont médiés par leur liaison aux *retinoic acid receptors* (RARs). Ils ont la capacité de s'hétérodimériser avec un autre type de récepteur nucléaire rétinoïde, les RXRs (RXR α, β ou γ) leur conférant une fonction de régulateur de la transcription ligand-dépendante (Figure 10) (Rochette-Egly and Germain 2009; Theodosiou et al. 2010).
Les RARs et les RXRs appartiennent à l'importante famille des récepteurs nucléaires pour certaines hormones (récepteur aux stéroïdes, récepteur aux hormones thyroïdiennes), vitamine (récepteur de la vitamine D) ou lipides (récepteur aux PPARs (*peroxisome proliferator–activated receptors*)), etc. Les RARs sont divisés en 3 sous-types : RAR α (NR1B1), β (NR1B2) et γ (NR1B3). Chaque sous-type des RARs est codé par un gène propre et se subdivise au moins en deux isoformes générées par un usage différentiel du promoteur et un épissage alternatif ne différant ainsi que par leur partie N-terminale (Germain et al. 2006).

Les RARs sont organisés selon des domaines bien définis avec une structure modulaire composée de six régions désignées par A pour la partie N-terminale jusqu'à F pour la partie C-terminale (Kumar and Thompson 1999).

- La région C correspond à une partie centrale liant l'ADN, le *DNA-binding domain* (DBD). Composée de deux modules en doigt de zinc, d'une double hélice α et d'une extension terminale COOH, elle confère au récepteur une capacité de liaison à un domaine spécifique de l'ADN, l'élément de réponse à l'atAR (RARE). Cette séquence est localisée au niveau du promoteur de gènes cibles. Classiquement, elle se compose de deux motifs directs répétés ou palindromiques espacés par 5 paires de bases ainsi désigné comme *direct repeat* DR5 (Lee et al. 1993). Mais l'hétérodimère RAR/RXR peut aussi se fixer sur les séquences répétées espacées par seulement une (DR1) ou deux (DR2) paires de bases (Balmer and Blomhoff 2005). Sur les éléments DR2 et DR5, RXR occupe la partie 5' du motif et le RAR la partie 3' (5'-RXR-RAR-3'). Sur les éléments DR1, la polarité est inversée (5'-RAR-RXR-3'). Cette différence d'orientation basculerait l'activité coactivateur du RAR à une activité corépresseur en présence du son ligand (Predki et al. 1994).
- La région E correspond à un domaine C-terminal liant le ligand, le *ligand-binding domain* (LBD) (Moras and Gronemeyer 1998). Il s'agit, avec la région précédemment décrite, de régions très conservées. Le domaine LBD est formé de douze hélices α et d'une boucle β. Il contient la poche de fixation du ligand, *ligand-binding pocket* (LBP), un domaine de dimérisation et un

domaine d'activation ligand-dépendante (AF-2). La LBP est une zone hydrophobe permettant la fixation des ligands physiologiques mais également des agonistes ou antagonistes. Elle est directement en lien avec les deux autres zones car elle permet de manière ligand-dépendante de stabiliser l'interaction entre les hétérodimères RAR-RXR et de modifier la structure du récepteur lui permettant le recrutement de coactivateur. Ainsi RAR-RXR joue le rôle de répresseur transcriptionnel en l'absence de ligand par le recrutement de corépresseurs et d'activateur transcriptionnel en présence du ligand par recrutement de coactivateurs.

- Un autre domaine d'activation ligand-dépendante (AF-1) est présent au niveau N-terminal (correspondant à la région A et B). Il est également impliqué dans le contrôle de la transcription mais sans l'intervention de corépresseur ou coactivateur. Le mécanisme, non totalement élucidé, ferait intervenir des modifications de stabilité de liaison à l'ADN (Nagpal et al. 1993).
- Enfin la région D est un domaine charnière entre DBD et LBD permettant une rotation des deux domaines et donc un changement de conformation impliqué dans le mécanisme d'activation du récepteur.
- Le rôle de la région F est encore mal connu (Rochette-Egly and Germain 2009).

Les RARs sont aujourd'hui décrits comme des récepteurs nucléaires dont l'activité transcriptionnelle est très fortement régulée par un ensemble de corépresseurs et coactivateurs (Al Tanoury et al. 2013). Les premiers incluent le corépresseur nucléaire NCoR/NCoR1/RIP13 et le *silencing mediator for retinoid and thyroid hormone receptor* (SMRT/NCoR2/TRAC). Les seconds incluent la famille p160 de coactivateurs des récepteurs aux stéroïdes *steroid receptor coactivators* (SRC-1/NCoA-1, SRC-2/TIF-2/GRIP-1 et SRC-3/ACTR/AIB1/TRAM1/RAC3)

En l'absence de ligand, NCoR ou SMRT recrutent des complexes protéiques à activité histone déacétylase (HDACs) maintenant la chromatine sous sa forme condensée (Al Tanoury et al. 2013). Inversement, en présence du ligand, les coactivateurs p160 viennent remplacer les corépresseurs et recrutent des complexes décompactant la chromatine (les histones acétyltransferases, HATs comme *CREB binding protein* CBP/p300 et *p300/CP-associated factor* p/CAF) pour rendre possible la transcription du gène. Il est à noter que les ligands spécifiques du RXR ne permettent pas à eux seul le recrutement des coactivateurs. L'action concomitante d'agonistes RAR et RXR permet une synergie de dissociation des corépresseurs du dimère et une coopération pour la fixation des coactivateurs (Lefebvre et al. 2005).

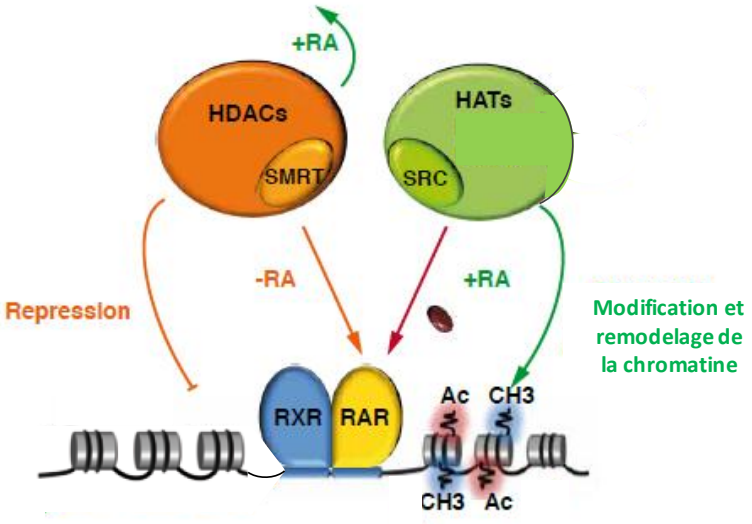

Figure 10 : Voie d'activation génomique de l'activité transcriptionnelle du récepteur à l'acide rétinoïque RAR. Adapté de Al Tanoury et al. 2013.

HATs : histone acétyltransferase ; SRC : *steroid receptor coactivators* ; HDACs: histone désacétyltransférase ; SMRT : *silencing mediator for retinoid and thyroid hormone receptor* ; RAR : retinoic acid receptor ; RXR : retinoid X receptor.

D'autres corégulateurs, dits non-conventionnels, sont également décrits comme participant à la modulation de la transcription par RAR (par exemple le *receptor interacting protein of 140 kDa* (RIP140/NRIP1) (Hu et al. 2004), le *preferentially expressed antigen in melanoma* (PRAME) (Epping et al. 2005) et le *transcription intermediary factor-1α* (TIF1α/Trim24)) (Le Douarin et al. 1995). Ils jouent essentiellement un rôle répressif malgré la présence du ligand.

b) Voie non génomique

De récentes publications développent le concept d'effets non génomiques de l'atAR (Figure 11) (Al Tanoury et al. 2013). Ces effets sont alors indépendants des RARs mais résultent de l'activation par l'atAR de kinases : *p38 mitogen-activated protein kinase* (p38MAPK), p42/44MAPKs, *cyclin-dependent kinase 7 et 5* (CDK7 et CDK5) ou *calmodulin-dependent kinase via* l'activation de la *phosphoinositide 3-kinase* (PI3K) ou de la *protein kinase C delta type* (PKCδ) (Bastien et al. 2006; Chen and Napoli 2008). Cette activation a des conséquences sur d'autres voies d'activation comme celle de la *mitogen and stress-activated kinase*, MSK1 pour provoquer *in fine* des effets transcriptionnels (Bruck et al. 2009).

Surtout, une interaction entre ces effets non-génomiques kinases-dépendantes et la voie génomique

classique des RARs est montrée. En effet, les RARs possèdent plusieurs sites de phosphorylation (deux sur des résidus sérine du domaine LBD et une sur une sérine du domaine NTD). Sans que le mécanisme soit parfaitement élucidé, il apparaît que ces phosphorylations participent à l'état d'activation des RARs notamment par le recrutement des coactivateurs(Rochette-Egly 2005). Or ces phosphorylations sont les conséquences de voies d'activation directement ou indirectement activées par l'atAR comme MSK1 et CDK7. A ceci s'ajoute la phosphorylation par ces mêmes kinases de coactivateurs tels que p160 SRC-3 ainsi que celle des histones.

Enfin, il semble maintenant évident que les RARs peuvent exercer une action de transrépression. L'exemple le mieux décrit est celui de l'inhibition du facteur de transcription AP-1 de manière ligand-indépendant par le RARβ. Plusieurs hypothèses sont avancées pour expliciter cet effet comme la compétition pour des coactivateurs communs tels que CBP (Lefebvre et al. 2005).

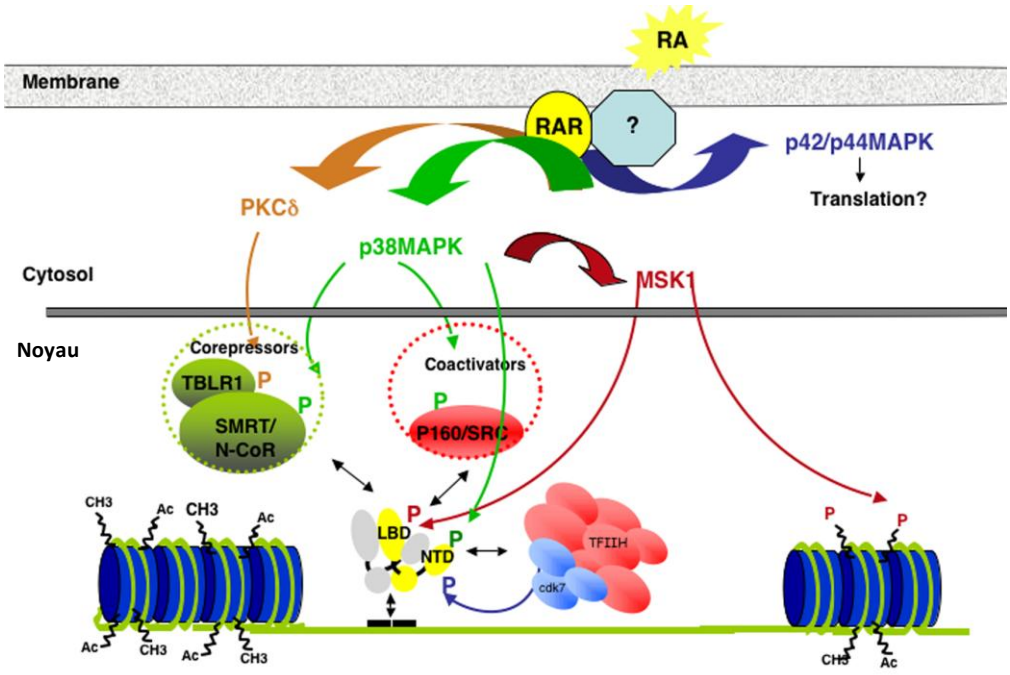

Figure 11 : Effets non génomiques de l'acide rétinoïque. Adapté de Rochette-Egly and Germain 2009.

RA*: retinoic acid*; p160/SRC : *steroid receptor coactivators* ; HDACs: histone désacétyltransférase ; SMRT : *silencing mediator for retinoid and thyroid hormone receptor* ; N-CoR : *Nuclear co-repressor* ; RAR : retinoic acid receptor ; RXR : retinoid X receptor ; p38MAPK *p38 mitogen-activated protein kinase*, p42/44MAPKs : *p42/44 mitogen-activated protein kinase*, CDK7 : *cyclin-dependent kinase* 7 ; PKCδ : *Protein kinase C delta type* ; MSK1 : *mitogen and stress-activated kinase* ; TFIIH : *Transcription factor II Human*.

c) Autres ligands des RARs

Les RARs sont capables de lier l'atAR et le 9-*cis* avec une affinité importante similaire mais des cinétiques de liaison différentes pour chaque RAR. Cependant, les RARs sont aussi capables d'être activés par certains dérivés rétinoïques physiologiques comme le all-*trans*-4-oxo AR, le all-*trans*-4-

oxo retinaldehyde, le all-*trans*-4-oxo rétinol et le all-*trans*-3,4-didehydro AR. Plusieurs études montrent leur liaison *in vivo* aux RARs sans que les conséquences physiologiques ne soient encore bien comprises (Achkar et al. 1996).

A côté de ces ligands physiologiques, un grand nombre de ligands synthétiques, agonistes ou antagonistes, a été développé. Le plus connu et le plus utilisé des agonistes pan-RAR (c'est-à-dire capable d'activer les trois types de RARs) est le (E)-4[2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphtalenyl)-1-propenyl]benzoic acid ou TTNPB. Il lie les RARs avec une affinité plus faible que l'atRA mais sa faible affinité pour les CRABPs et son faible catabolisme cellulaire expliquent ses effets similaires voire supérieurs à celui du ligand naturel (Pignatello et al. 1997). Ensuite, il existe des ligands plus ou moins spécifiques d'un type de RAR, c'est-à-dire ayant une affinité au moins cent fois supérieure pour l'un des récepteurs (par exemple le Am580). Les antagonistes empêchent l'action du ligand sur son récepteur et donc le recrutement des coactivateurs. Il peut s'agir de pan-antagonistes ou d'antagonistes sélectifs d'un type de RAR. Les agonistes inverses quant à eux favorisent l'interaction des corépresseurs (Rochette-Egly and Germain 2009).

d) Autres récepteurs à l'acide rétinoïque

L'atAR pourrait également lier et activer d'autres récepteurs comme les récepteurs orphelins PPARβ/δ et RORβ et le *chicken ovalbumin upstream promoter-transcription factor* (COUP-TFI et II). Ces phénomènes, sous réserve d'être confirmés, ouvrent des voies nouvelles concernant la compréhension des mécanismes d'action et des effets de l'atAR *in vivo* (Al Tanoury et al. 2013).

E. Rôles physiologiques de la vitamine A dans l'organisme

Le contrôle de très nombreux gènes par la vitamine A et l'atAR explique les actions multiples de ce métabolite dans l'organisme. Une preuve indirecte est apportée par les effets d'une carence en vitamine A. Celle-ci touche encore aujourd'hui 200 millions de personnes dans le monde et se traduit par des lésions cutanées, une xérophtalmie (ramollissement de la cornée pouvant conduire à la cécité) accompagnée d'héméralopie, un ralentissement de la croissance chez l'enfant ainsi que des troubles du système immunitaire et reproductif. Outre ses effets sur l'embryogenèse, la morphogenèse, la différenciation tissulaire (Maden 2000), la vitamine A et l'atAR sont largement étudiés pour leur implication dans la vision notamment crépusculaire, l'activité cardiaque (D'Ambrosio et al. 2011), les fonctions hématopoïétiques, la trophicité de la peau et des muqueuses et enfin dans les cancers pour leurs effets suppresseurs de tumeurs (Duong and Rochette-Egly 2011; Tang and Gudas 2011).

Depuis quelques années, le rôle capital de l'atAR dans les fonctions cérébrales a été mis en évidence. Il était déjà bien connu que l'atAR est essentiel dans le développement du système nerveux central mais aucune étude n'avait été menée chez l'adulte. Or l'atAR est synthétisé au niveau du cerveau adulte et les RARs y sont exprimés (Fragoso et al. 2012). Les premières études ont montrées qu'une carence en rétinoïdes était impliquée dans le ralentissement des processus d'apprentissage (Cocco et al. 2002). D'autre part, il a été montré que le métabolisme de la vitamine A est perturbé au cours du vieillissement et que les effets cognitifs lié à cette relative carence sont réversibles avec une supplémentation en atAR (Enderlin et al. 1997; Etchamendy et al. 2001; Feart et al. 2005; Mingaud et al. 2008). Plusieurs études *in vitro* ont montré que l'atAR intervient dans différentes étapes de la neurogenèse (Wohl and Weiss 1998; Takahashi et al. 1999; Jacobs et al. 2006), dans la différentiation neuronale et la formation et la croissance des dendrites, (Corcoran and Maden 1999), dans la plasticité neuronale (Fragoso et al. 2012). Cette différentiation s'accompagne de l'expression atAR-dépendante de certaines protéines comme la *neurogranin* (RC3) et la *neuromodulin* (GAP43), classiquement décrites comme étant le reflet d'une certaine plasticité cérébrale. L'expression du récepteur au BDNF et la sécrétion du BDNF sont également sous la dépendance de l'atAR (Katsuki et al. 2009; Kurauchi et al. 2011). Enfin les processus de génération de potentialisation à long terme PLT ou dépression à long terme DLT des neurones sont également sous la dépendance de l'action des rétinoïques (Misner et al. 2001).

Les récepteurs RARα et RARβ semblent plus particulièrement impliqués dans le mécanisme moléculaire de ces effets. Deux équipes ont également montré que l'effet de l'atAR sur la plasticité neuronale emprunte une voie non génomique de RARα (Chen and Napoli 2008; Maghsoodi et al. 2008; Chen et al. 2012).

A côté de ces effets mnésiques et cognitifs, l'atAR joue également un rôle dans le système dopaminergique. Les animaux mutants pour les gènes de RARβ-RXRβ ou RARβ-RXRγ ou RXRβ-RXRγ présentent des déficits moteurs importants avec une expression des récepteurs dopaminergiques D1 et D2 diminués de 40% (Krezel et al. 1998).

Enfin, l'atAR est impliqué dans la maladie d'Alzheimer (Goodman and Pardee 2003; Lane and Bailey 2005) en particulier dans la formation des plaques amyloïdes caractéristiques de cette pathologie (Sahin et al. 2005; Ding et al. 2008)

II. Rôle de la vitamine A dans le fonctionnement des glandes endocrines

Pour inscrire notre travail dans un cadre plus général, nous nous sommes intéressés à l'implication de la vitamine A dans la physiopathologie des systèmes endocriniens. Excluant les effets embryologiques des rétinoïques de notre propos, nous avons réalisé une revue de la littérature sur les effets de la vitamine A sur les tissus endocriniens. Les axes thyréotropes et corticotropes semblent les plus affectés par la carence ou l'apport de ce métabolite (PUBLICATION N°4).

A. PUBLICATION N°4 Interaction de la vitamine A dans la physiopathologie des tissus endocriniens et des hormones

Vitamin A, endocrine tissues and hormones: interplay & interactions

J Brossaud, V Pallet, J-B Corcuff

Soumis à Journal of Endocrinology

Vitamin A, endocrine tissues and hormones: interplay & interactions

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Running title: Vitamin A and hormones

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Abstract

Vitamin A (retinol) is a micronutrient critical for cell proliferation and differentiation**.** In adults, vitamin A and metabolites such as retinoic acid (RA) play major roles in vision, immune and brain functions, and tissue remodelling and metabolism. This review presents the physiological interactions of retinoids and endocrine tissues and hormonal systems.

Two endocrine systems have been particularly studied. In the pituitary, retinoids targets the corticotrophs with a possible therapeutic use in corticotropinomas. In the thyroid, retinoids interfere with iodine metabolism and vitamin A deficiency aggravates thyroid dysfunction caused by iodine-deficient diets. Retinoids use in thyroid cancer appears less promising than expected.

Recent and still controversial studies investigated the relations between retinoids and metabolic syndrome. Indeed, retinoids contribute to pancreatic development and modify fat and glucose metabolism. However, more detailed studies are needed before planning any therapeutic use.

Finally, retinoids probably play more minor roles in adrenal and gonads development and function apart from their major effects on spermatogenesis.

Introduction

Vitamin A (retinol) is a lipophilic micronutrient that is critical for embryo and child development (Gutierrez-Mazariegos *et al.* 2011; Tang and Gudas 2011). In adults, vitamin A and metabolites are critical for the control of cell proliferation and differentiation, and for the maintenance of some very specific cell functions such as photo-transduction. Vitamin A deficiency is a marker of malnutrition that correlates with infection and mortality in children and possibly in childbearing women. Vitamin A is present in foods of animal origin such as liver, eggs and dairy products. An alternative source of vitamin A is the absorption of pro-vitamin A – carotenoids – from plants. However, although carotenoids are abundant, their absorption is about one order of magnitude less efficient compared to vitamin A. Thus, in populations from low-income countries, to depend solely on vegetable intakes for vitamin A sources increases the risk of vitamin A deficiency. The latter causes anomalies of development such as childhood blindness as vitamin A and metabolites act as morphogens that modulates gene transcription during embryogenesis. In developed adults, vitamin A and metabolites are also play key roles in vision, immune and brain functions, and tissue remodelling and metabolism.

To paraphrase Shearer *et al.*, (Shearer *et al.* 2012) is vitamin A a vitamin for the glands? To endocrinologists, vitamin A presents some similarities to vitamin D: it is a necessary lipophilic nutrient, it is transported by carrier proteins in the blood, it may be metabolised in the organism according to its needs, and it acts on nuclear receptors to modify gene transcription. Conversely, it is not regulated by a specific endocrine system (such as the calcium-PTH duet for vitamin D) and thus less famous in the endocrinologist community than vitamin D. This review will present the known physiological interactions of vitamin A and endocrine tissues and hormonal systems in normal adults with minor incursions in childhood or pathology when appropriate (see Figure 1).

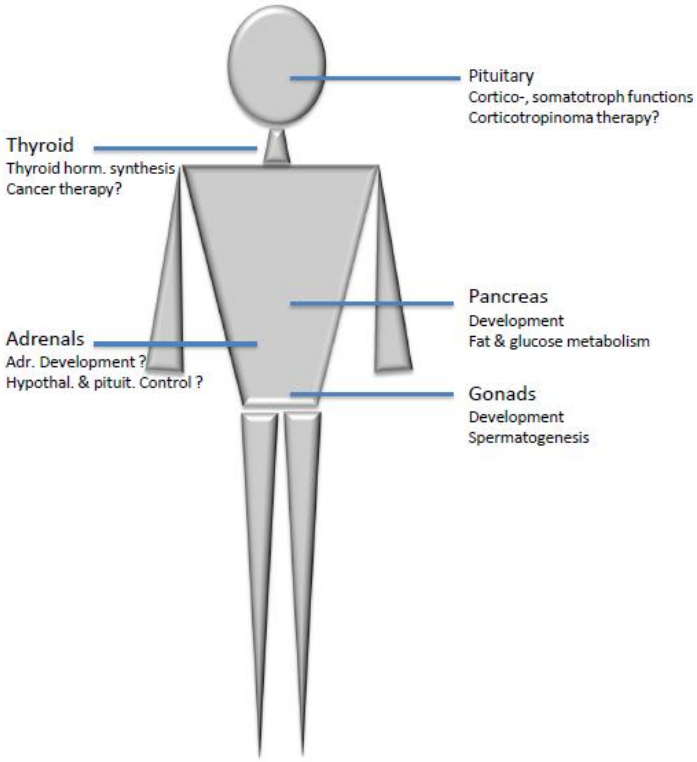

Figure 1 Main retinoids endocrine target tissues

Pituitary: retinoids mainly targets the corticotrophs with a possible therapeutic use in corticotropinomas. Thyroid: retinoids interfere with iodine metabolism; their possible use in thyroid cancer seems less promising than expected. Pancreas: apart from the gland development, retinoids modify fat and glucose metabolism. Adrenals: retinoids may play a role in adrenal development and function. Gonads: retinoids play a minor role in steroidogenesis but a major one in spermatogenesis.

When available, we cited reviews; we thus apologize not to be able to cite multiple pertinent references as space is constrained.

Brief physiology of the retinoids

Natural retinoids absorption

Retinoids constitute the family of molecules that includes both naturally occurring compounds with vitamin A activity as well as synthetic analogues of retinol or retinoic acid. Some of the latter are clinically used. Vitamin A in the body derives from the diet either of animal sources (all-*trans* retinol or retinyl esters) or plants sources (carotenoids). A simplified view of natural retinoids

metabolism is presented here but detailed reviews can be found (D'Ambrosio *et al.* 2011; Harrison 2012; O'Byrne and Blaner 2013). To summarise, retinol, retinyl esters and carotenoids absorption depends both on common lipid absorption and on specific enzymes, binding proteins and transporters. Retinol is directly taken up by enterocytes whereas retinyl esters must be hydrolysed by retinyl ester hydrolases (REH) within the lumen. These REH are gatekeepers of vitamin A/retinyl esters absorption as their expression is controlled by vitamin A metabolites (Lobo *et al.* 2010). Retinoids are hydrophobic and thus are usually bound in the cells to specific retinoid-binding proteins. For instance, cellular retinol-binding protein Type II (CRBPII) is expressed in the intestinal mucosa to facilitate retinol and retinal uptake and enterocyte storage (O'Byrne and Blaner 2013). In enterocytes, retinol is esterified by lecithin:retinol acyltransferase with long chain fatty acids to retinyl esters that are delivered *via* chylomicrons to hepatocytes that thus up-take about 70% of dietary retinol. Retinyl esters are hydrolysed in hepatocytes and transferred – possibly *via* cellular retinol-binding protein (RBP) Type I – for re-esterification and storage into hepatic stellate cells. *Via* yet unknown sensing mechanisms, when retinol is needed in other tissues, the stellate cells hydrolyse retinyl esters. Retinol is then back-transferred to hepatocytes and liberated along with (RBP). In plasma, retinol and RBP form a ternary complex with transthyretin that may also transport thyroxin to tissues. These non-hepatic tissues may also incorporate the ingested retinol not up-taken by hepatocytes (about 30%).

Carotenoids are incorporated intact by enterocytes with a membrane scavenger receptor and enzymatically converted to retinoid or incorporated unmodified into chylomicrons. Although abundant in food, the amount of retinol originating from carotenoids is limited by the conversion of 12 µg beta-carotene to only 1 µg vitamin A (Allen *et al.* 2002).

Thus, the delivery of retinoids to the cells depends on the temporal distance from the previous meal: in fasting state cells are mainly delivered retinol bound to RBP and transthyretin, in

post-prandial state retinyl esters are mainly delivered by lipoproteins.

Retinoids cellular effects

In tissues, retinol cellular uptake can depend on passive diffusion. In cells that have high needs of retinol is usually facilitated by a RBP-binding transporter, STRA6 (STimulated by Retinoic Acid 6)(Kawaguchi *et al.* 2007)(see Figure 2).

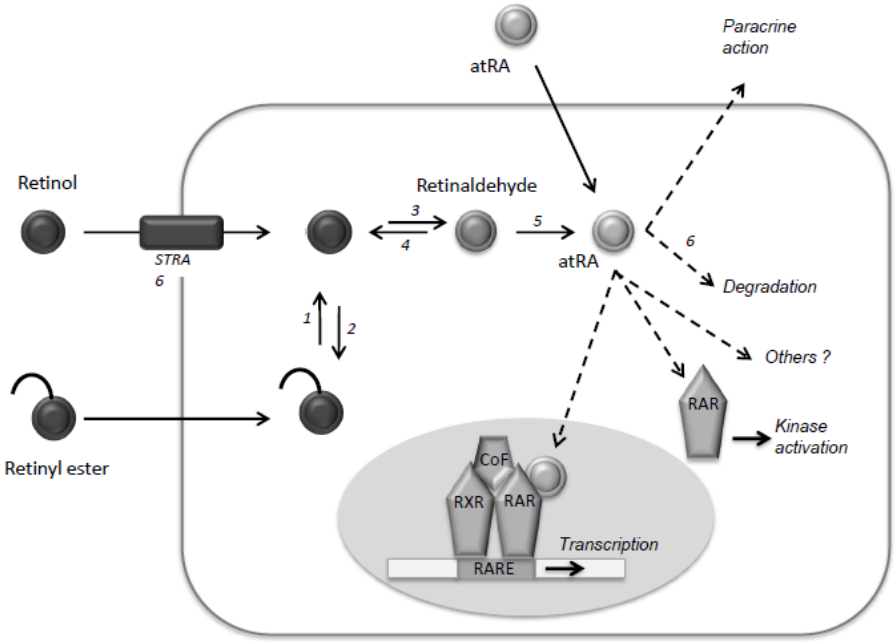

Figure 2 Retinoids cellular effects

Retinol bound in the plasma to retinol-binding protein and transthyretin is up taken by an RBP-binding transporter, STRA6. Retinyl esters freely cross the cell membrane to be transformed into retinol by retinyl ester hydrolase (1). This step can be reversed by lecithin:retinol acyltransferase (2). All-trans retinol is metabolised by retinol dehydrogenases (3) into all-trans retinaldehyde. This step can be reversed by retinal reductase (2). all-trans retinaldehyde is metabolised into all-trans retinoic acid (atRA) by retinaldehyde dehydrogenase (5). atRA is degraded by oxidation/catabolised by cytochrome P450 enzymes (6, mainly CYP26A1). atRA actions are mediated primarily by RA receptors *via* heterodimers of retinoic acid receptor (RAR) and retinoic X receptors (RXR) acting with cofactors (CoF) on RA response elements (RAREs) of target genes. In the absence of retinoid ligand, RAR/RXR heterodimers are bound to transcriptional repressors. Upon retinoid ligand binding, the heterodimers are bound to coactivator proteins. atRA may also exert non-genomic effects through cytoplasmic kinases.

STRA6 can also facilitate retinol efflux. Mutations of this protein cause severe and often lethal development abnormalities. (Slavotinek 2011) Within cells, retinol is metabolised as most of its

functions are in fact exerted by its metabolites. The intracellular concentrations of these retinoids are controlled by the activities of several metabolic enzymes. The expression and activity of the latter vary among cell types and differentiation. Partially redundant dehydrogenases metabolise all-*trans* retinol in all-*trans* retinaldehyde and the latter in all-*trans* retinoic acid (atRA) by retinaldehyde dehydrogenases (RALDH) (see Figure 2). (D'Ambrosio *et al.* 2011) atRA is degraded by hydroxylation by cytochrome P450 enzymes mainly by CYP26A1, a RA-induced p450 enzyme (Thatcher and Isoherranen 2009). Mutations of this enzyme are possibly one cause of sirenomelia through RA excessive signalling at the caudal end of the embryo (Garrido-Allepuz *et al.* 2011). Various isomers of RA exist. The major one is atRA (tretinoin); a minor one is 13-*cis* RA (isotretinoin) and a possible one is 9-*cis* RA (alitretinoin). There is however no large body of evidence of the natural occurrence of 9-*cis* RA, a potent RXR agonist. To assay sub-picomolar amounts of RA isomers is indeed technically challenging and 9-*cis* RA has yet to be found in tissues other than the pancreas (Kane *et al.* 2010). Thus, it is difficult to be precise about the relative concentrations of these isomers in given tissues. Indeed, it seems that different equilibria occur as a function of "isomerisation chaperones" such as glutathione S-transferase that can act as isomerases (Armstrong *et al.* 2005). Finally, the retinol metabolite 11-cis-retinal is essential for photo-transduction in the retina (Parker and Crouch 2010).

The physiological actions of atRA are mediated primarily by its binding to RA receptors (RAR α, β & γ isoforms) and subsequent formation of heterodimers of RAR and retinoic X receptors (RXR α, β & γ isoforms) (Rochette-Egly and Germain 2009). RAR and RXR are members of the large family of hormones, vitamins, and lipid receptors: receptors for steroids, thyroid hormones, vitamin D and peroxisome proliferator–activated receptors (PPAR) that act as ligand-dependent transcription factors. RAR and RXR form heterodimers that regulate the transcriptional activation on the RA response elements (RAREs) of retinoids target genes. Most tissues are targets of retinoids through

different heterodimeric complexes. There is apparently a large degree of functional redundancy between the various heterodimers of RAR α, β & γ and RXR α, β & γ. Interestingly, in the absence of retinoid ligand, RAR/RXR heterodimers act as transcriptional repressors *via* a corepressor complex that includes N-CoR1 or N-CoR2 (SMRT, Silencing Mediator of Retinoic acid and Thyroid hormone receptors) and proteins with histone deacetylase activity. Upon retinoid ligand binding, the RAR/RXR heterodimers modify their structure and interact with a higher affinity with coactivator proteins that include SRC 1, 2 & 3 and proteins with histone acetyl-transferase activity such as p300 (Rochette-Egly and Germain 2009; Tang and Gudas 2011). Of note, Dax1 (NR0B1) a critical developmental transcription factor in steroidogenic tissues has initially been described as a competitor of RAR/RXR heterodimers on the RAREs (Zanaria *et al.* 1994).

RA action through so-called nuclear receptors may not be limited to RAR-induced transcriptional effects. Firstly, RA could act on RXR receptors through one of its metabolite: 9-cis RA. However, although 9-cis RA is a powerful agonist of RXR, extensive proof of its presence within cells is largely lacking apart from few publications (Kane *et al.* 2010). Secondly, RA may exert non-genomic effects through receptors present in the cytosol or in membranes (Rochette-Egly and Germain 2009). Indeed, RA can rapidly modulate MAP kinases, phosphatidylinositol 3-kinase, calmodulin-dependent kinases, etc (Liu *et al.* 2009; Rochette-Egly and Germain 2009). This could establish crosstalks between kinase cascades and RAR-activated genomic pathways leading to coordinated phosphorylations targeting RAR themselves, other receptors, coregulators and histones (Rochette-Egly and Germain 2009).

In conclusion, vitamin A metabolites act as intracellular ligands on identified receptors and other cellular targets. Unknowns or controversial steps persist such as: what are the sensor mechanisms promoting the liberation of stocks of retinol from the liver, are the oxidised

metabolites of RA really inactive, are there mechanisms specifically responsible of isomerisation of RA, are there specific cellular actions of the RA isomers, are there hormonal controls of RA signalling, etc.?

Keeping these interrogations in mind, we will present the known interactions of Vitamin A and metabolites with endocrine tissues and hormones action?

Vitamin A and the hypothalamo-pituitary-peripheral gland axes

There are arguments for a role of retinoids in the development and function of the hypothalamus, the pituitary and the peripheral glands they act upon. Indeed, in these tissues, there are RAR and RARE bearing genes, (Nogami *et al.* 2000; Nogami *et al.* 2002; Chen *et al.* 2009; Meng *et al.* 2011) and retinoids metabolising enzymes (Fujiwara *et al.* 2007; Fujiwara *et al.* 2009; Muhlbauer *et al.* 2010). *In vivo* studies also show modifications of hypothalamo-pituitary-peripheral gland axes upon retinoid deprivation or treatment (Haider 2004; Angioni *et al.* 2005; Zimmermann 2007; Hess 2010; Clagett-Dame and Knutson 2011).

Vitamin A and the hypothalamo-pituitary-thyroid axis

RA does not seem to be involved in thyroid organogenesis (De Felice and Di Lauro 2011). Conversely, RA appears involved in maintaining developed a thyroid cell phenotype both in animals and humans. In animals, vitamin A deficiency causes thyroid hypertrophy with a reduction of iodine uptake, of thyroglobulin and of thyroid hormones synthesis ((Zimmermann *et al.* 2004) and older publications within). Combined iodine and vitamin A deficient diets produce greater impairments in thyroid metabolism than either isolated iodine or vitamin A deficient diets. In children with moderate vitamin A deficiency, TSH concentrations, thyroid volume and total T4 are increased (Zimmermann *et al.* 2004). A very important point about vitamin A and thyroid

metabolism is the possible co-existence of iodine and vitamin A deficiencies because of their high prevalence in developing countries: more than 30% children had simultaneous vitamin A deficiency and goitre in Côte d'Ivoire (Zimmermann *et al.* 2007). There are interactions between vitamin A and iodine metabolism as indicated by observational and interventional studies. In iodide deficient children, vitamin A increases TSH stimulation and thyroid size but reduces risk of hypothyroidism (Zimmermann 2007; Hess 2010). In these children, vitamin A supplementation improves iodide efficiency (Zimmermann 2007; Zimmermann *et al.* 2007);

Thus, various works investigated the actions of RA on thyrocytes (mainly expression and function of key proteins). Retinyl palmitate administration decreases thyroid gland size and serum thyroid hormones and conversely increases thyroidal iodine uptake and hepatic conversion of T4 to T3 in rats (Morley *et al.* 1980). Such an increase of iodine uptake has not been reproduced with a low dose of atRA (decreased iodine uptake) but the same dose of 13-cis RA increased iodine uptake (Muhlbauer *et al.* 2010). RA isoforms may thus have different consequences on thyrocyte functions. TSH-induced thyroid hormones synthesis requires the incorporation of iodide into the thyrocytes *via* the sodium–iodide symporter (NIS), its transport through the cell with pendrin to thyroglobulin in the lumen. Iodide oxidation and organification are catalysed by thyroperoxidase (TPO) with H_2O_2 produced by a dual oxidase. Various steps in this process depend on RA with noticeable differences related to the different cell models used complicating the description of the physiological role of RA. RA reduces TSH receptor mRNA levels (Tuncel *et al.* 2007). The dual oxidase is up-regulated in animal treated by an isomer of RA, 13-*cis* RA, but down-regulated by atRA (Muhlbauer *et al.* 2010). As 13-*cis* RA does not bind efficiently to RAR or RXR, this suggests either different cellular targets for these two retinoids or a critical role for the inter isomer conversion. RA may suppress the accumulation of TPO and TG mRNA stimulated by TSH in a time- and dose-dependent manner in cultures of human thyrocytes (Namba *et al.* 1993; del Senno *et al.*

1994).

The main recent reason for the interest about the role of RA in the thyroid is probably its potential therapeutic use in thyroid cancer. The rational for this interest is precisely the potential ability of RA isoforms to sustain cell differentiation or to reverse cell dedifferentiation in various models of cancer. With regards to a potential use to eradicate cancer cells with 131I, NIS was particularly investigated. In rat follicular cell lines, RA increases NIS mRNA levels and iodide uptake but this effect was not seen in untransformed cells (Schmutzler *et al.* 1997; Kogai *et al.* 2006). More importantly an increase of iodide uptake was described in human thyroid cancer (Schmutzler *et al.* 1997; Kogai *et al.* 2006). Thus, the use of RA in thyroid through the increased expression of NIS to increase 131I uptake has been under careful investigation in human cancers (Grunwald *et al.* 1998; Simon *et al.* 1998; Schmutzler and Kohrle 2000; Coelho *et al.* 2005). Unfortunately, different studies did not report a clear usefulness for such a treatment in thyroid cancer (O'Neill *et al.* 2010). Though, for a similar expected action on NIS, RA is under consideration for the treatment of breast cancer (Kogai and Brent 2012).

RA can also modulate the effects of thyroid hormones on target tissues. Firstly, RA induces the expression of the thyroid hormone transporter, monocarboxylate transporter (Kogai *et al.* 2010). This is responsible of a cross talk between RA and thyroid hormones signalling at least during critical steps of embryo brain development (Bernal 2011). Secondly, although RAR and thyroid receptors do not seem to directly physically interact, they share some cofactors such as CART1 a de-repressor in the cytoplasm and NCoR2 a corepressor in the nucleus (Chen and Evans 1995; Park *et al.* 2010). It is then likely that some form of competition occurs between the two ligands and their receptors. Subsequent consequences would then depend on resulting gene trans-activations and trans-repressions. Lastly, there are interferences between thyroid and retinoid signalling. For

instance, during a vitamin A-deficient diet or in aged rats, retinoid and thyroid nuclear receptor expressions decrease. This can be corrected by either thyroid hormone or RA treatments (Feart *et al.* 2005). In humans, such a link is likely since a decreased expression of RAR occurs in mononuclear cells of hypothyroid patients (Feart *et al.* 2005). Conversely, an increased concentration of retinol was seen in hypothyroidism. Currently, it is not known if a thyroid hormone replacement therapy restores RA signalling back to a status seen in euthyroidism.

In conclusion, there are many levels where RA can interact with the physiology of hypothalamo-pituitary-thyroid axis including through vitamin A and iodine co-deficiency in low-income living conditions. Unfortunately, the hopes raised by early work in thyroid cancers are probably dashed now because of the absence of clear usefulness.

Vitamin A and the hypothalamo-pituitary-adrenal (HPA) axis

There are arguments for an action of RA on the HPA axis. For instance, chronic treatment of young rats by RA increases basal corticosterone concentration (Cai *et al.* 2010). However, most of the recent literature refers to its possible use or role in pituitary or adrenal tumours.

Firstly, RAR-α is co-localised with corticotrophin-releasing hormone and vasopressin in neurons of the hypothalamic paraventricular nucleus suggesting a regulation of these cells by RA (Chen *et al.* 2009; Meng *et al.* 2011). Furthermore, RA is localised in hypothalamic neurons although it is not yet known whether these neurons regulate the HPA axis (Mangas *et al.* 2012). Retinaldehyde dehydrogenase enzymes are also localised in the hypothalamus (Mangas *et al.* 2012). Altogether these data strongly supports a role of RA in regulating hypothalamic functions.

Secondly, RA could act on the secretion of corticotrophs, but there are apparently conflicting data. In normal rat, atRA administration increases basal serum corticosterone concentration

possibly through the increased mRNA expression of corticotrophin release factor and RAR-α in the hypothalamus (Cai *et al.* 2010). No *in vitro* data is available about RA and normal corticotrophs. An apparently opposite effect has been shown in tumoural cells as RA reduces growth and secretion of AtT20 cells (Paez-Pereda *et al.* 2001) possibly through bone morphogenic protein 4 action (Paez-Pereda *et al.* 2001; Giacomini *et al.* 2006; Labeur *et al.* 2010; Yacqub-Usman *et al.* 2013). This explains many recent papers about the possible use of retinoids to treat Cushing's disease (Pecori Giraldi *et al.* 2012). Ectopic ACTH secretion may also be affected by retinoids. Indeed, the nuclear co-repressor SMRT is over-expressed in ACTH-secreting thymic carcinoids suggesting that aberrant expression might be involved in the pathogenesis of tumoural cortisol resistance (Jiang *et al.* 2010).

Thirdly, RA could possibly act on adrenals and especially adrenal ontogeny, physiology and tumorigenesis through SMRT (Clipsham and McCabe 2003) and Bone Morphogenic Proteins (BMP) signalling (Johnsen and Beuschlein 2010). BMP are known modulators of different hormonal systems including the adrenal. On one hand, RA regulates BMP signalling by promoting the degradation of phosphorylated Smad1. On the other hand, RA promotes the transcription of GATA-6 that in turn promotes BMP2 transcription. Whether reactivating BMP signalling in adrenocortical tumour tissues by therapeutic retinoids is yet unknown (Johnsen and Beuschlein 2010). Finally, a meta-analysis of adrenocortical tumour genomics data also revealed a putative role of RA signalling (Szabo *et al.* 2010).

Lastly, part of the interaction between vitamin A and glucocorticoid action may occur downstream of adrenal hormone production as vitamin A and glucocorticoid receptors may interact directly or indirectly. As a consequence, RA is for instance able to decrease glucocorticoid receptor expression and modify glucocorticoid signalling in a neuronal model (Brossaud *et al.* 2013). In addition, RA may modulate local glucocorticoid activation by 11β-hydroxysteroid dehydrogenase 1 (HSD1). This has been shown, *in vitro* in muscle cells in which RA exerts a dose-

dependent down regulation of 11β-HSD1mRNA expression and activity (Aubry and Odermatt 2009). Similarly, in the liver of obese rats 11β -HSD1 activity and gene expression are significantly reduced by vitamin A supplementation (Sakamuri *et al.* 2011). Similarly, in vitamin A-deficient LOU/C rats the expression of 11β-HSD1 is increased in the hypothalamus and the hippocampus. This increase, as well as the associated increased HPA axis activity, is normalized by RA administration (Marissal-Arvy *et al.* 2013).

Vitamin A and the hypothalamo-pituitary-gonads axis

RA is a critical factor for the formation of the gonads in man and one of the major consequences of vitamin A deficiency apart from blindness is infertility (Haider 2004; Clagett-Dame and Knutson 2011). In Leydig as well as ovarian cells, RA stimulates Steroidogenic Acute Regulatory protein (StAR) and P450 17α hydroxylase expression and thus steroidogenesis (Wickenheisser *et al.* 2005). The endocrine role of RA in the production of gonadic hormones of developed gonads appears however less important although RA stimulates steroid hormone synthesis.

It has been suggesting that there is an interesting interplay between RA and oestrogen signalling in breast cancer cells particularly with opposite actions on cell proliferation. RARα could be an integral part of the ERα transcriptional complex (Tang and Gudas 2011; Ombra *et al.* 2013). Whether this is true in normal cells is yet unknown.

Vitamin A and the somato-lactotroph axis

RA probably plays a role in the differentiation of somatotrophs through pit-1 transcription factor (Cheng *et al.* 2011) but also in the expression of GHRH receptors in somatotrophs as there is a RARE in the promoter of Growth Hormone-Releasing Hormone (GH-RH) receptor gene (Mallo *et*

al. 1992). In developed somatotrophs, retinoids affect basal and GH-RH induced GH secretion (Mallo *et al.* 1992). Insulin Growth Factor 1 and 2 (IGF1 and IGF2) synthesis is increased by retinoids in some skin models (Shim *et al.* 2012) but no data is available about the most important GH-induced hepatic IGF 1 production. In pituitary tumours including somatotroph tumours, RA increases the expression of type 2 dopamine receptors hence a possible therapeutic use to control these tumours (Bondioni *et al.* 2008).

RA action has also been described in prolactin secreting cells either normal or tumoural as demonstrated for corticotrophs. Again, a possible mechanism of action is the role of BMP-4, a member of the transforming growth factor β (TGFβ) family, overexpressed in different prolactinoma models and induces the development of these lineage adenomas (Giacomini *et al.* 2006).

Vitamin A and glucose and fat metabolism

Vitamin A and the pancreas

RA receptor signalling is required in early pancreatic progenitor cells for pancreatic development (Kam *et al.* 2012).

There are different lines of arguments linking endocrine β cells function to RA. Hereafter are some of the main arguments developed in recent years (Yang *et al.* 2005; O'Byrne and Blaner 2013). Vitamin A plasma concentrations are higher in subjects with glucose intolerance and the RBP/retinol ratio is elevated in patients with type 2 diabetes. RA restores an insulin-secreting function of vitamin A-deprived rats. In pancreatic β cells, atRA increases the transcription of glucokinase, glucose transporter 2 and pre-pro-insulin genes and promotes insulin secretion. Furthermore, some RXR-γ haplotypes are associated with indicators of pancreatic β-cell function.

Conversely, 9-cisRA, the ligand of RXR receptors, decreases glucose-induced insulin secretion

(Miyazaki *et al.* 2010). Interestingly, the pancreas is one of the few (if any others) tissues where endogenous 9cisRA has been detected (Kane *et al.* 2010; Kane 2012). RXR agonists have been proposed to improve insulin-sensitivity as 9cisRA/RXR might inhibit excessive insulin release under – only under – high-glucose conditions. This action may be obtained through PPAR/RXR heterodimerisation. 13-cis RA may also be a player as it seems to alter pancreatic cell viability (Shimamura *et al.* 2010). Furthermore, Raldh3 (retinaldehyde dehydrogenase 3) is present in the pancreas and promotes the formation of 13-cis RA from 13-cis retinal (Graham *et al.* 2006). In diabetic mice, Raldh3 expression is increased and this is correlated with reduced insulin and increased glucagon secretions. Thus in the pancreas, unusual RA isomers may play a role in pancreatic function but confirmation of these studies has to be obtained.

Vitamin A and glucose and fat metabolism

Vitamin A is involved in lipid metabolism and cell response to insulin. Many studies point toward a vitamin A-induced reduction of fat accumulation but there are some conflicting results that likely come from the large number of animal models to study fat metabolism (for detailed data see recent reviews (Berry and Noy 2009; Bonet *et al.* 2012; Yasmeen *et al.* 2012)). To summarise, atRA binding RAR and possibly PPARβ/δ (not α nor γ) regulate lipolysis, energy dissipation, fatty acid oxidation, and glucose transport (Berry and Noy 2009; Bonet *et al.* 2012; Yasmeen *et al.* 2012). RA regulates the transcription of many genes encoding proteins involved in lipid metabolism (Miller *et al.* 1997; Zolfaghari and Ross 2003; Cadoudal *et al.* 2008). Indirect activation of PPARα target genes in fatty acid catabolism occurs through promoters containing RARE and PPAR response elements (Sohlenius *et al.* 1995; Iacobazzi *et al.* 2009). Globally, in the liver, RA seems to favour fatty acid catabolism possibly through an increased RA-dependent expression of PPARα.

Other reports evidenced various intriguing observations about retinol binding protein, RBP. Firstly, i) plasma RBP concentration is increased in obese and ii) insulin-resistant animals and RGP administration leads to insulin-resistance and iii) animals lacking RBP are protected against fat diet-induced insulin-resistance. Secondly, RBP-retinol activates JAK/STAT kinase pathway up-regulating genes that inhibit insulin signalling and that control lipid homeostasis. It thus appears that RBP acts as an adipokine that contributes to obesity-induced insulin resistance (Yang *et al.* 2005; Berry *et al.* 2011; Berry and Noy 2011). This is possible as STRA6, the retinol transporter, acts as a membrane receptor to RBP activating JAK/STAT pathway. Targets of the latter include: i) SOCS3, an inhibitor of cytokine receptors signalling (including insulin and leptin receptors, and ii) PPARγ, a regulator of adipocyte differentiation (including lipid storage). Thus, vitamin A could be involved in the regulation of insulin response and lipid homeostasis through a membrane-activated kinase cascade inducing gene transcription and not only through the more commonly described RA-activated RAR-dependent gene transcription (Ziouzenkova and Harrison 2012).

A last and interesting point is the role of vitamin A metabolism in sex- and depot-specific fat formation. Indeed, high-fat diets as well as menopause-related estrogen deficiency alter adipose tissue distribution. It has been shown that a high-fat diet or estrogen deficiency induce autocrine RA signalling by increasing aldehyde dehydrogenase expression. RA, in turn, governs fat formation in a depot- and sex-specific fashion: the retinaldehyde-mediated lipolysis in visceral adipocytes is replaced by a RA-mediated lipid accumulation (Yasmeen *et al.* 2013).

Various

Vitamin A and renin-angiotensin-aldosterone axis

There are *in vivo* and *in vitro* arguments in animals reporting the effects of RA on renin or angiotensin production (Zhong *et al.* 2004; Guleria *et al.* 2011; Zhou *et al.* 2012; Glenn *et al.* 2013).

atRA treatment increased the expression of angiotensin converting enzyme 2 with a subsequent reduction of blood pressure in hypertensive rats. (Zhong *et al.* 2004) To our knowledge, no clinically useful data is available neither for renin or aldosterone levels nor for the effect of RA on blood pressure.

Vitamin A and the adrenal medulla

RA seems to play neither a remarkable role in adrenal medulla organogenesis nor function in adults. *In vitro*, RA could initiate neuronal differentiation in PC12 cells eliciting the expression of a nerve growth factor receptor as well as tyrosine hydroxylase expression (Cosgaya *et al.* 1996). This is usually considered as a differentiating action on cells sharing a common origin with neurons.

Vitamin A and thyroid C cells

No information has been reported about a role of RA and calcitonin secretion by normal thyroid C cells. *In vitro*, 9-cis RA decreases the release of calcitonin in the rat C cell line CA-77 (Lamari and Garel 1997) but no data is available about the spontaneous presence of 9-cis RA in C cells. atRA had no significant effect on a human medullary thyroid carcinoma cell line (Elisei *et al.* 2005).

Vitamin A and the pineal gland

One publication (Fu *et al.* 1998) relates a role of RA on the pineal gland of the quail! Another publication reports the treatment of a pinealoblastoma by RA (DeBoer *et al.* 2009) but no other human data reports any physiologic function of RA neither on the pineal gland nor on melatonin production.

Vitamin A and erythropoietin

RA stimulates the erythropoietin synthesis in foetal rats *via* a RARE in the erythropoietin gene dependent on RAR/RXR receptors (Makita *et al.* 2005; Brade *et al.* 2011). In adult rats however, this effect disappears and adult erythropoiesis takes place. The RAR/RXR complex is replaced by an orphan receptor, Hepatocyte Nuclear Factor 4, which binds to the same *cis* element to facilitate an interaction with the Hypoxia-Inducible Factor 1 bound to an adjacent site (Evans 2005).

Conclusion

Vitamin A and RA metabolisms and mechanisms of action are progressively deciphered. RA is also for some glands development and/or function. One of the most critical roles is its effect on thyroid function as simultaneous iodine and vitamin A deficiencies potentiate to affect thyroid function. To achieve adequate intake of these micronutrients among others is indeed a challenge in developing countries. Conversely, in well-fed developed countries, RA role in fatty acids and glucose metabolism appears important. However, despite clear advances, the complex interplay of lipid agonists on the large panel of receptors involved is not yet fully understood. Therapeutic use of agonists or antagonists of these pathways is not available yet. In the endocrine field, the use of retinoids in pituitary tumours especially corticotroph adenomas is emerging. Well-tolerated, clinically available retinoids used for skin or hematologic diseases, renders clinical studies comparing other medical options readily possible.

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B. Conclusion

Les rôles de la vitamine A dans les systèmes endocriniens sont multiples. Certains aspects de la question sont cependant mal ou partiellement connus. Dans le cadre de la thématique de notre laboratoire, il nous est apparu intéressant de préciser les mécanismes impliqués dans la modulation de l'imprégnation et de la réponse cellulaire aux GC par la vitamine A. Nous avons focalisé notre travail sur l'effet post-récepteur de l'exposition aux GC par l'atAR.

III. Modifications post-récepteurs de l'exposition aux GC : données bibliographiques

La modification de l'activité transcriptionnelle du récepteur aux GC, après fixation du ligand, est largement impliquée dans la modulation de la sensibilité tissulaire aux GC. L'aspect moléculaire de cette régulation post-récepteur est bien décrit dans le cadre de l'inflammation. En effet, de nombreux travaux montrent que les cytokines peuvent influencer la fonction du GR. Elles modifient la translocation du GR vers le noyau et son interaction avec le GRE et avec les autres facteurs de transcription. Ces modifications sont liées à l'action modulatrice des cytokines sur la voie de signalisation de plusieurs kinases entraînant une modification de la phosphorylation de GR (Hu et al. 2009; Pace and Miller 2009). D'autres facteurs de modulation de l'activité GC - le stress cellulaire (Galliher-Beckley et al. 2011), les hormones (exemple des estrogènes (Zhang et al. 2009b; Miranda et al. 2013), la vitamine D (Obradovic et al. 2006; Zhang et al. 2013) - jouent un rôle dans l'activation post-récepteur des GC.

Au regard de plusieurs publications, il semble que les dérivés rétinoïques participent à la modulation de l'exposition tissulaire aux GC et exercent leur action à plusieurs niveaux.

- Au niveau de la sécrétion des GC. Certains auteurs montrent l'effet modulateur de l'atAR sur la sécrétion tumorale d'ACTH par l'hypophyse (Paez-Pereda et al. 2001; Giacomini et al. 2006; Yacqub-Usman et al. 2013). Ces travaux justifient les essais récents de traitement de la maladie de Cushing par l'atAR (Pecori Giraldi et al. 2012). Néanmoins, il s'agit d'études menées sur des tumeurs corticotropes et il semble que l'effet de l'atAR sur des cellules non tumorales puisse être différent (Cai et al. 2010)

- Au niveau de l'exposition pré-récepteur des tissus aux GC. Aubry et son équipe ont été les premiers à montrer une diminution de l'activité de la 11βHSD1 dans un modèle de cellules myoblastiques par l'acide rétinoïque (Aubry and Odermatt 2009). *In vivo*, dans

l'hippocampe de rat, la carence en atAR augmente l'expression et l'activité de l'enzyme et cette augmentation est réversible par une supplémentation en atAR (Marissal-Arvy et al. 2013)(Bonhomme *en préparation*). Enfin l'atAR diminue l'activité de la 11βHSD1 dans le foie et la graisse viscérale de rats obèses (Sakamuri et al. 2011).

- Au niveau de l'activité transcriptionnelle du GR. Plusieurs publications suggèrent qu'il existe une interaction entre la voie de la vitamine A et celle des GC. Les conséquences de cette interaction semblent très variables selon le contexte dans lesquelles elles sont observées. L'action conjointe de l'atAR et des GC sur une même cible peut être sans effet ou être antagoniste (Grummer and Zachman 1998; Ubels 2005; Zhang et al. 2009a; Conaway et al. 2011), additive voire synergique (Nogami et al. 2000; Schneider et al. 2001; Rowling and Schalinske 2003; Subramaniam et al. 2003; Wang et al. 2004; Unterholzner et al. 2006; Fukui et al. 2009; Cheng et al. 2011; Toth et al. 2011). L'effet de l'atAR sur la voie de signalisation du GR n'a été évoqué que de manière récente. Deux mécanismes ont été proposés. Wang *et al.* proposent un recrutement synergique du cofacteur p300 en présence de dexaméthasone et d'atAR (Wang et al. 2004). Toth *et al.* montrent une interaction RAR-RXR-GR responsable d'une augmentation de la fixation du GR au GRE potentialisant l'action de la dexaméthasone sur l'apoptose des lymphocytes T (Toth et al. 2011).

Alors que les modifications de la voie des kinases par l'atAR ont été largement démontrées (Zassadowski et al. 2012; Al Tanoury et al. 2013), aucun auteur ne suggère une modification de la phosphorylation de GR par l'atAR. Pourtant, celle-ci modifie largement sa fonction. *In vivo*, des rats carencés en atAR présentent une capacité de liaison du GR cytoplasmique au niveau hépatique augmentée par rapport aux rats non carencés. Sous traitement par la dexaméthasone, le GR se transloque et la capacité de liaison du GR augmente dans le noyau. Lors d'un co-traitement dexaméthasone + atAR, la capacité de liaison du récepteur au niveau nucléaire double par rapport au simple traitement par la dexaméthasone. Ces modifications de la localisation du récepteur suggèrent une modification de son statut phosphorylé par l'atAR (Audouin-Chevallier et al. 1995).

IV. Modifications post-récepteurs de l'exposition aux GC : résultats

De nombreuses publications montrent des effets antagoniste, neutre, additif ou synergique des rétinoïques et des GC selon le modèle d'étude et les conditions expérimentales sur des cibles communes. Aucune n'a été réalisée sur un modèle neuronal. Alors que ces deux métabolites

présentent sur le SNC et en particulier au niveau de l'hippocampe, des effets respectifs opposés très importants, rien n'est connu de l'action concomitante des rétinoïques et des GC. Ainsi, les effets bénéfiques de l'atAR sur les fonctions centrales pourraient être en partie expliqués par une modulation de l'activité post-récepteur des GC. D'autre part, seuls deux auteurs proposent une explication mécanistique impliquant une modification de l'activité transcriptionnelle de l'activité du GR en aval de la fixation du ligand sur son récepteur.

Il nous est donc apparu intéressant de choisir une lignée de cellules hippocampiques murines, les HT22, comme modèle d'étude de la modulation post-récepteur de l'exposition aux GC par la vitamine A et son métabolite actif.

Ainsi, le second objectif de ce travail de thèse a été de préciser les effets du traitement concomitant de l'atAR et de la dexaméthasone sur des cibles hippocampiques communes et notamment leur effet réciproque sur leur récepteur nucléaire (PUBLICATION N°5). Nous avons choisi pour cela deux cibles communes classiquement décrites comme impliquées dans la différentiation neuronale pour la tissue transglutaminase 2 (tTG) et dans la plasticité et la neuroprotection pour le BDNF. Il nous a fallu préciser également par quel(s) récepteur(s) RARs l'atAR exerçait son effet. Puis, nous avons exploré différents mécanismes intracellulaires potentiellement impliqués dans la modification de la phosphorylation du récepteur aux GC et responsables d'une modulation des effets GC par l'atAR (PUBLICATION N°6). Le choix de cette voie mécanistique nous a semblé intéressant à étudier au vu de la littérature récente des effets non-génomiques de l'atAR sur l'activation de nombreuses kinases et sur les récupérations génomiques sur l'activité transcriptionnelle de ces propres récepteurs.

Dans ce modèle de cellules hippocampiques traitées pendant quatre jours par l'atAR et/ou la Dex à 10^{-6}M, nous montrons que l'atAR et la dexaméthasone présentent des effets antagonistes à la fois sur l'expression de tTG et de BDNF. Le récepteur RARβ apparait comme le meilleur candidat pour expliquer l'effet de l'atAR dans ce modèle. L'atAR et la dexaméthasone modulent réciproquement l'expression de leur récepteur nucléaire : l'atAR diminue l'expression du GR et la dexaméthasone augmente celle du RARβ.

Enfin nos résultats indiquent que le traitement par l'atAR augmente la phosphorylation Ser 220 du GR par rapport au traitement dexaméthasone seul. Cela se traduit par une augmentation (pour tTG et BDNF) ou une diminution (promoteur du GRE couplé au gène de la luciférase) de la réponse aux GC en fonction de la cible et donc du promoteur étudié. Cette augmentation de la phosphorylation du GR est sous la dépendance de la CDK5 et de son activateur, le peptide p35.

A. PUBLICATION N° 5 : Interaction entre la voie des rétinoïques et des glucocorticoïdes sur des cibles communes dans les cellules hippocampiques HT22

Retinoids and glucocorticoids target common genes in hippocampal HT22 cells

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ORIGINAL ARTICLE

Retinoids and glucocorticoids target common genes in hippocampal HT22 cells

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Abstract

Vitamin A metabolite retinoic acid (RA) plays a major role in the aging adult brain plasticity. Conversely, chronic excess of glucocorticoids (GC) elicits some deleterious effects in the hippocampus. We questioned here the involvement of RA and GC in the expression of target proteins in hippocampal neurons. We investigated proteins involved either in the signaling pathways [RA receptor β (RARβ) and glucocorticoid receptor (GR)] or in neuron differentiation and plasticity [tissue transglutaminase 2 (tTG) and brain-derived neurotrophic factor (BDNF)] in a hippocampal cell line, HT22. We applied RA and/or dexamethasone (Dex) as activators of the pathways and investigated mRNA and protein expression of their receptors and of tTG and BDNF as well as tTG activity and BDNF secretion. Our results confirm the involvement of RA- and GC-dependent pathways and their interaction in our neuronal cell model. First, both pathways regulate the transcription and expression of own and reciprocal receptors: RA and Dex increased RARβ and decreased GR expressions. Second, Dex reduces the expression of tTG when associated with RA despite stimulating its expression when used alone. Importantly, when they are combined, RA counteracts the deleterious effect of glucocorticoids on BDNF regulation and thus may improve neuronal plasticity under stress conditions. In conclusion, GC and RA both interact through regulations of the two receptors, RARβ and GR. Furthermore, they both act, synergistically or oppositely, on other target proteins critical for neuronal plasticity, tTG and BDNF.

Keywords: brain neurotrophic factor, glucocorticoids, hippocampus, retinoic acid, retinoic acid receptor, tissue transglutaminase 2.

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Retinoic acid (RA) plays a major role in adult brain plasticity through various processes such as neurite outgrowth and neuronal differentiation but, unfortunately, retinoid signaling decreases with age (Etchamendy *et al.* 2001; Maden 2007; Mingaud *et al.* 2008; Shearer *et al.* 2012). RA main effects are mediated by the formation of heterodimers of RA receptors (RARα, β, or γ) and retinoid-X receptor (RXRα, β, or γ) which bind RA-responsive elements in the regulatory region of target genes. On the other hand, glucocorticoids (GC) elicit harmful cellular and behavioral effects on the hippocampus (Alfarez *et al.* 2002; Krugers *et al.* 2006; Sousa *et al.* 2008). These effects are mediated by GC binding to glucocorticoid or mineralocorticoid receptors (GR and MR, respectively) eliciting receptors nuclear translocation (Bamberger *et al.* 1996). Following activation, GR forms homodimers that interact with target genes GC responsive elements. Interestingly, both RA and GC regulate genes involved in the cerebral plasticity and memory processes. Moreover, in neuronal cells simultaneous activation of RA and GC signaling pathways results in synergic or opposite effects, suggesting that their pathways interact. In thymocytes, RA enhances GC-induced cell death by improving GC-induced transcriptional activity (Toth *et al.*

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Abbreviations used: BDNF, brain-derived neurotrophic factor; Dex, dexamethasone; GC, glucocorticoids; GRE, glucocorticoids responsive element; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RARE, retinoic acid responsive element; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoic X receptor; tTG, tissue transglutaminase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

2011). In skeletal muscle, RA strongly inhibits the expression of a GC-induced gene by inhibiting GR transactivation (Aubry and Odermatt 2009). In hepatocytes, dexamethasone (Dex) enhances the RA-dependent increase of RARβ expression (Yamaguchi *et al.* 1999). Conversely, in human myeloma cells, Dex inhibits RA-dependent induction of tissue transglutaminase (tTG) (Lefebvre *et al.* 1999). Despite the known involvement of RA and GC in neuronal plasticity, there is no data concerning possible interactions of their pathways in the hippocampus.

We questioned the involvement of RA and GC in the expression of target genes and proteins involved either in the signaling pathways or in neuron differentiation and plasticity. The receptors involved were investigated: RAR, RXR, GR, and MR. Indeed, for instance, the expression of RARβ is RA-dependent through a positive feedback loop (Ballow *et al.* 2003; Latasa and Cosgaya 2011). Two target proteins were also chosen to investigate neuronal plasticity. First, the tissue transglutaminase 2 (tTG) was chosen because of its involvement in cell survival and differentiation, and its dependence on RA and GC control (Lefebvre *et al.* 1999; Campisi *et al.* 2008; Fahey *et al.* 2009; Garabuczi *et al.* 2011; Hodrea *et al.* 2012). Second, brain-derived neurotrophic factor (BDNF) was chosen because GC downregulates BDNF expression in the hippocampus (Murakami *et al.* 2005; Duman and Monteggia 2006), and conversely, RA enhances BDNF expression in midbrain cells (Katsuki *et al.* 2009).

Our results confirm the involvement of RA- and GC-dependent pathways in our neuronal cell model. First, both pathways regulate the transcription and expression of their own and reciprocal receptors. Second, upon simultaneous activation, Dex did not always oppose RA action in the same way. Dex reduced the expression of tTG when associated with RA, whereas it stimulated its expression when used alone. Conversely, Dex reduced BDNF expression, both when used alone and combined with RA.

Materials and methods

Cell cultures

Immortalized brain cell lines that retain parental cell characteristics have been generated from neuron/glial precursors, astrocytes, and microglia (Lendahl and McKay 1990). HT-22 cells are immortalized mouse hippocampal-neuronal precursor cells that were subcloned from their parent HT-4 cells and keep the parents' characteristics (Liu *et al.* 2009a). We choose this cell line rather than the other main hippocampal line, H19-7, of rat origin because we wished to more easily relate to prior work with mice in the laboratory. The clone of HT22 cells was kindly provided by Dr. E. Maronde (Frankfurt am Main, Germany) (Benz *et al.* 2010). To allow cells to expand, they were grown under 5% CO_2 at 37°C in (Dubelcco's modified Eagle medium, Life Technologies, Van Allen Way Carlsbad, CA, USA) with pyruvate supplemented with 10% fetal bovine serum (Life Technologies) and 1% streptomycin sulfate/phenoxypenicilinic acid (stock solution 50 mg/mL and 10 000 UI/mL, respectively). The culture medium was changed every other day (including during experiments).

The cells were seeded in plates (density: 1500 cells/cm² except for immunocytochemistry) and cultured for 4 days in Dubelcco's modified Eagle medium with antibiotics and pyruvate supplemented with 10% charcoal-depleted fetal bovine serum. Except for the dose–response experiments (10^{-10} to 10^{-6} M), treatments consisted in all-trans RA (final concentration 10^{-6} M), Dex (final concentration 10^{-6} M), or RA+Dex (final concentrations 10^{-6} M for both). RA and Dex stock solutions (Sigma Aldrich, St. Louis, MO, USA; 17.5 and 10 mmol/L, respectively) were diluted into ethanol:dimethylsulfoxide (50 : 50) (VWR International, West Chester, PA, USA) added as vehicle in controls.

MTT assay

Cell viability was determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 50 µL of MTT (0.5 mg/mL) were added in each well and incubated for 2 h at 37°C. The supernatants were removed and the formazan crystals were allowed to dissolve in 200 µL dimethylsulfoxide. The absorbance value was determined at 595 nm using a microplate spectrophotometer (Victor Multilabel plate reader; Perkin Elmer, Waltham, MA, USA).

In situ cell death detection

In situ nuclear DNA fragmentation was measured according to a method based on 3'OH end labeling of DNA breaks with deoxyuridine terminal deoxynucleotidyl transferase. Apoptosis was quantified with an *in situ* cell death detection kit (Roche, Boulogne-Billancourt, France). The cells were then washed with phosphate buffer saline (PBS) and peroxydases were blocked with 3% H_2O_2 in methanol. The cells were fixed (paraformaldehyde, 4%, 15 min) and permeabilized (1%Triton-X100 in PBS, 3 min). Two negative and one positive controls were included in each experiment. For the negative controls, fixed and permeabilized cells were incubated with the kit label solution instead of Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) reaction mixture (label solution + enzyme solution). For the positive control, cells were incubated with DNaseI recombinant, grade I (30 U/mL in Tris-HCl pH7.5 50 mM, $MgCl_2$ 10 mM, bovine serum albumin 1 mg/mL) (10 min). TUNEL reaction mixture was added (60 min, 37°C, dark humid atmosphere). After rinsing, cells were analyzed using an epifluorescence microscope (Nikon H600L, Champigny sur Marne, France).

Real-time PCR analyses

The cells were lysed with an extraction kit (Trizol reagent; Invitrogen, Van Allen Way Carlsbad, CA, USA). RNA concentration was determined by spectrophotometry on a NanoDrop ND-1000 spectrophotometer (Labtech, Palaiseau, France). RNA integrity was verified using the RNA-6000 NanoLabChip kit combined with a 2100 Bioanalyser (Agilent Technologies, Les Ulis, France). RNA integrity numbers (RIN) were greater than 8 indicating a good and comparable RNA quality across samples. RNA (1 µg) was reverse transcribed to cDNA using ImPromII reverse transcriptase (Promega, Charbonnières, France) (Bonnet *et al.* 2008). Total RNA mixed with RNAsin (Promega) and DNase (Roche) were incubated at 37°C

(15 min). OligodT and random primers (Promega) were added and incubated at 75°C (10 min). The reverse transcriptase reaction was performed at 42°C (60 min, final volume 20 µL).

Real-time quantitative PCR was performed using the LightCycler® 480II system (Roche). To detect target genes amplification products, a LC480 SYBER-GREEN I Master was used. PCR was performed in microtiter plates in a final volume of 20 µL containing 1X LC480 SYBER-GREEN I Master solution, 0.5 µM of each primer and 5 ng of cDNA. Specific primers were as follows: GAACATCATCCCTGCATCCA forward and CCAGTGAGCT TCCCGTTCA for Glyceraldehide 3 phosphate deshydrogenase (GAPDH); GCTGGGCAAGTACACTACGAAC and GGCGAA-CTCCACAGTCTTAATG for RARα; CAGCTGGGTAAATACA CCACGAA and GGGGTATACCTGGTACAAATTCTG for RARβ; CCCAAGGATGCTGATGAAAATC and GCCCTTT CTGCTCCCTTAGTG for RARγ; CCATCTTTGACAGGGTGCT AACA and ATCTGCATGTCACGCATCTTAGAC for RXRα; CCTGAAGATGTGAAGCCACC and CGTTGACGCTCC TCCTGAAC for RXRβ; CTCTGGTGAAACACATCTGTGCC and GGGGTATACCTGGTACAAATTCTGA for RXRγ; GTG GAAGGACAGCACAATTACCT and GCGGCATGCTGGA CAGTT for GR; GCCGTGGAAGGACAACACA and CCTA AGTTCATGCCGGCTTG for MR; AACAGCAACCTGCTCATC GAGTAC and TTCGCTCTTCTCCTGTGGTGTGGG for tTG; AACCATAAGGACGCGGACTTG and TTGACTGCTGAGCAT CACCC for BDNF. All these primers were generated from the respective mRNA sequences obtained from the National Center for Biotechnology Information (NCBI) gene bank. The following program was used: initial denaturation step for 10 min at 95°C, amplification for 45 cycles (10 s denaturation at 95°C, 6s annealing at 62°C, 10 s polymerization at 72°C), and melt-curve analysis (5 s at 95°C, 1 min at 65°C and 97°C, 0.1°C/s). The specificity and identity of the amplified products were verified as follows: (i) the melting curve analysis showed a single melting peak after amplification, and (ii) amplified products for each gene were verified by sequencing with the Big Dye Terminator v1.1 (Applied Biosystems, Foster City, CA, USA). The GAPDH housekeeping gene was used as reference gene for relative quantification, and sample equality was verified by analyzing the expression of GAPDH. The results were normalized by the ratio of the relative concentration of the target to that of GAPDH in the same sample. Quantification of the data was performed using the LightCycler480 Relative Quantification software (version 1.5). To compensate for differences in target and reference gene amplification efficiency, either within or between experiments, this software provides a calibrator–normalized relative quantification including a PCR efficiency correction. Therefore, the results are expressed as the target/reference ratio divided by the target/reference ratio of the calibrator. In our case, the calibrator was chosen among the cells in control conditions. We verified that the expression level of the reference gene GAPDH was unaffected by our different treatments.

Western blots

Protein extraction, electrophoresis, and transfer were performed as described (Mingam *et al.* 2008). The cells were washed with ice-cold PBS, scraped off, and centrifuged (285 g, 5 min, 4°C). The cell pellets were crushed in a lysis buffer [Tris-HCl pH7.5 20 mM, EDTA 1 mM, $MgCl_2$ 5 mM, Dithiothreitol (DTT) 1 mM, NaOV 1 mM, NaF 1 mM, and a protease mix inhibitor (Sigma-Aldrich P2714)] on ice (incubation 30 min). The samples were centrifuged (13 000 *g*, 20 min, 4°C). Protein concentration was assessed by bicinchoninic acid protein assay (Uptima, Montluçon, France). Proteins (40 µg) were loaded on Sodium dodecyl sulfate polyacrylamide gel electrophoresis gels (SDS-PAGE gels) (10%), transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and incubated overnight with primary and 1 h with appropriate secondary antibodies. Primary antibodies were diluted as follows: rabbit anti-actin (Sigma Aldrich, A2066) 1 : 2500; mouse anti-RARβ (Santa Cruz Biotechnology, Dallas, Texas, USA, sc-56864) 1 : 500; rabbit anti-GR (Santa Cruz Biotechnology, sc-1004) 1 : 10 000; mouse anti-tTG 2 (Abcam, Cambridge, UK, ab2386) 1 : 500; rabbit anti-BDNF (Abcam, ab108383) 1 : 5000. Secondary horseradish peroxidase-conjugated antibodies were diluted as follows: donkey anti-mouse (Jackson Immunoresearch, Westgrove, PA, USA) 1 : 5000; donkey anti-rabbit (Jackson Immunoresearch) 1 : 5000. The blots were developed using Western Lighting Chemiluminescence Reagent Plus (PerkinElmer). Actin was used as a housekeeping gene. The density of the band on the membranes was quantified with a Syngene (Saint Quentin en Yvelines, France) detection system.

Quantification of tTG activity

The activity of tTG was quantified on cytosolic protein by detecting the incorporation of [3H]-putrescine (PerkinElmer) into NN-dimethylcasein (Sigma Aldrich) as previously described (Piacentini *et al.* 1986; Alfos *et al.* 1996). The cells were washed with ice-cold PBS, scraped off, and centrifuged (285 *g*, 5 min, 4°C), lysed in an extraction buffer (saccharose 0.25 M, trisma pH7 50 mM, EDTA 1 mM), and centrifuged (105 000 *g*, 1 h, 4°C). The lysate was mixed with the reaction buffer (Trisma buffer pH 8.3 50 mM, $CaCl_2$ 5 mM, DTT 10 mM, NaCl30 mM, NN'-dimethylcasein 3 mg/mL, putrescine 0.2 mM and [3H]-putrescine 1 µCi to 10-300 µg of protein) and incubated for 30 min, at 37°C, in a shaking water bath. Hundred microliter samples were spotted on Whatman 3MM filter paper moistened with 20% trichloroacetic acid (TCA). Free [3H]-putrescine was eliminated by washing with large volumes of cold TCA 5% containing KCI 0.2 M. Filters were transferred into vials with scintillation fluid and radioactivity was quantified using a βcounter.

Enzyme-linked immunosorbent assay of BDNF concentration

BDNF concentrations in the cell culture medium were quantified following manufacturer's instructions with the ChemiKine™ BDNF Sandwich ELISA Kit (Millipore).

Immunolocalization and immunofluorescence quantification

After 4 days treatments (seeding density: 750 cells/cm²), the cells were fixed (paraformaldehyde 4%, 15 min) and permeabilized (Triton X-100 1%, 3 min). Specificity was prevented by incubation (1 h) with PBS/bovine serum albumin (3%). tTG, BDNF, RARβ, and GR were detected using corresponding antibodies diluted as follows: mouse anti-tTG2 (Abcam, ab2386) 1 : 50; rabbit anti-BDNF (Abcam, ab108383) 1 : 100; mouse anti-RARβ (Santa Cruz Biotechnology, sc-56864) 1 : 50; mouse anti-RARβ (Abcam, H00005915-B01P), rabbit anti-GR (Santa Cruz Biotechnology, sc-1004) 1 : 50; incubation time was 3 h. The cells were subsequently incubated with appropriate secondary Alexa fluor

antibodies for 1 h (Molecular Probes, Van Allen Way Carlsbad, CA, USA, A11005, A21206, A11005, A21206; 1 : 1000). Next, cells were observed using an epifluorescence microscope (Leica AF DMI6000, Nanterre, France). The results were analyzed and quantified using the Metamorph software (Molecular Device, St. Grégoire, France).

Background was subtracted in each treated image using Metamorph software. All images were thresholded (using inclusive threshold). Cytosolic and nuclear compartments were distinguished by mounting the blades on slides with Vectashield with, 6-diamidino-2-pnenylindole (DAPI) (data not shown). Antibody specificity in immunochemistry was verified with data obtained from a commercial supplier or by previous publications (tTG (Scarpellini *et al.* 2009), BDNF (commercial supplier of www.labome.com), GR (Mikkonen *et al.* 2010). We used two antibodies directed against RARβ (one polyclonal and one monoclonal); both elicited similar staining.

Data analysis

Unless otherwise indicated, all data were expressed as mean ± SEM, calculated for at least three independent experiments. The statistical significance of the differences between multiple groups was determined using the non-parametric Kruskal–Wallis' test. When the statistic was associated with a $p < 0.05$ probability, intergroup comparisons were conducted using the Mann–Whitney *U*-test.

Results

RA and Dex modify RARβ, GR, tTG and BDNF, mRNA expression

RARα, RARβ, RARγ, RXRα, RXRβ, RXRγ, MR, GR, tTG, and BDNF mRNA were quantified after a 4d treatment with RA or Dex, concentrations from 10^{-10} to 10^{-6} M.

RA significantly increased RARβ mRNA expression even at the lowest RA concentration (more than fivefold at 10^{-6} M) (Fig. 1a, left panel). Dex increased RARβ mRNA expression only for the highest concentrations (10^{-7} and 10^{-6} M, more than threefold at 10^{-6} M) (Fig. 1a, right panel).

RA did not significantly affect RARα, RXRα, and RXRβ mRNA expression even at 10^{-6} M but increased RARγ mRNA expression (increase: 54.7 ± 6.3% at 10^{-6} M) (not shown). Dex did not affect RXRβ mRNA expression but

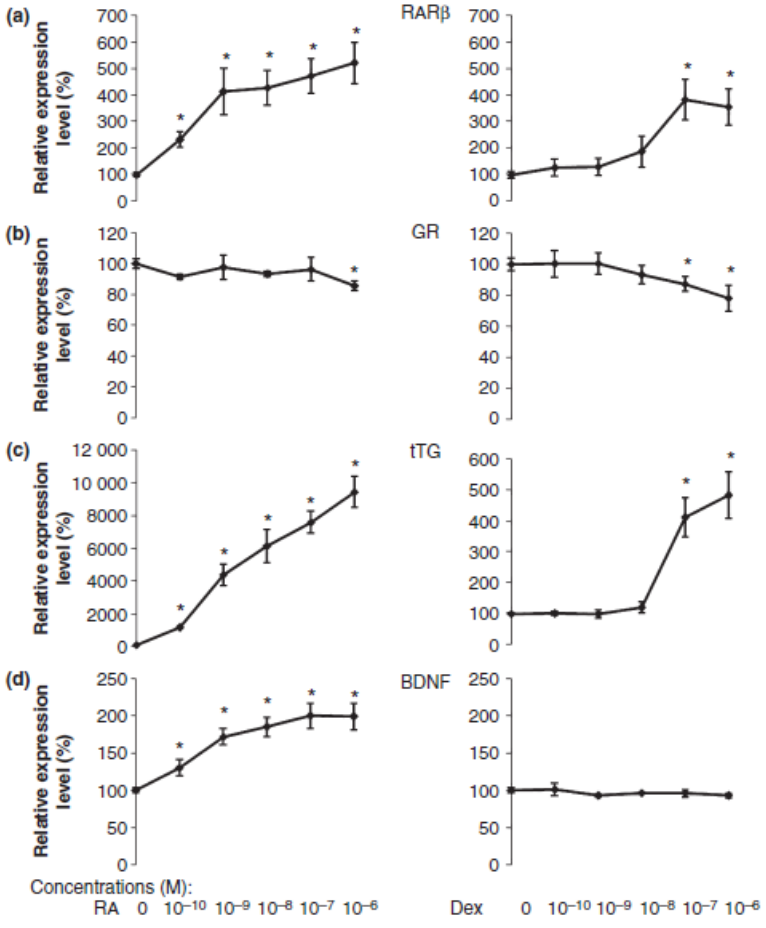

Fig. 1 Effects of retinoic acid (RA) and dexamethasone (Dex) on retinoic acid receptor (RAR)β, glucocorticoid receptor (GR), tissue transglutaminase (tTG), and brain-derived neurotrophic factor (BDNF) mRNA expression. RARβ (a), GR (b), tTG (c), BDNF (d) mRNA expressions upon increasing concentrations of RA or Dex (real-time PCR analyses, see Material and Methods). All mRNA were significantly modulated by RA or Dex treatments (10^{-6} M) except for BDNF in Dex-treated cells. Mean ± SEM from at least three independent experiments. *Significantly different from the control ($p < 0.05$).

significantly decreased RARα, RARγ, and RXRα mRNA expression at the highest concentration (decreases: 9.5 ± 3.4, 10.9 ± 3.4, and 8.4 ± 5.8%, respectively) (not shown). Finally, RXRγ mRNA was not detectable, regardless of conditions (not shown).

RA significantly decreased GR mRNA expression (decrease: 14.3 ± 3.3%) (Fig. 1b, left panel). Dex also decreased GR mRNA expression at the highest concentrations (10^{-7} and 10^{-6} M; decreases: 21.9 ± 8.5% at 10^{-6} M) (Fig. 1b, right panel). RA and Dex both increased MR expression (increases: 39.8 ± 8.0 and 45.5 ± 5.6%, respectively, at 10^{-6} M) (data not shown).

RA significantly increased tTG mRNA expression in a dose-dependent manner (10 to 100-fold) (Fig. 1c, left panel). An increased tTG mRNA expression was also observed with Dex, but only for the highest concentrations (10^{-7} and 10^{-6} M, about three-fold for 10^{-6} M Dex) (Fig. 1c, right panel).

RA significantly increased BDNF mRNA expression (about two-fold at 10^{-6} M), but Dex did not modify BDNF mRNA expression, whatever concentration was used (Fig. 1d).

RARβ, GR, and tTG mRNA were quantified after different durations of treatment (RA and/or Dex 10^{-6} M; 0.5 h, 1 h, 3 h, 1 day, and 4 day) (Fig. 2). As BDNF is a late gene, its expression was quantified at 24 h and 96 h). Some specific effects of RA alone versus Dex or RA+Dex were only seen after 96 h for RARβ and BDNF. On the basis of these results, a 96 h treatment and 10^{-6} M RA and Dex concentrations were selected to stimulate their respective pathways in our cell model.

HT22 cells viability, apoptosis and necrosis

After 4 day treatments by RA (10^{-6} M) and/or Dex (10^{-6} M), a MTT assay was performed to evaluate the proportion of viable cells under the different conditions. There was no significant difference of cell viability between control and RA- or Dex- or RA+Dex-treated cells (Fig. 3a).

Cell apoptosis and necrosis were evaluated by TUNEL after the same treatments (Fig. 3b and c). Control TUNEL-positive cells corresponded to 2.6 ± 0.6% total cells. There was no significant difference between the percentage of TUNEL-positive cells in control and RA- and/or Dex-treated cells.

Modulation of RARβ expression, abundance, and localization by RA or Dex treatment

As RARβ was the most affected RA receptor by RA or Dex (Fig. 1a) and as RARβ is a major mediator of RA (Ballow *et al.* 2003; Latasa and Cosgaya 2011), we focused on their regulation of RARβ.

The RARβ mRNA expression was significantly increased by RA, Dex, or RA+Dex although the increase elicited by Dex alone (about threefold) was significantly lower than the one observed with RA or RA+Dex (Fig. 4a).

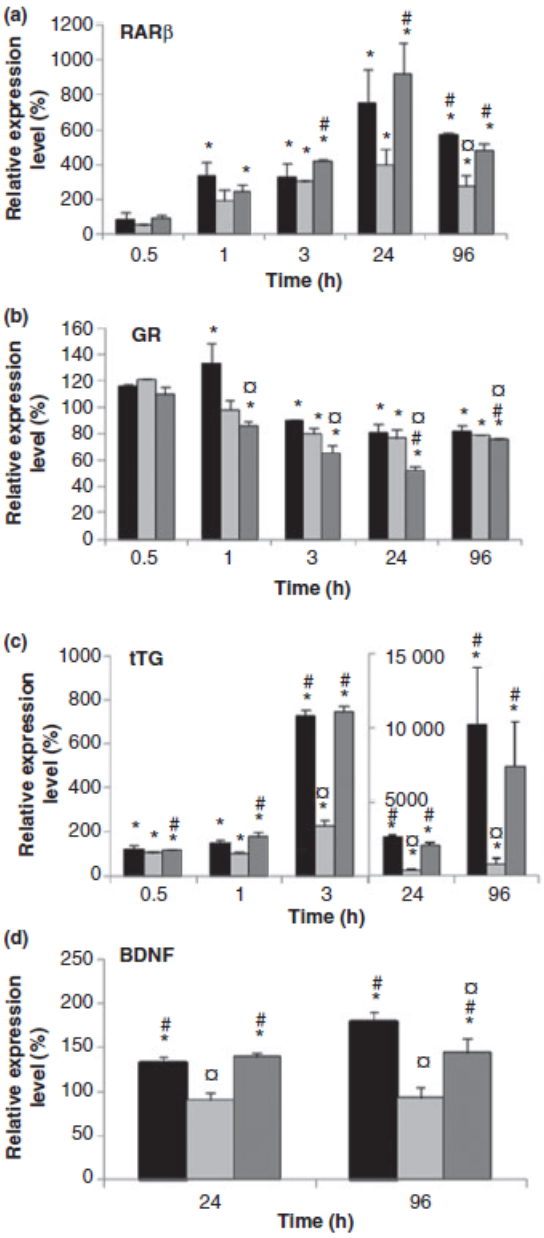

Fig. 2 Time-responses of retinoic acid receptor (RAR)β (a), glucocorticoid receptor (GR) (b), tissue transglutaminase (tTG) (c), and brain-derived neurotrophic factor (BDNF) (d) mRNA expression quantified by real-time PCR analyses. Effects of retinoic acid (RA) (10^{-6} M; black), dexamethasone (Dex) (10^{-6} M; light gray) or RA+Dex (dark gray) on retinoic acid receptor (RAR)β, GR, tTG, and BDNF mRNA expression. In this study, 96 h treatments best highlighted the specific effects of the three treatments on BDNF and RARβ expressions. Results are mean values ± SEM from at least three independent experiments. *Significantly different from the control (cells treated with vehicle); #significantly different from Dex treatment effect; ¤ significantly different from RA treatment. ($p < 0.05$).

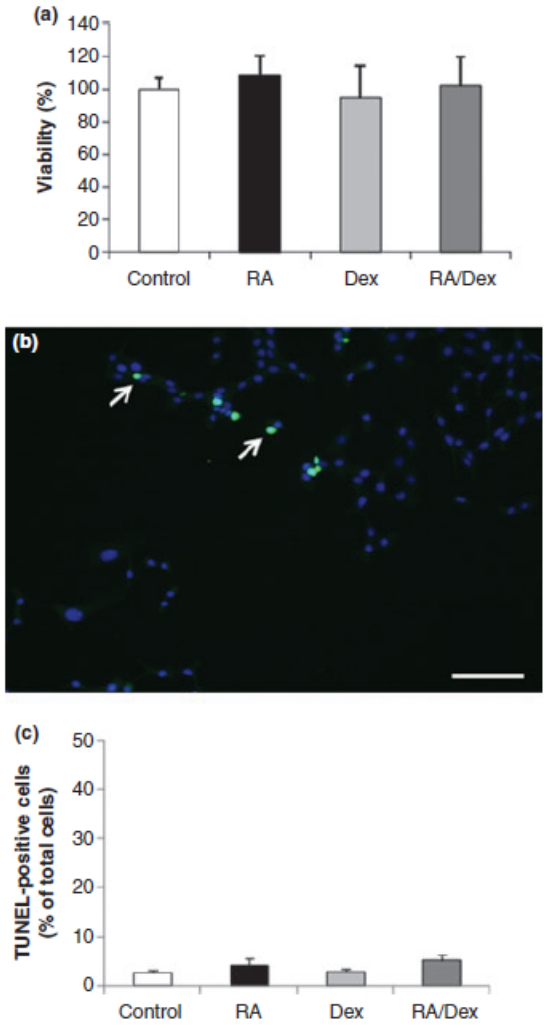

Fig. 3 Quantification of apoptotic and necrotic cells after treatment with retinoic acid (RA) or dexamethasone (Dex). Results of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on *RA* (10^{-6} M; black) Dex (10^{-6} M; light gray) or RA+Dex (dark gray) treated cells. No significant difference of cell viability was evidenced between control and treated cells (a). Apoptotic and necrotic cells detection (white arrows) after treatment with RA (10^{-6} M) (b). *In situ* nuclear DNA fragmentation was measured by the Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) technique. The image is representative of at least three separated sets of culture. Scale bar: 100 μm. The percentage of TUNEL-positive cells was determined in triplicate counts of 200 cells treated by RA (10^{-6} M) or Dex (10^{-6} M) (c). There was no significant difference between the rate of TUNEL-positive cells in control and RA- and/or Dex-treated cells. Mean ± SEM from at least three independent experiments.

Immunofluorescence quantification on plated cells showed that RA and Dex increased RARβ compared to control (increases: 123.6 ± 16.6% and 19.5 ± 4.0%, respectively) (Fig. 4b). RA+Dex led to an intermediate increase in RARβ abundance (34.7 ± 6.1%).

The subcellular localization of RARβ investigated by immunocytochemistry showed that in control as well as RA- and RA+Dex-treated cells RARβ was mainly present around the nucleus with a decreasing gradient toward the cell periphery (Fig. 4c). This gradient was lower in Dex-treated cells where the cytoplasmic localization in these cells was more homogenous (Fig. 4c).

Modulation of GR expression, abundance and localization by RA or Dex treatment

The GR mRNA expression was decreased by RA, Dex and RA+Dex (decreases: 18.3 ± 4.7, 20.9 ± 0.1 and 24.9 ± 1.2%, respectively) (Fig. 5a). Moreover, the RA+Dex-dependent decrease was significantly greater than that because of RA or Dex treatments alone.

The GR abundance was quantified by Western blots (Fig. 5b). It was not affected by RA, but significantly decreased by Dex and RA+Dex when compared to control (decreases: 41.3 ± 5.8 and 25.2 ± 10.6%, respectively). There was no statistically significant difference between the effect of Dex and RA+Dex.

The GR subcellular localization showed that in control or RA-treated cells, GR displayed a perinuclear localization (Fig. 5c). In Dex- and RA+Dex-treated cells, GR was mainly translocated into the nucleus (Fig. 5c).

Effects of RA and Dex treatments on tTG expression, abundance, activity, and localization

The tTG mRNA expression was increased by RA (Fig. 6a) and similar results were obtained in the presence of RA+Dex (more than 100- and 70-fold, respectively). Dex increased tTG mRNA expression compared to control, but significantly less than RA or RA+Dex (eight-fold increase compared to the control).

The tTG abundance was significantly increased by RA (variation of 516.1 ± 51.6%) (Fig. 6b). The increase induced by Dex was more moderate but still different from control (variation 162.3 ± 23.5%). Finally, RA+Dex led to a significant intermediate increase (variation of 270.7 ± 39.9%).

The tTG activity evaluated by incorporation of [3H]-putrescine was significantly increased by RA and significantly less by RA+Dex (15699 ± 6803 and 7306 ± 225%, respectively) (Fig. 6c). The increased tTG activity observed with Dex was significant although smaller than with RA (521 ± 172%).

The tTG subcellular localization showed that in control, RA- and RA+Dex-treated cells, tTG was mainly concentrated around the nucleus with a decreasing gradient toward the cell periphery (Fig. 6d). In Dex-treated cells, tTG was localized differently; it was distributed throughout the cells (Fig. 6d).

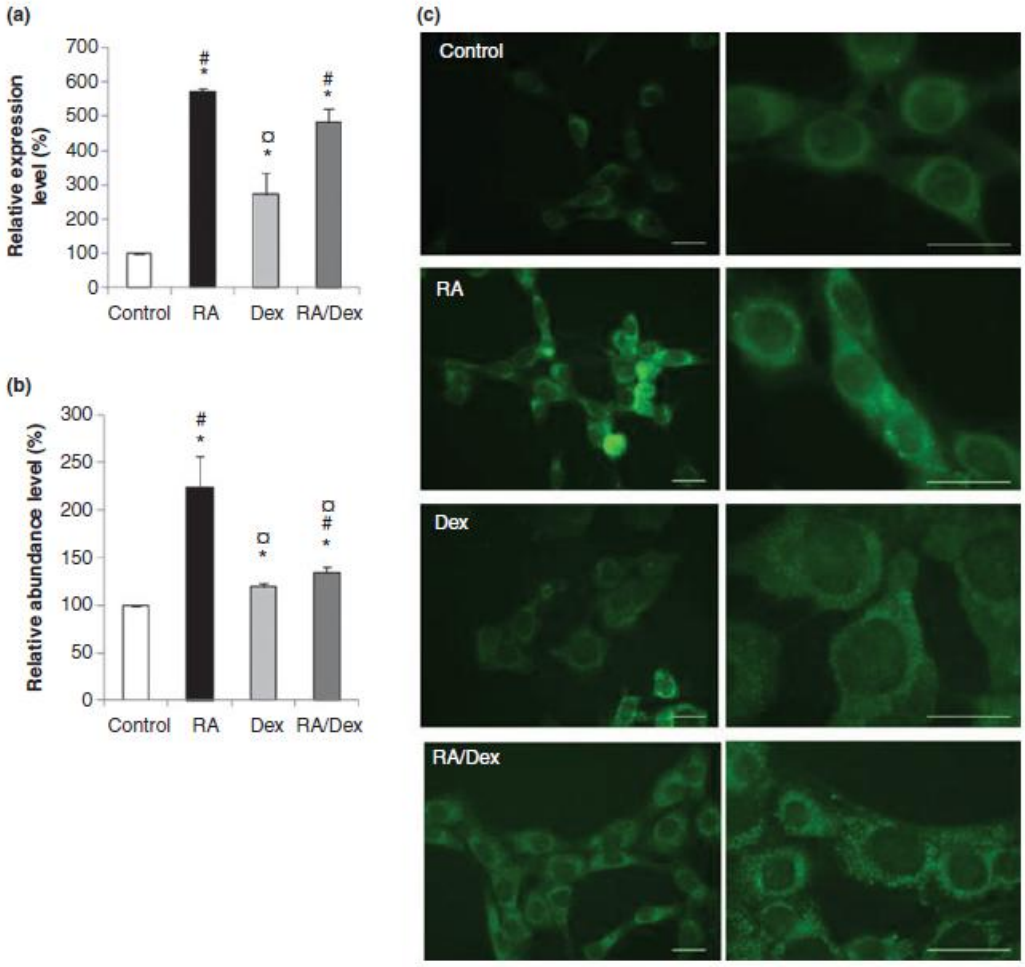

Fig. 4 Consequences of retinoic acid (RA) or glucocorticoid pathways activations in retinoic acid receptor (RAR)β regulation. Quantifications of RARβ expression (a), by real-time RT-PCR and abundance by immunofluorescence quantification on plated cells (b), after 4d treatments with RA (10^{-6} M) or dexamethasone (Dex) (10^{-6} M). Dex increased in RARβ mRNA and protein expression but less than with RA or RA+Dex. RA-Dex led to an intermediate level of RARβ abundance. Mean ± SEM from at least three independent experiments. *Significantly different from the control, # from Dex-treated cells; ¤ from RA-treated cells ($p < 0.05$). Immunocytochemical localization of RARβ (c) in untreated and RA- and/or or Dex-treated cells: for all conditions, RARβ was concentrated around the nucleus with a decreasing gradient toward the cell periphery. The images are representative of at least three separated sets of culture. Scale bar: 20 μm.

Effects of RA and Dex treatment on BDNF expression, abundance, secretion, and localization

The BDNF mRNA expression was only modulated by RA and RA+Dex (increases: 179.7 ± 10.8 and 144.3 ± 16.2% for RA- and RA+Dex-treated cells, respectively) (Fig. 7a). Interestingly, the increase in BDNF expression was significantly higher with RA alone than with RA+Dex. Dex tended to but did not influence BDNF mRNA levels.

The intracellular BDNF abundance assessed by Western blots was similar regardless of the treatment (data not shown). As BDNF is a secreted protein, the effect of these treatments was evaluated on the secretion of BDNF from these cells (Fig. 7b). RA and RA+Dex significantly increased BDNF secretion (157.8 ± 16.2 and 134.3 ± 5.4%, respectively). Conversely, Dex significantly decreased BDNF secretion when compared to controls (70.7 ± 5.4%).

The BDNF subcellular localization showed that in control cells, BDNF was peripheral to the nucleus in secretory vesicles (Fig. 7c). After RA, BDNF was localized in cells extensions (Fig. 7c). After Dex and RA+Dex treatments, the distribution of BDNF was less perinuclear than in control cells, more diffuse in the cytoplasm (Fig. 7c).

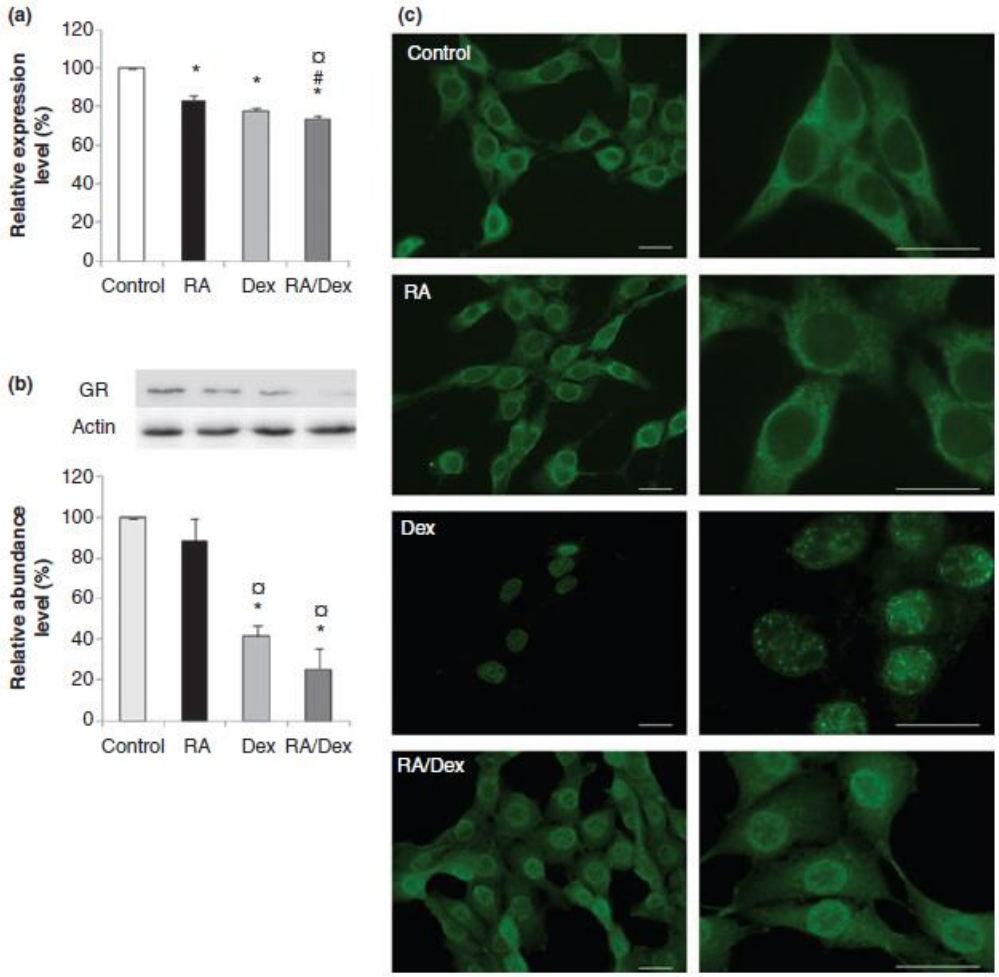

Fig. 5 Consequences of retinoic acid (RA) or glucocorticoid pathways activations in glucocorticoid receptor (GR) regulation. Quantification of GR: mRNA expression by real-time RT-PCR (a) and abundance by western blots (b), after 4 day treatments with RA (10^{-6} M) or dexamethasone (Dex) (10^{-6} M). RA+Dex treatment led to a greater decrease of GR mRNA level than RA or Dex treatments. GR abundance was significantly decreased in cells treated with Dex or RA+Dex. Mean ± SEM from at least three independent experiments. *Significantly different from the control, # from Dex-treated cells; ¤ from RA-treated cells ($p < 0.05$). Immunocytochemical localization of GR (c) in untreated and RA- and/or Dex-treated cells: in control cells and in cells RA-treated, GR was cytoplasmic. It translocated into the nucleus with Dex and RA+Dex. The images are representative of at least three separated sets of culture. Scale bar: 20 μm.

Discussion

We investigated genes involved in neuronal plasticity that can be used to decipher interactions between retinoids and glucocorticoids signaling pathways in hippocampal cells. As unavoidable when using cell lines in experiments, data will be best confirmed with *in vivo* studies. We used cells from the HT22 line because of its mouse hippocampal origin, which were of interest to us in relation with prior work on memory and aging in mouse (Etchamendy *et al.* 2001; Mingaud *et al.* 2008), and HT22 known sensitivity to glucocorticoids (Behl *et al.* 1997). The use of *in vitro*-grown fetal cells would not be suitable here because of the major role played by RA in neuronal differentiation (Maden 2007; Rhinn and Dolle 2012).

We first investigated a primary level of interaction between the retinoids and glucocorticoids signaling pathways by questioning the reciprocal influence of their ligands on the nuclear receptors. The up-regulated expression of RARβ, in agreement prior studies of neuronal or non-neuronal cells (Ballow *et al.* 2003; Coste and Labbe 2011; Latasa and Cosgaya 2011), is explained by the presence of a RA-responsive element in the RARβ gene (De The *et al.* 1990). This RA-induced increase of RARβ expression was reduced

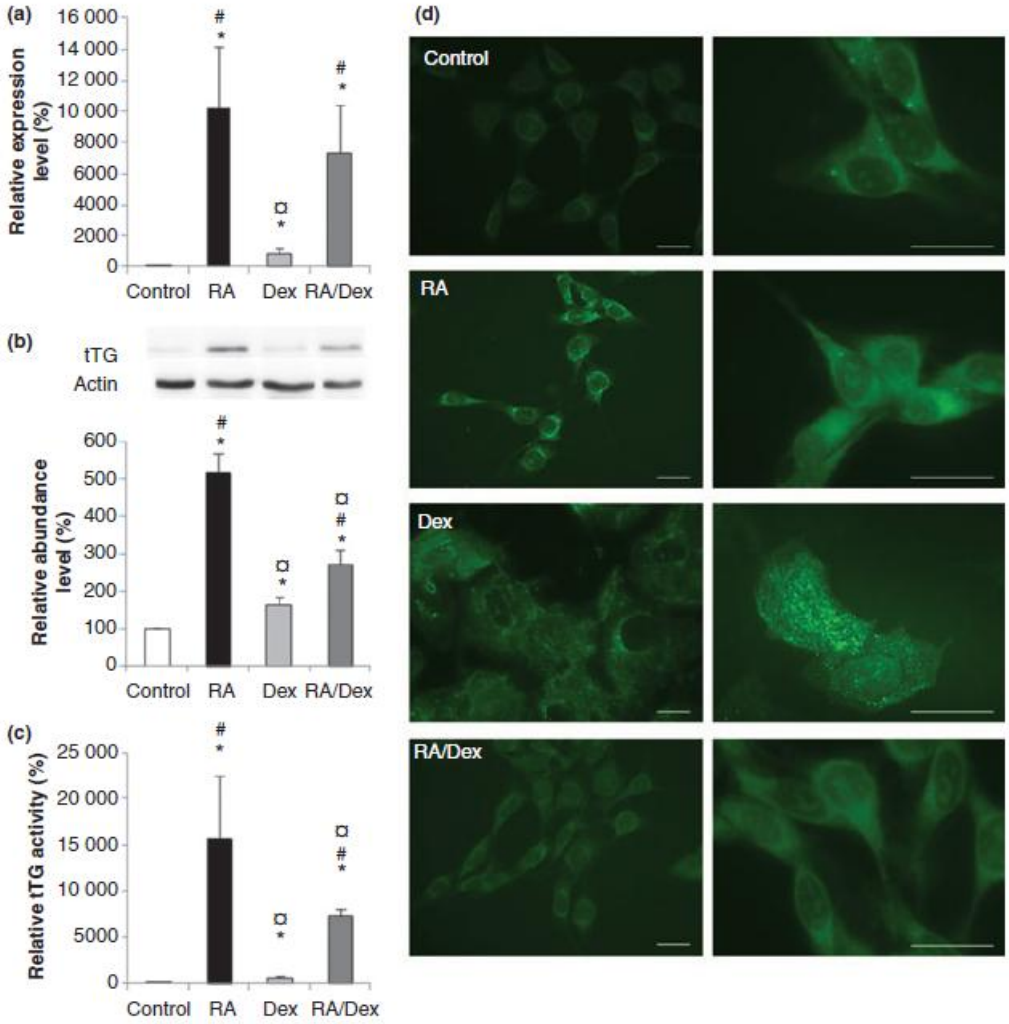

Fig. 6 Implication of retinoic acid (RA) and glucocorticoid pathways in tTG regulation. Quantification of tTG: mRNA expression by real-time RT-PCR (a), abundance by western blots (b) and activity (c) after 4 day treatments with RA (10^{-6} M) or dexamethasone (Dex) (10^{-6} M). All treatments significantly increase tTG expression, abundance, and activity. The RA-induced increase was higher than that induced by RA+Dex, itself higher than that induced by Dex. Mean ± SEM from at least three independent experiments. *Significantly different from the control, # from Dex-treated cells; ¤ from RA-treated cells ($p < 0.05$). Immunocytochemical localization of tTG (d) in untreated and RA- and/or Dex-treated cells: in control cells, RA- and RA+Dex-treated cells, tTG was concentrated around the nucleus. In Dex-treated cells, tTG was uniformly distributed throughout the cells. The images are representative of at least three separated sets of culture. Scale bar: 20 μm.

by Dex (although not significantly for RARβ mRNA). This contrasts with another study where Dex enhanced the RA-dependent increase in RARβ mRNA (Yamaguchi *et al.* 1999). Many negative interactions may occur, including a reduced half-life of RARβ mRNA induced by RA or Dex (George *et al.* 1998). Alternatively, the presence/absence of co-repressors or co-activators of RARβ and GR may explain the inverse effects of the combination of RA and Dex. Moreover, gene regulation in the brain subserves specific functions different from those of other cells. Finally, we observed RARβ in the cytoplasm as described in breast and neuroblastoma cells (Sommer *et al.* 1999; Dey *et al.* 2007; Masia *et al.* 2007). This suggests non-genomic pathways through: phosphatidylinositide-3-kinase (Ohashi *et al.* 2009), calmodulin kinase (Liu *et al.* 2009b), MAP kinase (Okamoto *et al.* 2000). This may be the basis for cross talks between RA-stimulated kinase cascades and genomic pathways to regulate myriads of proteins including the receptors themselves (Rochette-Egly 2005; Rochette-Egly and Germain 2009; Boldizsar *et al.* 2010; Galliher-Beckley *et al.* 2011).

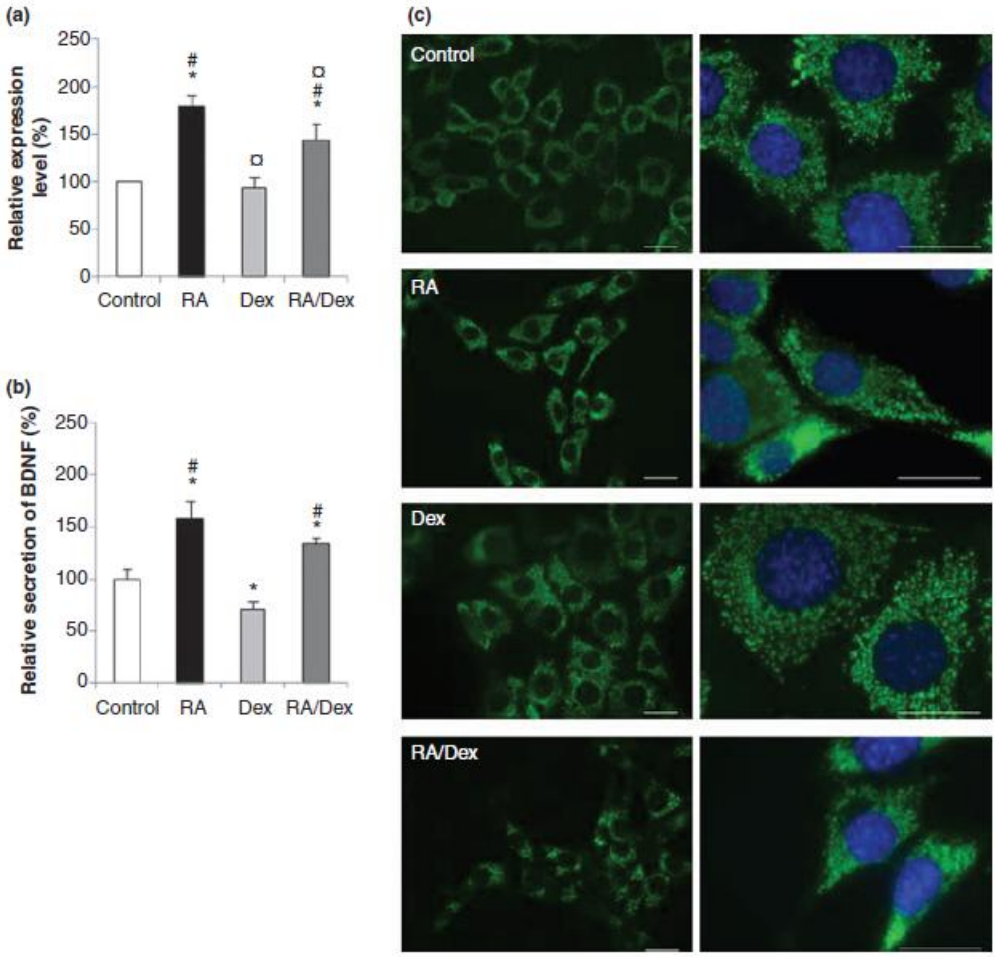

Fig. 7 Implication of retinoic acid (RA) and glucocorticoid pathways in brain-derived neurotrophic factor (BDNF) regulation. Quantification of BDNF: mRNA expression by real-time RT-PCR (a), secretion (b). BDNF expression was only modulated with RA or RA+dexamethasone (Dex) treatments which increase BDNF secretion. Mean±SEM from at least three independent experiments. *Significantly different from control, # from Dex-treated cells; ¤ from RA-treated cells ($p < 0.05$). Immunocytochemical localization of BDNF (c) in untreated and RA or Dex-treated cells: in control cells, BDNF had perinuclear localization. Treatment with RA led to BDNF migration to the cell extremities. In Dex- and RA+Dex-treated cells, BDNF was less perinuclear than in controls and more diffuse in the cytoplasm. The images are representative of at least three separated sets of culture. Scale bar: 20 μm.

Synaptic reorganization occurs after simultaneous MR and GR activation by endogenous glucocorticoids. Conversely, exclusive GR activation triggers neuronal abnormalities more deleterious than the dual activation (Oliveira *et al.* 2006; Sousa *et al.* 2008). Thus, we focused our attention on GR: RA and Dex decreased GR expression; the combined treatment elicited a more pronounced effect. A Dex-dependent GR expression decrease in the hippocampus was known (Reul *et al.* 1989; Numakawa *et al.* 2009). Conversely, data on down-regulation in HT22 cells seems less established. Wang *et al.* (2002) found that in their HT22 clone "GR levels remained relatively unchanged" after 1 μM Dex for 3 days (4 days here) contrasting with a major decrease in a hepatoma cell line. From the 1st to the 4th day in our conditions, this decrease reached a significant level but was more modest: about 40% on day 4. This could be in agreement with a negligible Dex-induced degradation of the GR protein (Wang *et al.* 2002) and a modest but continuous decrease of GR mRNA. A stable presence of GR in the nucleus under prolonged Dex treatment is found both by Wang *et al.* and ourselves. Wang *et al.* reported that the lack of major disappearance of GR could be related to proteasome-ubiquitine activity (Wang and DeFranco 2005). Whether this could explain these small differences between our subclones of HT22 is unknown.

Interestingly, the Dex-stimulated pathway has here a more prominent action on GR expression when co-stimulated by

RA: this is a different result from the one described above for RARβ. Thus, functionally, RA could contribute to reduce the deleterious effects of GR activation through amplification of GC-elicited GR negative feedback loop. As expected, Dex-induced GR translocation into the nucleus (Sarabdjitsingh *et al.* 2010).

Thus, retinoids and glucocorticoids signaling pathways interact by at least reciprocally influencing the expression of their receptors, RARβ and GR. We then investigated whether other genes expressed in neurons could be targeted to and differently regulated by these pathways. We focused on genes involved in neuronal plasticity: tTG and BDNF.

The former, tTG, plays a role in neuronal differentiation and function (Facchiano *et al.* 2010). The stimulation of tTG expression and function under RA or Dex treatments is consistent with results obtained in hepatoma cells (Fukuda *et al.* 1994). Interestingly, here, the RA+Dex treatment was reduced tTG expression and function compared with RA alone. Conversely, in hepatoma cells, the RA+Dex treatment increased both RA and Dex responses. However, results similar to ours were obtained in myeloma cells (Lefebvre *et al.* 1999). As described above for the receptors, it is possible that depending on the origin of the cells, factors – different from the receptors and targeted by RA and Dex – could be involved.

We observed a more diffuse cytoplasmic expression in Dex-treated cells compared to controls or RA-treated cells. This localization is consistent with descriptions of cytosolic tTG in various cells (Aeschlimann *et al.* 1995; Akimov and Belkin 2001; Scarpellini *et al.* 2009). Several studies have shown that RA stimulates the expression of tTG and elicits apoptosis despite being up-regulated in various malignant tissue (Chen and Mehta 1999; Pasquali *et al.* 1999; Ou *et al.* 2000; Fok *et al.* 2006; Verma *et al.* 2006; Yuan *et al.* 2007). The RA and Dex concentrations used here were used previously (Lefebvre *et al.* 1999; Yamaguchi *et al.* 1999; Ballow *et al.* 2003; Numakawa *et al.* 2009; Latasa and Cosgaya 2011) and were not deleterious despite massive increase of tTG. We observed neither modification of cell proliferation nor apoptosis possibly because tTG among other roles could act as a pro- or anti-apoptotic factor depending on multiple factors (cell type, localization).

The second target protein we investigated, BDNF, is a neurotrophin, essential to neuronal growth and differentiation (Lindvall *et al.* 1994; Korte *et al.* 1998; Ernfors and Bramham 2003). Changes in BDNF levels in the hippocampus are associated with pathological conditions, such as Alzheimer's disease and depression (Nagahara *et al.* 2009; Yulug *et al.* 2009). Here, in control HT22 cells, BDNF is cytoplasmic as described in hippocampal neurons (Tang *et al.* 2010; Park and Loh 2011). Dex decreases BDNF expression in colon carcinoma (Kino *et al.* 2010), cortical neurons (Numakawa *et al.* 2009) or in hippocampus (Murakami *et al.* 2005; Duman and Monteggia 2006; Wei *et al.* 2010). BDNF secretion was clearly reduced by Dex in HT22. Conversely, RA-stimulated BDNF secretion is in agreement with results found in midbrain neurons (Katsuki *et al.* 2009; Kurauchi *et al.* 2011). This RA-induced secretion was reduced by Dex to be intermediate between RA-stimulated and Dex-inhibited. Thus, here, RA and Dex have opposite effects on the secretion of a neurotrophin. As seen for GR, RA may minimize the potentially deleterious effect of a GC-decrease in the neurotrophin BDNF in the hippocampus. This restoration of normal BDNF levels may improve neuronal plasticity in stress condition. This result can be related to the fact that RA administration restores adult rat hippocampal plasticity (Bonnet *et al.* 2008). As during aging, there are decreased bioavailability of RA and increased GC levels, RA supplementation may represent a potential therapeutic tool to minimize the deleterious effects on memory and hippocampal plasticity during aging.

Interestingly there may be some relationships between tTG activity and neurotransmitter secretion, here BDNF. Indeed, tTG inhibits BDNF processing in primary rat neurons and tTG inhibitors stimulate catecholamine release from rat brain synaptosomes (Borrell-Pages *et al.* 2006). However, despite an increased tTG in Dex-treated cells, BDNF secretion was decreased. Multiple mechanisms must be involved including the above-mentioned variations of tTG targets. Thus, it would be of interest to correlate the effect of RA and Dex on phenotypic changes related to neuronal plasticity. *In vivo* and *in vitro* studies investigating cell phenotype, and gene involved in neurite growth, synapse genesis, etc., are being carried out presently in the laboratory.

In this study, we studied genes that could be used to investigate interactions between retinoids and glucocorticoids signaling pathways in a hippocampal cell line to better understand the mechanisms that occur during brain aging. A first level of interaction is modifications in the amount of molecules participating in the pathways (Grummer and Zachman 1998; Yamaguchi *et al.* 1999). Indeed, here, RARβ and GR are regulated by their own and reciprocal ligands, generating a closed feedback loop. A second level of interaction is direct or indirect (e.g., via co-factors) relationships between these receptors. This second level co-exists here as targets not involved in the pathways (involved in neuronal plasticity) were differentially affected by RA and Dex. A direct mechanism has been described; RAR/RXR heterodimers interact with a ligated GR, resulting in an enhanced transcriptional activity of GR (Toth *et al.* 2011). Alternatively, indirect mechanisms have been described through the regulation of the expression of proteins (Aubry and Odermatt 2009), action of co-factors of transcription (Wang *et al.* 2004), kinases pathways (Rochette-Egly 2005; Rochette-Egly and Germain 2009). This neuronal model could thus be valuable to understand GC-RA interactions in the hippocampus during cerebral plasticity aging.

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Conflict of interest

The authors declare that they have no conflict of interest.

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B. PUBLICATION N° 6 : Modification de la phosphorylation du récepteur aux glucocorticoïdes par l'acide rétinoïque

Retinoic acid modified phosphorylated status of the glucocorticoid receptor

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En préparation

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Running title: retinoids and phosphorylation of glucocorticoid receptor

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Keywords: retinoic acid, glucocorticoids, cyclin-dependent kinase 5, phosphorylation, brain neurotrophic factor, tissue transglutaminase 2, hippocampus.

Abbreviations used: BDNF, brain-derived neurotrophic factor; Cdk, cyclin-dependent kinase; Cmax, nuclear binding capacity; Dex, dexamethasone; Erk, extracellular signal-regulated kinase; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GC, glucocorticoids; GR, glucocorticoid receptor; GRE: glucocorticoids responsive element; hsp70, heat shock protein 70; LBD, ligand binding domain ; MAPK, mitogen-activated kinase; MR, mineralocorticoid receptor; PKA, protein kinase A; pp5, protein phosphatase 5; PI3K, phosphoinositide 3-kinase; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid responsive element; Rosco, roscovitine; RXR, retinoic X receptor; ser, serine; SNF2, sucrose nonfermentable protein 2; tTG, tissue transglutaminase.

Introduction

Glucocorticoids (GC), hormonal end-product of the hypothalamic-pituitary-adrenal axis, are a major component of the endocrine response to stress. While short-term exposure to stress affects brain functions to facilitate the adaptation to environmental challenges, long-term exposure may eventually results in maladaptation and ultimately in disease (McEwen 2007). In the central nervous system for instance, prolonged stress is known to elicit various harmful effects particularly in the hippocampus (Alfarez et al. 2002; Conrad 2006; Krugers et al. 2006). The effects of GC are mediated by GC binding to glucocorticoid or mineralocorticoid receptors (GR and MR, respectively) (Herbert et al. 2006; Sousa et al. 2008) (Harris et al. 2013). The GC-dependent GR activation elicits their nuclear translocation and homodimerisation. GR then interacts with target gene promoters on glucocorticoid-responsive elements (GRE) and recruit transcriptional machinery *i.e.* various coactivators and corepressors to regulate the transcription of GRE-containing genes (Nicolaides et al. 2010). Importantly, GR subcellular localization, transcriptional activity and turnover are largely modulated by several GC-induced phosphorylations on GR serine or threonine (Orti et al. 1989; Bodwell et al. 1991; Ismaili and Garabedian 2004; Galliher-Beckley and Cidlowski 2009). In mouse, phosphorylation of serine 220 (ser220) of mGR (232 on rGR or 211 in hGR), of ser212 in mGR (224 in rGR or 203 in hGR) and of ser234 (246 in rGR or 226 in hGR) are the most frequently studied (Chen et al. 2008b). GR phosphorylation of ser220 (ser220pGR) is implicated in the GR-dependent transcriptional activity and chronic GC effects (Wang et al. 2002; Chen et al. 2008b; Adzic et al. 2009) while phosphorylation of ser212 affects non-genomic activity. Ser234 has been described in acute GC effects and in the enhancement of nuclear ser220pGR export (Galliher-Beckley and Cidlowski 2009). The two major kinase families responsible of GR N-terminal phosphorylation are the mitogen-activated protein kinases (MAPK) and the cyclin-dependent kinases (CDK) (Krstic et al. 1997; Blum and Maser 2003). In the absence of ligand, GR is phosphorylated on three sites (S203, S211, S226) and then is rapidly dephosphorylated by protein phosphatase(s) associated with the ligand binding domain (LBD), including protein phosphatase (PP5) complexed with Hsp90. Ligand binding releases the LBD-associated protein phosphatase(s) and the kinases gain access to the GR serines to phosphorylate them (Wang et al. 2007).

Retinoic acid (RA) is crucial for fetal brain development but also for adult brain plasticity (Shearer et al. 2012). For instance RA modulates hippocampus plasticity through various processes such as neurite outgrowth and neuronal (Etchamendy et al. 2001; Maden 2007; Mingaud et al. 2008; Shearer et al. 2012). We previously hypothesised that *in vivo* beneficial effects of RA can be partly

explained by a modification of GC signalling pathway. We demonstrated that an interaction indeed exists between RA and GC pathways with, for instance, consequences on tissue transglutaminase 2 (tTG) and brain-derived neurotrophic factor (BDNF), two neuronal target proteins (Brossaud et al. 2013). Very few studies, investigated the mechanism involved in the interactions between RA and GC pathways (Subramaniam et al. 2003; Wang et al. 2004; Toth et al. 2011). Most known RA effects are mediated by nuclear heterodimers of RA receptors (RAR α, β or γ) and retinoid X receptor (RXR α, β or γ) which bind RA-responsive elements (RARE) in the regulatory region of target genes (Rochette-Egly and Germain 2009). In non-neuronal cells, two mechanisms of interaction have been respectively proposed: i) modification of cofactors recruitment on GR promoter by RA (Subramaniam et al. 2003; Wang et al. 2004) and ii) direct interaction between GR and RAR / RXR (Toth et al. 2011). However, recent studies describe the importance of non-genomic RA effects particularly in neurons (Canon et al. 2004; Chen et al. 2008b) Al Tanoury 2013). Indeed, RA is able to activate several kinase cascades such as p38 MAPK, extracellular-signal-regulated kinase p44/42 (Erk p44/42), PI3K or CDKs (Bour et al. 2007).

To better understand the interactions of GC and RA in neurons, we investigated here the effects of RA on the phosphorylation of GR and particularly on $_{pSer220}$GR. This led us to focus on the implication of a cyclin-dependent kinase, CDK5, in the RA-dependent effects. Indeed, CDK5 and its neuron-specific activators p35 and p25 are involved in $_{pSer220}$GR regulation particularly in the hippocampus (Kino et al. 2007; Adzic et al. 2009; Galliher-Beckley and Cidlowski 2009)., Furthermore, CDK5 plays an important physiological and pathological role in neuronal activity in other brain regions (Patrick et al. 1999; Cruz et al. 2003; Kumazawa et al. 2013). We show that i) ser220 phosphorylation status is modified both by RA and by a GR agonist, dexamethasone (Dex) and ii) CDK5 plays a key role in the RA-induced modulation of Dex-dependent GR transcriptional activity.

Material and methods

Cell Cultures

For experiments, HT-22 cells were cultured for 4d in Dulbecco's Modified Eagle Medium with antibiotics and pyruvate supplemented with 10% charcoal-depleted foetal bovine serum as previously described (Brossaud et al. 2013). The cells were seeded in plates (3000 cells/cm² for protein analysis, or 1500 cell/cm² for transfection or BDNF secretion experiments).Treatments consisted in all-trans RA (final concentration 10^{-6} M), Dex (final concentration 10^{-6} M) or RA+Dex (final concentrations 10^{-6} M for both). RA and Dex stock solutions (Sigma Aldrich, St. Louis, MO, USA; 17.5 and 10mmol/L, respectively) were diluted into ethanol:dimethylsulfoxide (DMSO, 50:50) (VWR International, West Chester, PA, USA) added as vehicle in controls.

Roscovitine (Rosco; a specific CDK5 inhibitor) (Cell Signalling, stock solution (2.8 mM) was prepared into ethanol:DMSO 50:50 and added to the culture medium to a 20 µM final concentration. When needed, roscovitine treatment was added for 2 d (after 2 d of Dex & RA treatments at the change of medium time-point). A brief, 1 h, incubation was also tested after 4 d of Dex & RA treatments.

Western blots

Protein extraction, electrophoresis and transfer were performed as described (Brossaud et al. 2013). Cytoplasmic and nuclear cells fractions were obtained using a centrifugation at 2500g during 5 min at 4°C. The supernatant (cytoplasmic protein extract) was separated from the pellet (nuclear protein extract). Nuclear fraction was recuperated in 50µL of the lysis buffer and sonicated few seconds. Primary antibodies were diluted as follows: rabbit anti-GADPH (Sigma Aldrich, A2066) 1:2500; mouse anti-RARβ (Santa Cruz Biotechnology, Dallas, Texas, USA, sc-56864) 1:500; rabbit anti-GR (Santa Cruz Biotechnology, sc-1004) 1:10000; rabbit anti-GR phosphorylated on serine 220 (Cell Signaling,. sc-1004) 1:500; mouse anti-tTG 2 (Abcam, Cambridge, UK, ab2386) 1:500; mouse anti-CDK5 (SantaCruz Biotechnology, , ab2386) 1:500; rabbit anti-p35/p25 (SantaCruz Biotechnology, , ab2386) 1:500.

Transfection and luciferase assay

Cells were transfected 18 h before the end of the 4 d experiment using Lipofectamine 2000 Reagent (Invitrogen, Van Allen Way Carlstbad, CA, USA), with (i) either 500 ng specific reporter vectors pTAL (control condition) or pGRE (Clontech, Mountain View, CA, USA) each containing a GRE cis-acting DNA response element upstream of the firefly luciferase reporter gene, and (ii) 25 ng of phRL-TK vector encoding the Renilla reniformis luciferase (Promega, Charbonnières,

France). The cells were lysed on day 4 and the enzymatic activities of the two separate reporter enzymes (renilla and firefly) were measured with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions, with a VICTOR 3V Multilabel Plate Readers (Perkin Elmer, Waltham, MA, USA).

Enzyme-linked immunosorbent assay (ELISA) of BDNF concentration

BDNF concentrations in the cell culture medium were quantified following manufacturer's instructions with the ChemiKine BDNF Sandwich ELISA Kit (Millipore).

Data analysis

Unless otherwise indicated, all data were expressed as mean ± standard error of the mean (SEM) calculated for at least three independent experiments. The statistical significance of the differences between multiple groups was determined using the non parametric Kruskal-Wallis' test. When the statistic was associated with a $p<0.05$ probability, intergroup comparisons were conducted using the Mann-Whitney's test.

Results

RA modulates the GR phosphorylation of Ser220 but not the phosphorylation of Ser234

We hypothesized that the modulation of the GC effects by RA was, at least in part, due to modifications of the GR phosphorylation status. We investigated the $_{pSer234}$GR expression but in the HT22 cells we found a low expression with no difference between the experimental conditions (data not shown). Conversely, exploration of total and phosphorylated GR level in the cytoplasm or the nucleus showed important modifications of $_{pSer220}$GR expression. In both cellular compartments, Dex treatment increased $_{pSer220}$GR expression (Figure 1A). In the cytoplasm, RA, with or without Dex, had no effect on $_{pSer220}$GR expression. By contrast, RA alone decreased nuclear $_{pSer220}$GR expression while RA/Dex increased the nuclear $_{pSer220}$GR expression. In the same time, total GR expression decreased after Dex treatment in both compartments (Figure 1B). RA treatment did not affect the cytoplasmic total GR expression while in nucleus RA decreased total GR expression to Dex level. In all conditions, there was no significant difference of total GR expression between Dex and AR/Dex treatments.

Finally, the nuclear $_{pSer220}$GR / total GR ratio expression was significantly higher in RA/Dex *vs.* Dex condition but there was no difference between control and RA treatment. In the cytoplasm compartment, there was no difference between control *vs.* RA treatments or between Dex *vs.* RA/Dex treatments (Figure 1C).

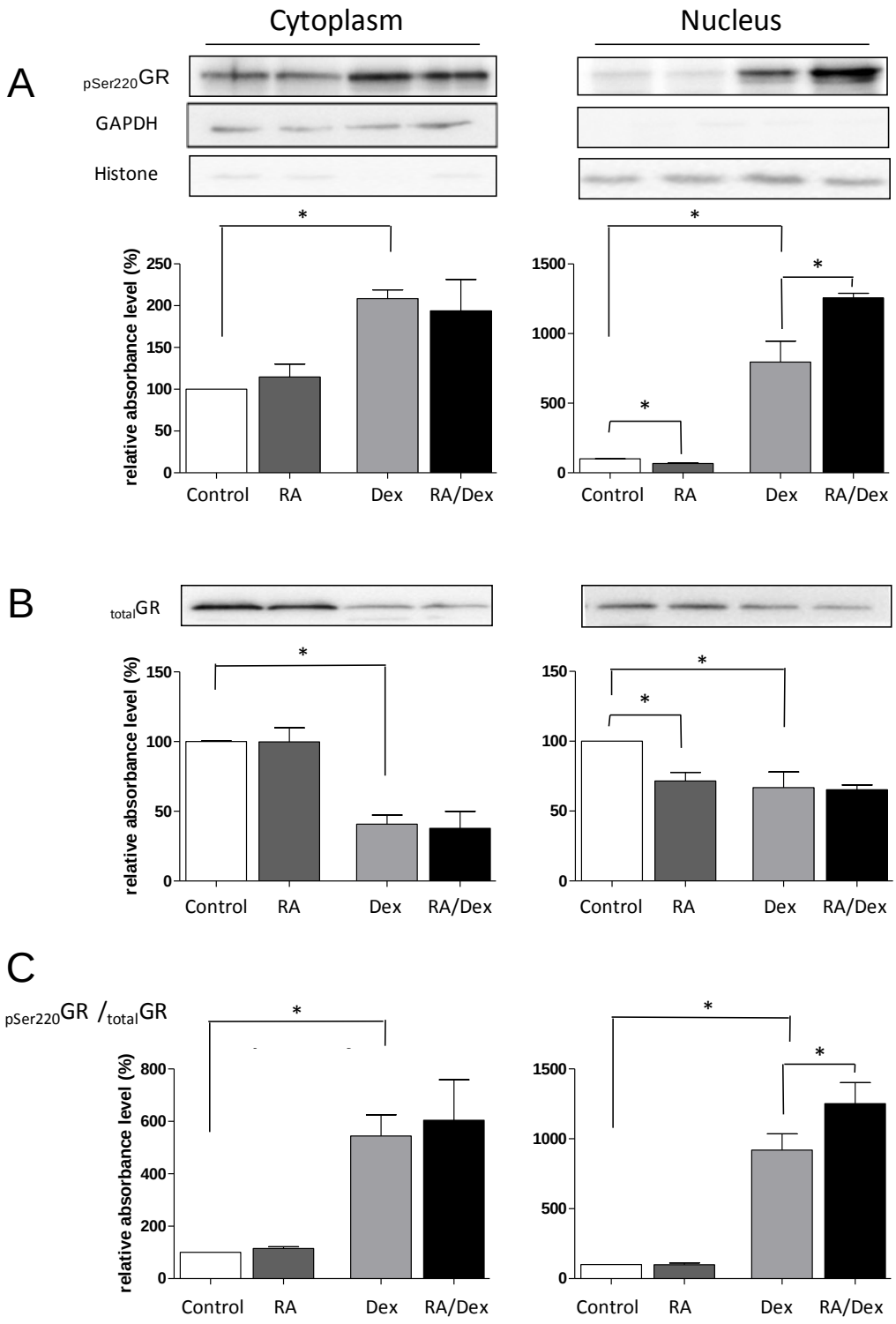

Figure 1

Effects of RA and Dex on the Ser220-phosphorylated GR expression ($_{pSer220}$GR) (A), on the total GR expression (GR) (B) and on the $_{pSer220}$GR / total GR expressions ratio ($_{pSer220}$GR/GR) (C) in the cytoplasm and in the nucleus analysed by Western Blot (WB) after 4 d treatments with vehicle (Control), 10^{-6} M retinoic acid (RA), 10^{-6} M dexamethasone (Dex) or a co-treatment with RA and Dex (RA/Dex) (10^{-6} M). Mean ± SEM from at least 3 independent experiments. * Significantly different from the controls ($p<0.05$).

RA modifies GR-induced transcriptional activity

We investigated whether conditions that modify $_{pSer220}$GR expression also modify GR-induced transcriptional activity using a reporter gene with GRE. Dex induced a large increase of GR-induced transcriptional activity. Conversely, RA decreased the transcriptional activity both in absence and in presence of Dex (Figure 2).

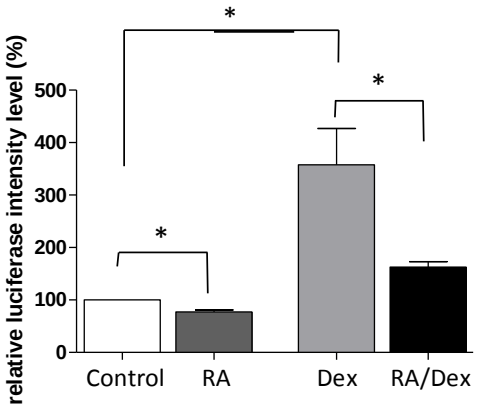

Figure 2

Effects of RA and Dex on the GR-induced transcriptional activity. HT22 cells were transfected with a reporter plasmid pGRE after 4 d treatments with vehicle (Control), 10^{-6} M retinoic acid (RA), 10^{-6} M dexamethasone (Dex) or a co-treatment with RA and Dex (RA/Dex) (10^{-6} M). Mean ± SEM from at least 3 independent experiments. * Significantly different from the controls ($p<0.05$).

RA effects differ in brief vs long cell exposure

The abovementioned RA-induced modulation of $_{pSer220}$GR expression after a 4 d treatment could depend on rapid or slow effects of RA e.g. the latter depending on RNA and protein synthesis (kinase, cofactors etc) and the former depending on enzyme activity (kinase, phosphatase etc). We thus investigated the effects of a 1 h RA ± Dex treatment on $_{pSer220}$GR expression. The consequences on nuclear $_{pSer220}$GR expression were clearly different from those with a 4 d treatment (Figure 1A). A brief exposure to RA had no effect on $_{pSer220}$GR expression whereas associated to Dex RA decreased nuclear $_{pSer220}$GR expression compared to Dex alone (Figure 3). As this effect was not seen after a 4 d exposure we hypothesized that RA could modify the expression/activity of intermediate factor(s) in these 4 d.

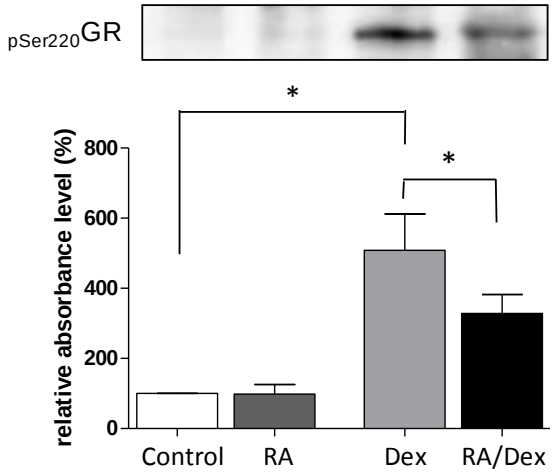

Figure 3

Effects of RA and Dex on the Ser220-phosphorylated GR expression ($_{pSer220}$GR) in the nucleus analysed by Western Blot (WB) after 1 h treatments with vehicle (Control), 10^{-6} M retinoic acid (RA), 10^{-6} M dexamethasone (Dex) or a co-treatment with RA and Dex (RA/Dex) (10^{-6} M). Mean ± SEM from at least 3 independent experiments. * Significantly different from the controls ($p<0.05$).

RA modulate the CDK5 expression and p35/p25 expression ratio in the cytoplasm

As $_{pSer220}$GR expression depends on CDK5 and p35/p25 expression (kino 2007, adkic 2009), we investigated if our experimental conditions could modify CDK5 or p35/p25 expression in the cytoplasm of HT22 cells. CDK5 expression was increased by RA and a milder but significant increase of CDK5 expression was elicited by Dex (Figure 4A). Conversely, RA failed to increase CDK5 levels in Dex-treated cells.

p35 expression tended to increase after RA treatment but this did not reach statistical significance (Figure 4B). Conversely, we observed a significant increase of the p35/p25 expression ratio after RA treatment both in absence and in presence of Dex.

Of note, in our Dex-treated cells, CDK5 and p35 mRNA expressions were not modified by RA (data not shown).

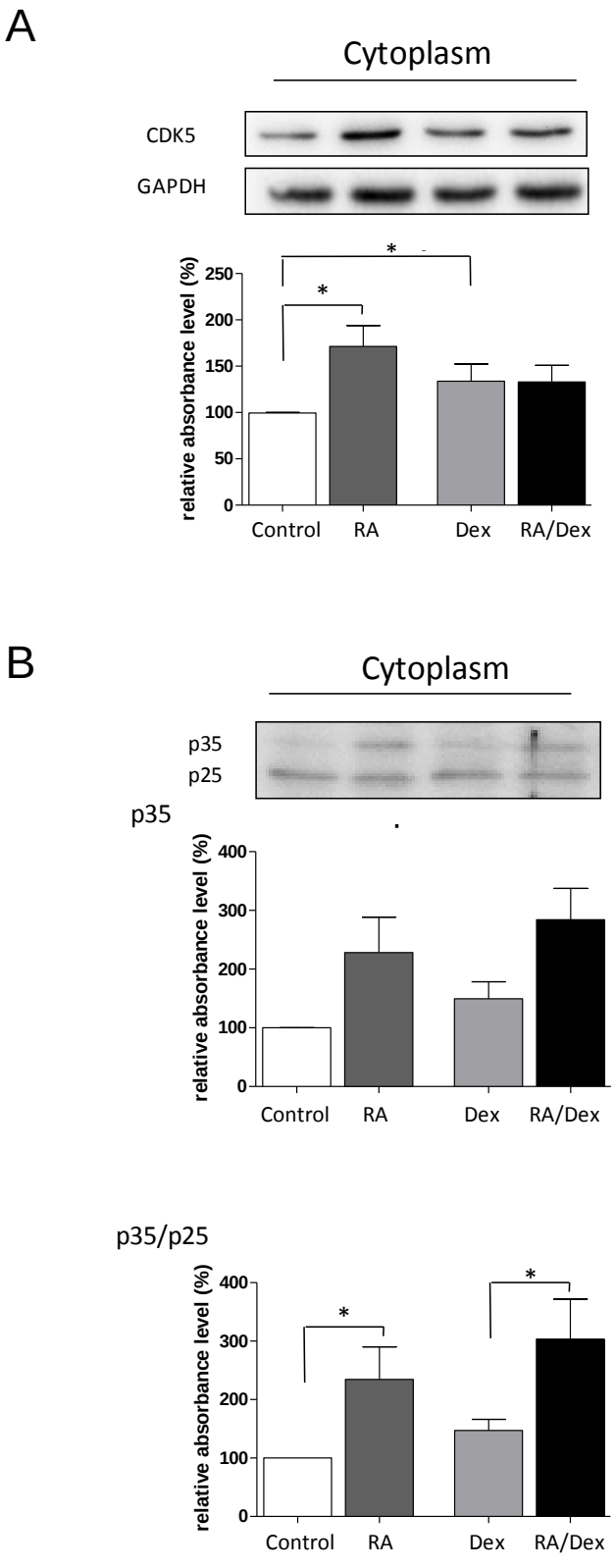

Figure 4

Effects of RA and Dex on the CDK5 (A) and on the p35 and p25 expression ratio (p35/p25) (B) in the cytoplasm analysed by Western Blot (WB) after 4 d treatments with vehicle (Control condition), 10^{-6} M retinoic acid (RA), 10^{-6} M dexamethasone (Dex) or a co-treatment with RA and Dex (RA/Dex) (10^{-6} M). Mean ± SEM from at least 3 independent experiments. * Significantly different from the controls ($p<0.05$).

CDK5 and p35/p25 are implicated in the RA-dependent $_{pSer220}$GR *expression*

To support the CDK5 and p35/p25 implication in the RA-dependent modulation of the $_{pSer220}$GR expression, we investigated the effect of roscovitine, a specific CDK5 inhibitor, in our experimental conditions. Roscovitine decreased the cytoplasmic $_{pSer220}$GR expression both in Dex and RA/Dex conditions (Figure 5). In the nuclear compartment, roscovitine suppressed the RA-dependent increase of the Dex-induced $_{pSer220}$GR expression. Unexpectedly, Rosco increased the nuclear Dex-induced $_{pSer220}$GR expression.

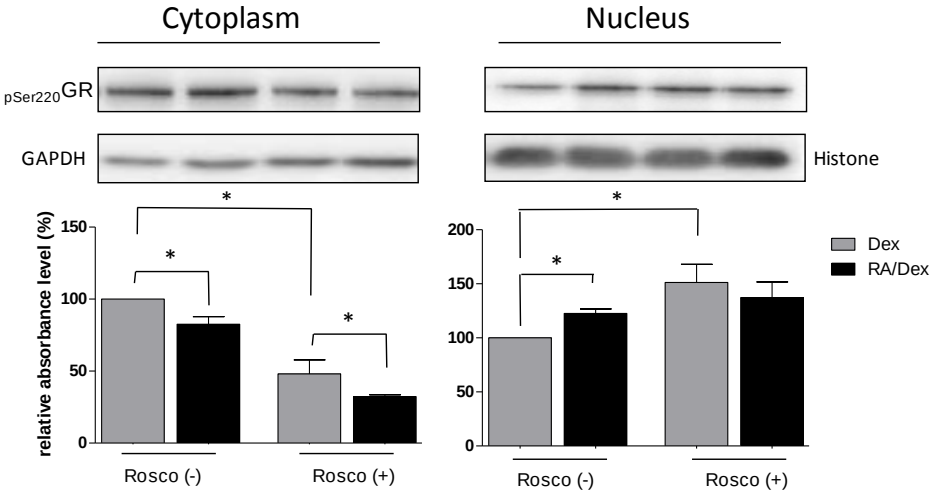

Figure 5

Effects of RA and Dex in presence of Roscovitine (Rosco), a CDK5 inhibitor, on the Ser220-phosphorylated GR expression ($_{pSer220}$GR) in the cytoplasm and in the nucleus analysed by Western Blot (WB) after 4 d treatments with 10^{-6} M dexamethasone (Dex) or a co-treatment with RA and Dex (RA/Dex) (10^{-6} M). Rosco (20 µM) was added after 2 d of cell culture. Mean ± SEM from at least 3 independent experiments. * Significantly different from the controls ($p<0.05$).

CDK5 and p35/p25 are implicated the modulation of GR-induced transcriptional activity by RA

We investigated whether roscovitine could modify GR-induced transcriptional activity using a reporter gene with GR response elements. roscovitine significantly reduced Dex-dependent GR-induced transcriptional activity (Figure 6A). roscovitine had no significant effect in RA/Dex-treated cells. However, a -60% decrease of GR-induced transcriptional activity was observed in RA/Dex condition *vs.* -34% decrease by RA/Dex + roscovitine. Thus, the RA-dependent decrease of GR-induced transcriptional activity was less important in presence of roscovitine.

We also investigated whether roscovitine could modify GR-induced transcription of tTg and BDNF. Roscovitine suppressed the RA-dependent increase of Dex-induced tTg expression. Indeed, there was no significant difference between tTG expressions after Dex +Rosco and RA/Dex + Rosco treatments (Figure 6B).

Similar results were obtained with BDNF. Roscovitine suppressed the RA-dependent increase of Dex-induced BDNF secretion (Figure 6C).

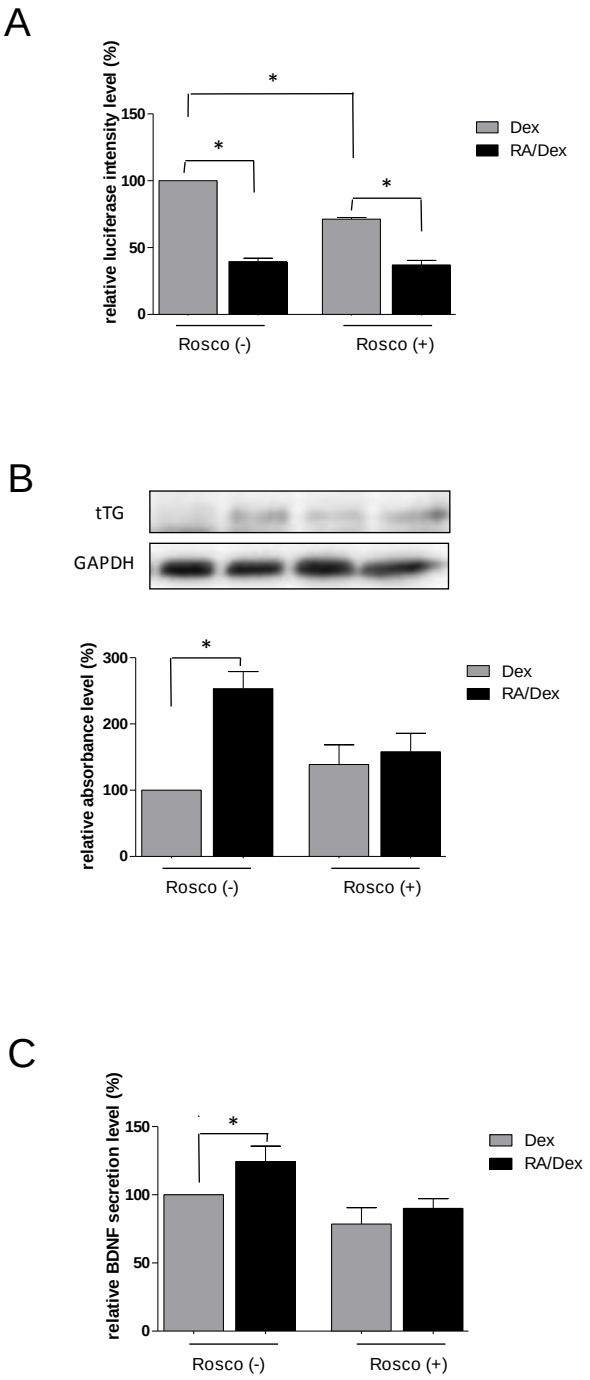

Figure 6

Effects of RA and Dex in presence of Roscovitine (Rosco), a CDK5 inhibitor, on the GR-induced transcriptional activity. HT22 cells were transfected with a reporter plasmid pGRE after 4 d treatments with 10^{-6} M dexamethasone (Dex) or a co-treatment with RA and Dex (RA/Dex) (10^{-6} M) (A), on the tissue transglutaminase 2 (tTG) (B) and on the brain-derived neurotrophic factor (BDNF) expression (C). tTG expression was analysed by Western Blot and BDNF expression by ELISA after 4 d with 10^{-6} M dexamethasone (Dex) or a co-treatment with RA and Dex (RA/Dex) (10^{-6} M). Rosco (20 µM) was added after 2 d of cell culture. Mean ± SEM from at least 3 independent experiments. * Significantly different from the controls ($p<0.05$).

Discussion

RA and GC pathways interact in hippocampal neurones. At least part of this interaction rests on reciprocal modulated transcription of their receptors, RARβ and GR (Brossaud et al. 2013). We however hypothesised that the modulation of the expression of these receptors may not fully explain the opposite effects of RARβ and GR agonists on proteins critical for neuronal plasticity. For instance, Dex reduces the expression of tTG when associated with RA despite stimulating its expression when used alone (Brossaud et al. 2013). Considering the known arguments for a cytoplasmic action of RAR (Rochette Egly 2009) and for GR sensitivity to phosphorylation (kino 2007, ismalli 2004), we investigated RA-dependent modifications of GR signalling through modification of GR phosphorylation status.

The expected increase of $_{pSer220}$GR in the presence of the GR agonist Dex was found both in the cytoplasm and the nucleus along with an increase of GR-induced transcriptional activity. While RA had no effect on cytoplasmic $_{pSer220}$GR expression, when added to Dex RA increased nuclear $_{pSer220}$GR expression above the effects of Dex alone. These results mirror the increased nuclear binding capacity of GR (Cmax) after a combined RA/Dex treatment obtained *in vivo* in rats (Audouin-Chevallier et al. 1995). Interestingly, GR Cmax directly depends on its phosphorylated status (Mendel et al. 1986). However, RA alone decreased nuclear $_{pSer220}$GR expression.

Thus, we evidenced an apparently paradoxical phenomenon as an increase of $_{pSer220}$GR expression in the nucleus can be in linked to an increase (Dex treatment) or a decrease (RA/Dex treatment) of the GR-induced transcriptional activity. Kino *et al* obtained very similar results in neuronal cells. An enhancement of the $_{pSer220}$GR expression was linked to an increase of the GR-induced transcriptional activity while, in presence of CDK35/p25, the increase of the $_{pSer220}$GR expression caused a decrease of the GR-induced transcriptional activity (Kino et al. 2007). Apart from Kino's study, the GR is described as more transcriptionally efficient when Ser220 is phosphorylated because of a conformational change and a subsequent increase of co-activators recruitment to GRE-containing promoters (Wang et al. 2002; Chen et al. 2008a; Chen et al. 2008b; Galliher-Beckley and Cidlowski 2009). Different kinases may be responsible of $_{Ser220}$GR phosphorylation: CDK5, Erk p44/42 or p38 MAPK (Krstic et al. 1997; Ismaili and Garabedian 2004; Miller et al. 2005; Kino et al. 2007). Although several publications show that RA is able to activate these kinases (Bour et al. 2007) RA does not seem to activate them directly in our model. Indeed, RA action appears after a long-lasting exposure to RA/Dex: a 1 h exposure actually decreased Dex-induced $_{pSer220}$GR expression. This suggests the existence of an intermediate factor

with a RA-dependent expression.

We focused here on CDK5 to explain the RA-dependent effect on the GC signalling for three reasons. Firstly, CDK5 is frequently described for its implication in the nervous system functions. Secondly, in the hippocampus $_{ser220}$GR phosphorylation is associated to chronic stress *via* CKD5 activation (Adzic et al. 2009). Thirdly, CDK5 modulated activity can explain our apparently paradoxical results of $_{ser220}$GR phosphorylation (Kino et al. 2007). Cdk5, a proline-directed serine/threonine kinase, is a member of CDK family (Lew et al. 1992). Cdk5 activity is tightly regulated by neuron-specific cyclin-related molecules p35 and p39 (Lew et al. 1994; Tsai et al. 1994). Regulated activity of Cdk5/p35 is essential for neuronal development and function (cytoskeleton/cell adhesion, neurogenesis, synaptic plasticity, cognition, and neuronal survival in embryo and adult) (Nikolic et al. 1996; Kumazawa et al. 2013; Sundaram et al. 2013) fisher j neurosc 2002). Under neuronal stress/insults, calpain, a Ca^{2+}-activated protease, cleaves p35 into two fragments (p10 & p25). p25 forms a more stable and hyperactive CDK5-p25 complex, causing aberrant hyperphosphorylation of tau and neurofilament proteins and induces neuronal cell death (Patrick et al. 1999; Lee et al. 2000). This phenomenon is linked to the development of neurodegenerative disorders, including Alzheimer's disease (Shukla et al. 2012).

Here, in Dex-treated cells, CDK5 expression was unchanged by RA. On the other hand, in Dex-treated cells, p35/p25 proteins ratio was increased by RA treatment. Some authors showed a similar increase of p35 by RA in neuronal cells supporting the effects of RA *via* CDK5 (Fu et al. 2002; Jamsa et al. 2004; Lee et al. 2004; Wang et al. 2006). Lee *et al*. proposed that the RA-induced neuronal differentiation can be caused by the activation of the non-genomic RA pathway. RA would increase Erk and cAMP-dependent protein kinase A (PKA) that in turn would increase CDK5 and p35 expressions. In our Dex-treated cells, CDK5 and p35 mRNA expression were not modified by RA. Thus, the RA-induced effects are probably not mediated by such an ERK activation (Lee et al. 2004).

We investigated CDK5 implication in RA effects by using roscovitine, a CDK5 inhibitor (ref). In Dex-treated cells, roscovitine suppressed the RA-induced increase of i) $_{Ser220}$GR phosphorylation, of ii) tTG expression and iii) BDNF secretion. Roscovitine failed however to totally suppress RA-induced effect on the GR-induced transcriptional activity on pGRE luciferase.

All together, these results suggest that CDK5 is implicated in RA-dependent modulation of GC pathway. The RA/CDK5-dependent GR phosphorylation cause a decrease of Dex effect: suppression of the inhibition of the BDNF expression, drastic inhibition of the luciferase expression and absence of an additive effect RA/Dex despite the stimulation observed when RA and Dex are

used alone.

Contrasting with a recent study (Kino et al. 2010), roscovitine had here no effect on the Dex-dependent decrease of BDNF or tTG protein expression. However roscovitine strongly decreased GR-induced transcriptional activity and concomitantly increased $_{Ser220}$GR expression in Dex-treated cells. A possible explanation to these paradoxical results may be the involvement of other phosphorylation sites on GR.

In conclusion, the results of this study demonstrate that RA modulates GC signalling by increasing $_{Ser220}$GR phosphorylation. This effect is mediated by the p35/CDK5 pathway with an increase of cytoplasmic p35/p25 ratio under RA stimulation. The increase of $_{Ser220}$GR phosphorylation might modify the recruitment of co- activators such as p300 and SNF2 (Kino et al. 2007).

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C. PUBLICATION N°7 : Effets des rétinoïques et des glucocorticoïdes sur la morphologie des cellules hippocampiques HT22

Introduction

Brain aging is marked by a decline of cognitive functions probably related to a decrease neuronal plasticity, the basis of learning and memory processes. Retinoic acid (RA, vitamin A metabolite) plays a major role in brain development [1] but its implication is still crucial in the adult brain through its involvement in cellular and synaptic plasticity [2-5]. Of note, it has been shown that retinoid signalling decreases with age [6-7]. RA main but not sole role is to regulate gene expression by binding heterodimers of nuclear receptors: RA receptors (RARα, β or γ) and retinoid X receptors (RXRα, β or γ) to RA-responsive elements (RARE). By contrast, aging is correlated to an increased signalling by glucocorticoids (GC) [8-10]. In humans, this is correlated with hippocampus-dependent memory impairment [9]. In mice, corticosterone affects synaptic potentiation and plasticity in the hippocampus [11-12]. These deleterious effects are mediated by GC binding to glucocorticoid and mineralocorticoid receptors (GR and MR, respectively) eliciting receptors nuclear translocation [13]. GR homodimers regulate gene expression by binding to GC-responsive elements (GRE) of target genes.

Some studies describe interactions between AR and GR signalling pathways in thymocytes cell death [14], in skeletal muscle where RA reduces GC sensitivity [15] or in hepatocytes where dexamethasone (Dex – a specific agonist of GR) enhances the RA-dependant increase in RARβ expression [16]. The opposite effects of RA and Dex on plasticity were both linked to non genomic pathway [17-18].

Neuronal plasticity is characterized by morphological changes directly dependent on the cytoskeleton dynamics. The cytoskeleton is composed of three major elements: microtubules, intermediate filaments and actin microfilaments. The latter are formed by polymerisation of

globular actin (G-actin) into filamentous actin (F-actin). The turnover of actin (polymerisation and depolymerisation) is – at least in part - under the control of calpains, calcium-dependent ubiquitous endoproteases [19]. Calpains are heterodimers encoded by capn1 and capn2, for the μ- and m-catalytic subunit (80 kDa), respectively and by the css1 and css2 genes for the small regulating subunit (30 kDa). Their activity is mainly regulated by calpastatin, their specific endogenous inhibitor.

To evaluate the interaction of RA and GC on a neuronal cytoskeleton target we questioned the effect of RA and Dex treatments on the morphology of hippocampic cells. We studied the phenotype of actin cytoskeleton and questioned the involvement of a modulated activity of calpains.

Materials and Methods

Cell cultures.

Cell cultures were performed as described previously [20]. Briefly, HT-22 cells were grown under 5% CO_2 at 37°C in DMEM (Dubelcco's modified Eagle medium, Life Technologies, Van Allen Way Carlsbad, CA, USA) with pyruvate supplemented with 10% fetal bovine serum (FBS – Life Technologies) and 1% streptomycin sulfate/phenoxypenicilinic acid.

For all experiments, the cells were placed for 48h in a quiescent medium (DMEM supplemented with 0.1% FBS).

Cells treatments.

RA and Dex treatments. Treatment consisted in all-trans RA (final concentration 10^{-6} M), Dex (final concentration 10^{-6} M), or RA/Dex (final concentration 10^{-6} for both). RA and Dex stock solutions (Sigma Aldrich, St Louis, MO, USA; 17.5 and 10 mM, respectively) were diluted into ethanol:dimethylsulfoxide (50:50) (VWR International, West Cheter, PA, USA) added as vehicle in

control). The duration treatment was 4 d [20].

Calpain inhibitor treatment. The chemical calpain inhibitor Calpeptin (Z-Leu-Nle-CHO) was obtained from Merck (Nottingham, UK) and used at final concentration of 50 µM. Calpeptin was added to culture medium 4 h before the end of experiments [21].

Morphological characterization and actin cytoskeleton organization analysis.

After 4 d treatments (seeding density: 7500 cells/cm²), the cells were fixed (paraformaldehyde 4%, 15 min) and their morphologies were observed in phase contrast. Images were analyzed using ImageJ software. Cell areas, nuclear area, nucleus/cytoplasm ratio and the degree of circularity were determined.

For actin quantification, F-actin was stained as previously described [21]. Briefly, control and RA, Dex and RA/Dex treated cells with or without Calpeptin were fixed (paraformaldehyde 4%, 15 min), permeabilized (Triton X-100 1%, 3 min) and F-actin was stained with phalloidin-fluorecein-5-isothiocyanate (FITC, 0.5 µM) and nucleus with 4'-6-diamino-2-phenylindole (DAPI, 1.5 µg/ml) for 40 min. After three washings, the cells were observed using an epifluorescence microscope (Leica AF DMI6000, Nanterre, France) or confocal microscopy (Olympus, Fluoview). Images were taken at 40x magnification and analyzed using ImageJ software. Tiff images were converted to 8-bit grayscale, calibrated in ImageJ to optical density (OD) values, and the background was subtracted. The threshold on the image was adjusted until actin fluorescence was excluded from the threshold limits [22]. The following measurements were made to quantify F-actin: F-actin area, cortical actin along cell borders, stress fibres that traverse the cell and stress fibres/cell area ratio. Cortical actin was detected and analyzed using the freehand selection tool. For stress fibres quantification, a line selection perpendicular to the stress fibres orientation was made (Fig. 1A). The ImageJ plot profile was used to obtain the stress fibres density (Fig. 1B).

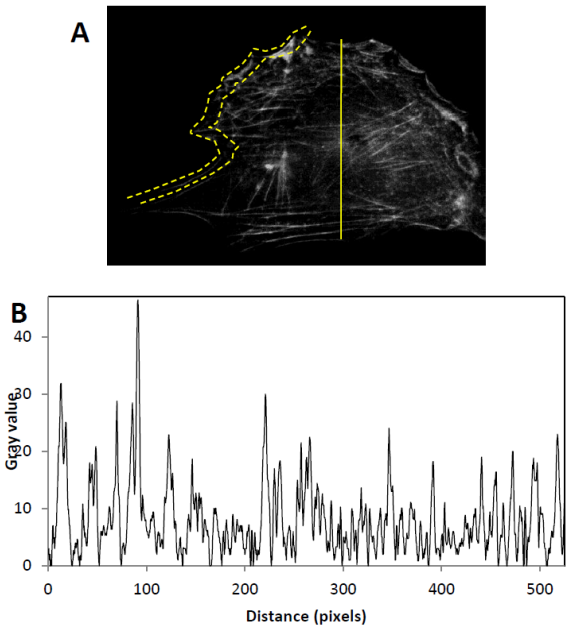

Figure 1. Example of cortical actin and stress fibres quantification. Cortical actin was detected and analyzed using the freehand selection tool ((A) dotted line). For stress fibres quantification, a line selection perpendicular to the stress fibres orientation was made ((A) solid line). The ImageJ plot profile was used to obtain the stress fibres density (B).

Real-time PCR analyses. Real-time PCR analyses were performed as described previously [20]. Specific primers were as follows: GAACATCATCCCTGCATCCA and CCAGTGAGCTTCCCGTTCA for Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) GGCTGTATTCCCCTCCATCG and CCAGTTGGTAACAATGCCATGT for Actin; TCCTGCAGATTGGTAAGTGC and GTGCAACCCTTTCAGCTCTC for Arc; ATGCGGTGGGCTCTATGGCTTCTG and TGCACACTTCCCTCTGCATGCACG for CaMKII.

Data analysis. Unless otherwise indicated, all data were expressed as mean + SEM, calculated for at least three independent experiments. The statistical significance of the differences between multiple groups was determined using non-parametric Kruskal-Wallis'test. When the statistic was associated with a $p < 0.05$ probability, intergroup comparison were conducted using Mann-Whitney U-test.

Results

Activation of GC signalling pathways leads to drastic morphological changes in hippocampal cell line and is modulated by RA treatment.

Previous studies showed that stress is associated in neurons with reduced dendritic branching and decrease in spine density [23-24]. By contrast, some studies showed that in hippocampal neurons, spine formation is induced by RA [17] and that BDNF enhances hippocampal dentritic branching [25]. Moreover, we have demonstrated that in hippocampal cells, RA treatment thwarted the deleterious effect of Dex treatment on BDNF [20]. To determine whether activation of RA and GC signalling pathways modify hippocampal cell morphology, we treated HT-22 cells with RA (10-6) and/or Dex (10-6) during 4 d and analyzed several morphological characteristics.by evaluating total cell and nucleus areas, the nucleus/cytoplasm ratios and the degree of the circularity of the cells.

The total area of RA-treated cells was significantly higher than control cell area but smaller than that of Dex treated cells (Fig. 2B). In Dex-treated cells, RA reduced cell area to control levels. Dex treatment also significant increased the nucleus area (Fig. 2C). RA suppressed (down to control level) this latter increase. RA treatment alone did not affect the nucleus/cytoplasm ratio (Fig. 2D), which was increased by Dex treatment and was intermediate when AR and Dex were combined.

In view of those morphological changes, we evaluated the degree of circularity of the cells (Fig. 2E). Total circle represents “1”: a perfect circle: the larger the shaded area of the disk, the larger the population of rounded cells and conversely, the smaller the white area, the larger the population of elongated cells. Compared to control cells, RA-treated cells exhibited a more elongated shape whereas Dex-treated cells exhibited a rounder shape. RA/Dex treated cells presented an intermediate morphology.

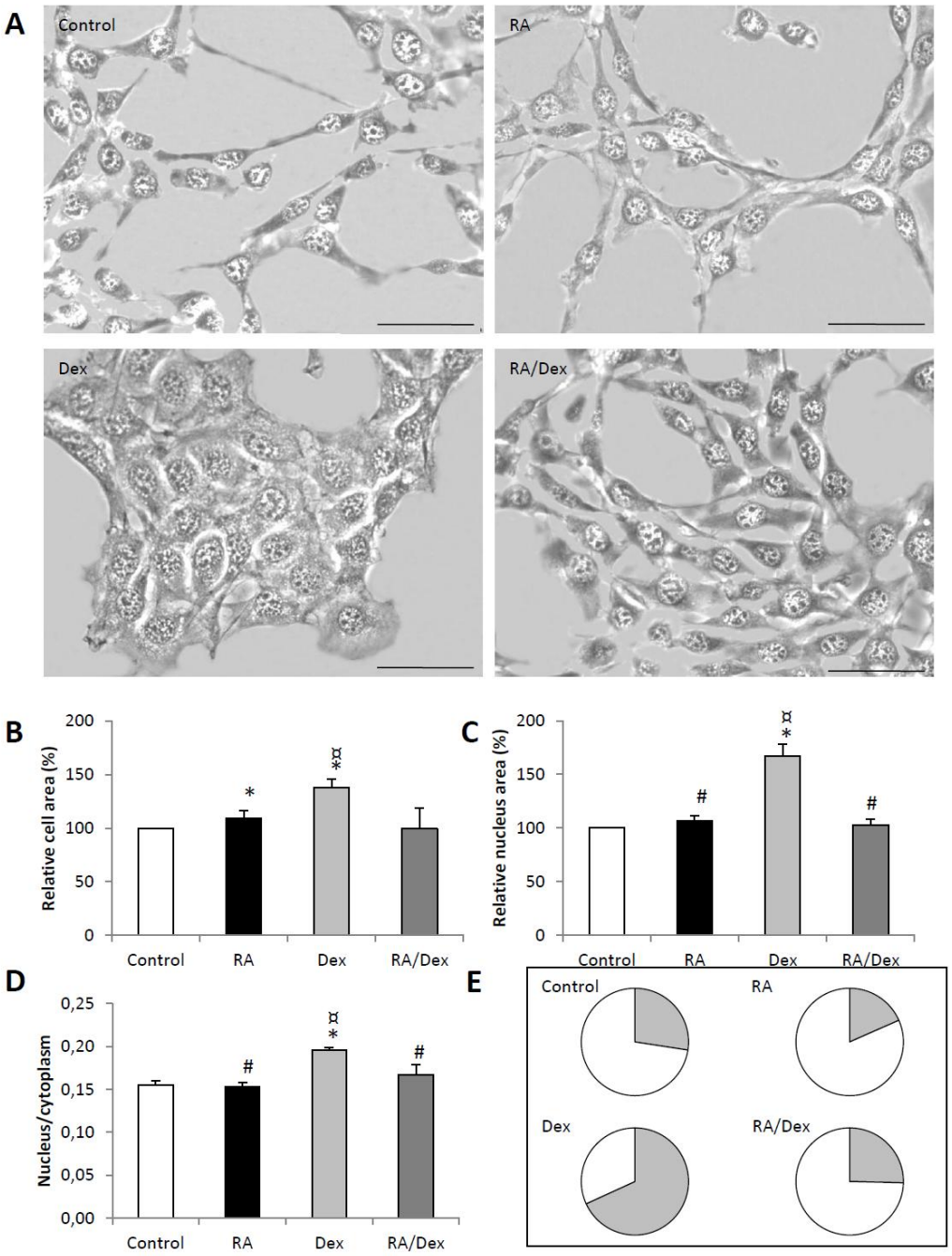

Figure 2. Consequences of RA (10^{-6} M) and/or GC (Dex 10^{-6} M) pathways activation in morphological characteristics of HT-22 cells. Phase contrast images of untreated, RA and/or Dex treated cells (A). Relative cell area (B), nuclear area (C) and nucleus/cytoplasm ratio (D) of untreated, RA and/or Dex treated cells. Evaluation of the circularity degree of cells (E) for each condition. Total circle represents "1": a perfect circle: the larger the shaded area of the disk, the larger the population of rounded cells & conversely, the smaller the shaded area, the larger the population of elongated cells. Mean ± SEM from at least three independent experiments. * Significantly different from the control, # from Dex-treated cells, and ¤ from RA-treated cells ($p<0.05$). The images are representative of at least three separated sets of culture. Scale bar: 50 µm.

Activation of RA and GC signalling pathways leads to actin cytoskeleton reorganization but did not change actin expression or abundance levels.

Since actin cytoskeleton affects cell morphology and is a major actor of cell movements including synaptic remodelling [26], we studied its organization. Quantification of actin expression and abundance (by RT-qPCR and Western blots, respectively) did not show any effects of RA or Dex treatments (data not shown). RA treatment led to a minor but significant increase in total phalloidin- stained polymerized F-actin (Fig. 3A&B). A large increase was induced by Dex treatment whereas RA/Dex treatment did not affect the global level of actin polymerization compared to basal condition.

Cellular actin further evaluated by separatedly quantifiying cortical actin from stress fibres. All treatments increased significantly cortical actin. Dex treatment produced the larger increase (Fig. 3C). RA added to Dex reduced this effect to the level obtained with RA alone. Dex treatment also significant increased actin stress fibres (Fig. 3D). RA and RA/Dex treatments had no effect on actin stress fibres.

The stress fibre/cell area ratio was calculated (Fig. 3E). This ratio was decreased by RA treatment and significantly increased by Dex treatment. The co-treatment induced a reduction of Dex effect by RA.

To summarise, for all studied parameters, RA reduced Dex treatment effects on the polymerisation and organisation of actine in hippocampal cells.

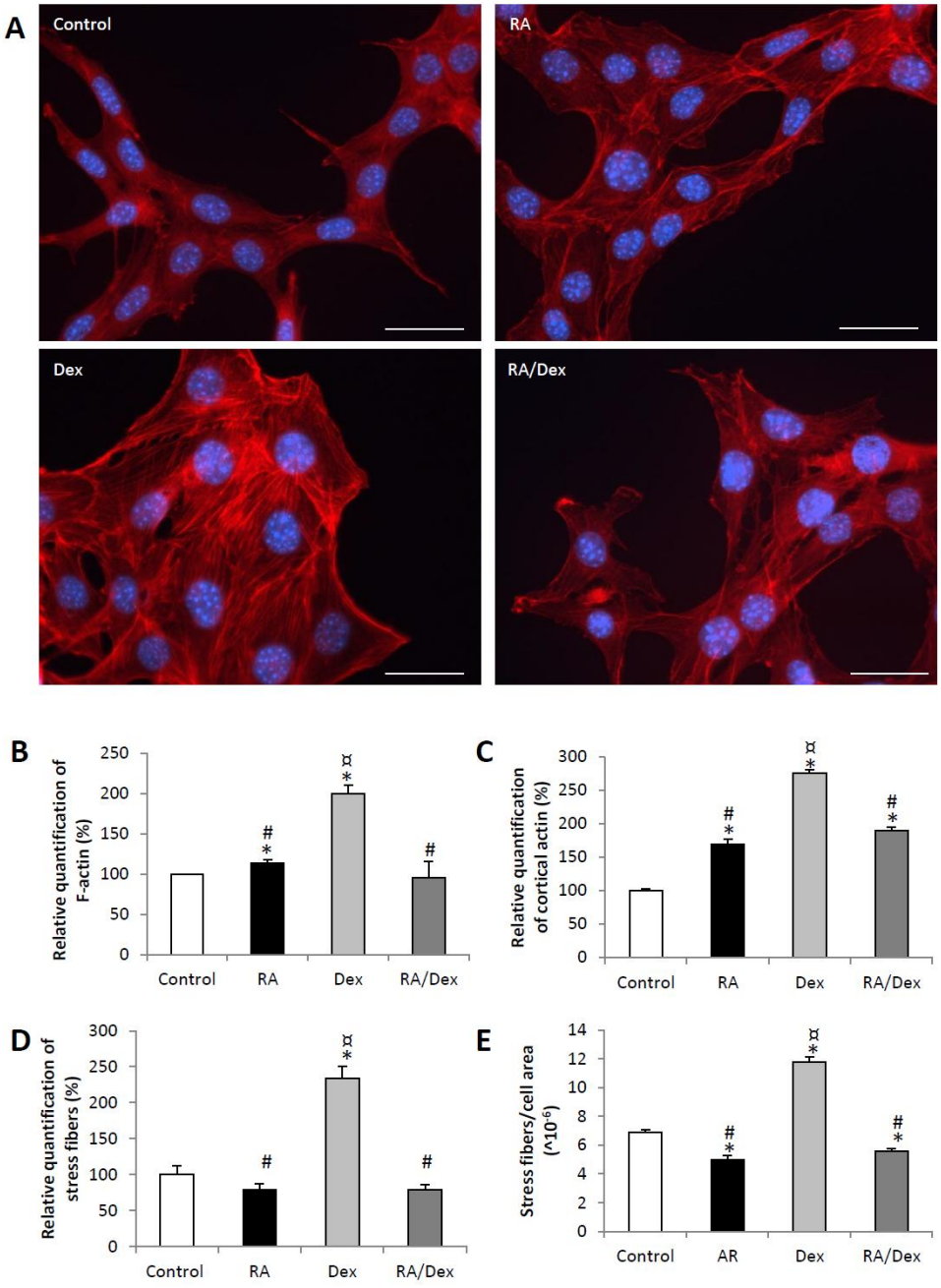

Figure 3. Consequences of RA (10^{-6} M) and/or GC (Dex 10^{-6} M) pathways activation in actin cytoskeleton organization of HT-22 cells. F-actin organization of cells for each condition (A). F-actin was stained with phalloidin-FITC (0.5 µM) and nucleus with DAPI (1.5 µg/ml). Relative quantification of total F-actin (B), cortical actin (C) and stress fibres (D) of untreated and treated cells. Stress fibres/cell area ratio (E) for each condition. * Significantly different from the control, # from Dex-treated cells, and ¤ from RA-treated cells ($p<0.05$). The images are representative of at least three separated sets of culture. Scale bar: 20 µm.

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D. Conclusion

Le deuxième volet de ce travail confirme l'existence d'une modulation transcriptionnelle et post-transcriptionnelle des GC par l'atAR.

Cette modulation est montrée au travers d'une interaction de la dexaméthasone et de l'atAR sur l'expression de cibles protéiques de lignées cellulaires hippocampiques : alors que les deux agonistes augmentent l'expression et l'activité de la tTG, sous le co-traitement atAR/Dex, la tTG présente un niveau d'expression et d'activité intermédiaire par rapport à celui de l'atAR ou de la Dex seuls. Sans interaction, le co-traitement aurait été associé à une expression supérieure ou égale à l'effet de l'atAR seul. Concernant l'expression de BDNF, l'atAR et la Dex seul montrent des effets opposés puisque l'expression et la sécrétion de BDNF sont augmentées par l'atAR et diminué par la Dex (pour cette dernière condition, seule la différence de sécrétion est significative). En co-traitement, l'atAR semble annuler les effets de la Dex car la sécrétion de BDNF n'est pas significativement différente après traitement par l'atAR ou l'atAR/Dex.

Ces effets antagonistes sur des tTG et BDNF sont expliqués par l'augmentation de la phosphorylation de GR en position Ser220 lors du traitement des cellules par l'atAR. En effet, comme attendu, $_{pSer220}$GR est augmenté lors du traitement par la Dex. Par contre, l'atAR seul diminue l'expression nucléaire de $_{pSer220}$GR alors qu'associé à la Dex, l'atAR augmente significativement $_{pSer220}$GR.

Dans notre modèle, nous montrons que l'expression de CDK5 est augmentée par l'atAR. Il en est de même pour le ratio p35/p25 avec une différence significative entre la condition Dex seule et atAR+Dex. En présence de roscovitine, inhibiteur de l'activité CDK5, l'atAR n'est plus capable d'augmenter $_{pSer220}$GR, ni l'expression de tTG ou de BDNF.

Au vue de ces résultats, il apparait que les effets bénéfiques de l'atAR au niveau du système nerveux central puissent être en lien avec une diminution de l'activité transcritionnelle de GR *via* une augmentation de la phosphorylation Ser220. Cette phosphorylation s'explique par l'effet modulateur de l'atAR sur l'activité CDK5 en protégeant la coupure de p35 en p25.

Enfin, l'interaction atAR et GC est mise également en évidence dans notre modèle par des modifications morphologiques des cellules en lien avec des changements phénotypiques du cytosquelette d'actine.

DISCUSSION ET PERSPECTIVES

Les GC modulent environ 10% des gènes de notre organisme. Ils interviennent pour une part importante dans le contrôle de nos fonctions vitales. L'action physiologique des GC est de mobiliser certaines ressources de l'organisme pour surmonter et s'adapter à des changements internes ou externes susceptibles de perturber l'homéostasie de l'organisme. Leurs effets bénéfiques peuvent néanmoins devenir néfastes lorsque l'exposition au GC est trop importante (concentration excessive et/ou exposition prolongée). Leur action nécessite donc une régulation permanente et la plus adaptée possible à l'environnement dans lequel évolue l'individu. La perception d'un stress par le SNC et le rétrocontrôle négatif qu'exercent les GC sur celui-ci constituent le système régulateur de la sécrétion des GC le plus connue et le mieux décrit. Mais la littérature scientifique des quinze dernières années prouve qu'il est loin d'être le seul. D'autres systèmes de régulation existent et ils interviennent après la sécrétion hormonale par les glandes surrénales. Ainsi, trois niveaux de régulation peuvent être décrits :

- La modulation de la sécrétion des GC, comprenant la régulation de la libération de l'ACTH par l'hypophyse et la stimulation de la stéroïdogenèse par les glandes surrénales ainsi que l'auto-régulation de l'axe corticotrope par les GC.
- La modulation de la biodisponibilité des GC aux tissus avec les propriétés régulatrices de la CBG et aux cellules avec l'action des 11β-HSD1 et 2.
- La modulation de l'activité transcriptionnelle et non génomique des GC et de leurs récepteurs faisant intervenir à la fois des modifications post-traductionnelles du GR tel que la phosphorylation mais aussi des interactions avec des voies de signalisations de kinases et avec des facteurs de transcription.

Alors que le SNC intervient explicitement dans la régulation de la sécrétion de GC par son implication dans la perception du stress et dans le rétrocontrôle négatif des GC, il est important de noter ici que les deux niveaux de régulation suivants ne doivent pas être entendus comme exclusivement périphériques. Le SNC est un tissu exposé aux GC sur lequel la régulation pré et post-récepteur joue un rôle capital dans la mesure où elle conditionne l'auto-régulation des GC. Le SNC constitue ainsi un modèle d'étude privilégié dans lequel les trois niveaux de régulation interviennent.

Pour ce travail de thèse, notre attention s'est portée sur la régulation de la biodisponibilité cellulaire des GC et de l'activité transcriptionnelle des GR afin de mieux comprendre leur implication et les mécanismes qui interviennent à ces niveaux.

Le dosage par spectrométrie de masse du cortisol urinaire et de ses métabolites (PUBLICATION N°1), constitue un outil d'importance capitale dans l'exploration de la biodisponibilité et la clairance des GC. Il permet une approche plus dynamique de l'exposition aux GC dans l'organisme que le simple dosage d'un GC. Les résultats obtenus lors de la validation de cette méthode témoignent d'un système enzymatique de métabolisation robuste. En effet, lors de sécrétion massive de cortisol, les concentrations des métabolites sont globalement toutes augmentées de manière homogène sans qu'un déséquilibre important ne soit mis en évidence. Nous n'observons qu'une augmentation de l'activité de la 11β-HSD1 et une diminution de celle de la 11β-HSD2 dans le groupe atteint de maladie de Cushing (résultats non montrés). Cette observation, déjà décrite dans la littérature (Mazzocchi et al. 2002), est en lien avec l'effet des GC sur l'expression de ces enzymes (Chapman et al. 2013a). L'ensemble des enzymes participant à la fois à la biodisponibilité du cortisol et à sa clairance ne semble pas saturé lorsque le cortisol est en excès. Par ailleurs, ce dosage permet la discrimination de faibles différences de concentrations des métabolites justifiant son intérêt dans le diagnostic différentiel d'hypersécrétion cortisolique (Arlt et al. 2011; Kotlowska et al. 2011). Il est également utilisé pour étudier l'influence des facteurs métaboliques sur la régulation pré-récepteur des GC comme l'obésité (Stewart et al. 1999; Rask et al. 2013), le diabète (Tomlinson et al. 2008; Stimson et al. 2011), l'alimentation et les principes actifs végétaux (PUBLICATION N°3)(Frey and Ferrari 2000; Basu et al. 2004; Schloms et al. 2013; Stimson et al. 2013), l'activité physique (Gatti et al. 2005; Dovio et al. 2010), la grossesse et le développement *in utero* (Wyrwoll et al. 2011).

L'un des objectifs principaux de ce travail était d'explorer le métabolisme du cortisol dans les urines de sujets diabétiques traités par insulinothérapie pour évaluer l'activité des deux enzymes responsables du métabolisme du cortisol, les 11β-HSD1 et 2. Le but était d'établir s'il existe un lien entre l'équilibre diabétique ou l'état inflammatoire des patients diabétiques traités par insulinothérapie sous-cutanée et le métabolisme du cortisol (PUBLICATION N°2).
Le modèle d'étude était le jeune enfant diabétique de type I. Le choix d'une étude chez l'enfant nous est apparu particulièrement intéressant puisque ce modèle correspond à un stade relativement précoce de la maladie pour lequel les complications microvasculaires et cardiovasculaires ne sont pas encore apparues. Ainsi, ces complications n'interférent pas dans les hypothèses mécanistiques que soulèvent une telle étude. De plus, il s'agit d'un travail préliminaire s'inscrivant dans un objectif de compréhension plus large de la pathologie. Dans ce cadre, nous avons envisagé la mise en évidence de perturbations des concentrations urinaires des métabolites du cortisol comme des marqueurs potentiels précoces de la survenue de ces complications.

Nous observons une augmentation significative de l'activité de la 11β-HSD1 chez les enfants diabétiques. De plus, cette augmentation est corrélée aux valeurs de la CRP chez les enfants diabétiques contrairement à la population contrôle. Ces résultats suggèrent un lien entre le diabète et l'existence d'une inflammation à bas bruit et l'augmentation de l'exposition cellulaire aux GC.

Pour expliquer ces résultats, plusieurs hypothèses peuvent être envisagées :

- La première hypothèse serait un effet direct de l'hyperglycémie des enfants diabétiques sur l'activité de la 11β-HSD1. Fan *et al.* montrent une augmentation de l'activité de cette enzyme dans les hépatocytes de rats présentant une hyperglycémie (Fan et al. 2011). Dans nos conditions expérimentales, nous n'observons pas de corrélation du ratio THFs/THE avec l'hémoglobine glyquée, témoin de l'hyperglycémie chronique. Il est toutefois possible que cela soit plutôt les variations importantes de la concentration de la glycémie qui puissent être en cause dans l'augmentation de l'activité de la 11β-HSD1 comme le suggère Qi *et al.* (Qi et al. 2012).
- L'existence d'hypoglycémies nocturnes non identifiées chez les enfants diabétiques (les hypoglycémies avérées étant un critère d'exclusion), serait responsable d'une stimulation de l'axe corticotrope. Cette hypothèse n'a pas été privilégiée car nous n'observons pas de différence significative du cortisol urinaire entre nos deux groupes. De plus, pour un faible nombre supplémentaire d'enfants diabétiques (n=5), des capteurs glycémiques sous-cutanés ont enregistré en continu les concentrations du glucose sanguin des enfants 24h/24h pendant cinq jours. Nous n'avons alors pas mis en évidence de lien entre hypoglycémie (ou variations importantes de la glycémie) et la perturbation de la métabolisation du cortisol (résultats non publiés).
- La stimulation de l'activité de la 11β-HSD1 par l'élévation des cytokines pro-inflammatoires (Chapman et al. 2013b). Nous n'observons de différence significative ni de la CRP et ni de l'interleukine 6 entre les deux populations. Cependant, cet effet est peut-être masqué dans la circulation générale par l'augmentation locale par la 11β-HSD1 du cortisol qui, par ses propriétés anti-inflammatoires, modère l'élévation de ces cytokines.
- L'insulinopénie relative au niveau hépatique des enfants diabétiques en lien avec l'apport exclusivement sous-cutané du traitement par insuline (*a contrario* d'un apport physiologique par la voie portale de l'insuline pancréatique) (Shishko et al. 1992) entrainerait une augmentation de l'activité de la 11β-HSD1, l'insuline ayant été décrite comme inhibitrice de cette activité (Voice et al. 1996). Cette augmentation se traduirait par une augmentation relative de la disponibilité aux tissus du cortisol expliquant, par son effet anti-inflammatoire, l'absence de corrélation entre l'hémoglobine glyquée et l'IL6.

Des travaux menés au laboratoire sur l'animal (résultats non publiés) orientent notre réflexion vers cette dernière hypothèse. En effet, de jeunes rats ont été rendus diabétiques par la streptozotocine et ont été traités par insulinothérapie selon deux modes d'administration : soit par voie sous-cutanée soit par voie péritonéale. Les rats recevant l'insuline par voie sous-cutanée présentent une augmentation significative de l'expression de la 11β-HSD1 hépatique *vs* une administration péritonéale. Néanmoins, ces résultats nécessitent d'être confirmés notamment par la mise au point du dosage de la corticostérone urinaire et de ses métabolites par spectrométrie de masse. Un autre angle d'approche serait l'étude des métabolites du cortisol dans une population de diabétiques de type I traités avec une pompe délivrant l'insuline par voie péritonéale en comparaison avec d'autres sujets appariés traités par voie sous-cutanée.

De manière intéressante, plusieurs équipes ont montré récemment que la vitamine A et son métabolite actif l'atAR, facteurs métaboliques d'origine alimentaire, modulent également l'activité de la 11β-HSD1 (Aubry and Odermatt 2009; Sakamuri et al. 2011; Marissal-Arvy et al. 2013). Il semble en effet que les voies de métabolisation et de signalisation de l'atAR interfèrent au niveau périphérique comme au niveau central avec celles des GC. Cette modulation de l'exposition pré-récepteur aux GC est explorée au laboratoire (Marissal-Arvy et al. 2013 ; Bonhomme *et al.*, en préparation) comme l'une des explications possibles de la modulation des effets des GC par l'atAR. Pour ce travail de thèse, nous nous sommes intéressés non pas aux effets pré- mais post-récepteurs de l'atAR sur la réponse cellulaire aux GC.

De nombreuses publications montrent des effets antagoniste, neutre, additif ou synergique des rétinoïques et des GC, selon le modèle d'étude et les conditions expérimentales, sur des cibles communes. Aucun travail n'avait été réalisé sur un modèle neuronal. Alors que ces deux types de composés présentent sur le SNC, et en particulier au niveau de l'hippocampe, des effets respectifs opposés très importants, rien n'est connu de l'action concomitante des rétinoïques et des GC. Pourtant, les effets bénéfiques de l'atAR sur les fonctions centrales pourraient être en partie expliqués par une modulation de l'activité post-récepteur des GC. Dans ce contexte, il nous est apparu intéressant de choisir une lignée de cellules hippocampiques murines, les cellules HT22, comme modèle d'étude de la modulation post-récepteur de l'exposition aux GC par le métabolite actif de la vitamine A (PUBLICATION N°5).

Nous avons tout d'abord montré qu'il existe une interaction dans notre modèle de cellules hippocampiques entre la voie des rétinoïques et celle des GC. Il en résulte des effets antagonistes entre l'atAR et la dexaméthasone après quatre jours de traitement.

Dans la littérature deux hypothèses sont évoquées pour expliquer une interaction entre les deux voies de signalisation et comprendre par quel mécanisme l'atAR module la réponse des GC. La première est développée par Wang en 2004 dans un modèle de cellules hépatiques (Wang et al. 2004). L'auteur propose que l'atAR, *via* l'hétérodimère RAR/RXR, modifie la liaison du GR aux coactivateurs et augmente ainsi son activité transcriptionnelle. La deuxième hypothèse suggère la possibilité d'une liaison RAR/RXR/GR, augmentant l'activité transcriptionnelle du GR dans les lymphocytes T (Toth et al. 2011). Dans ces deux publications, contrairement à notre modèle, l'interaction GR et RAR conduit à une synergie d'effet entre les agonistes. Par ailleurs, la présence de RARβ et la modification du phénotype du cytosquelette d'actine dans le cytoplasme des cellules HT22, nous ont orientés vers un effet non-génomique de l'atAR. En effet, les modifications non-génomiques de la voie des kinases par l'atAR ont été largement démontrées (Zassadowski et al. 2012; Al Tanoury et al. 2013). Elles ont même un rôle clé dans l'action de l'atAR sur la plasticité neuronale (Canon et al. 2004). Alors que cette voie non-génomique contribue à la modulation transcriptionnelle des RARs par modification des phosphorylations du récepteur et de ces facteurs de transcription, aucun auteur ne suggère une modification de la phosphorylation de GR par l'atAR.

Dans la publication n°6, nous montrons que l'expression cytoplasmique et nucléaire de $_{pSer220}$GR est fortement modifiée par nos différents traitements. En particulier, l'atAR augmente l'expression nucléaire de $_{pSer220}$GR en présence de la Dex. Lorsque la Dex est seule, l'augmentation de l'expression nucléaire de $_{pSer220}$GR est associée à une augmentation de l'expression de tTG et à une diminution de BDNF en lien avec la présence d'un GRE dans le promoteur de tTG (Johnson et al. 1998) et d'un nGRE dans celui de BDNF (Schaaf et al. 1997; Suri and Vaidya 2013). En présence du co-traitement atAR+Dex, cette augmentation de $_{pSer220}$GR se traduit par une augmentation de l'expression de tTG et de BDNF. Le promoteur de tTG et peut-être celui de BDNF (mais, à notre connaissance, cela n'a pas pu être démontré) comporte également un RARE (Johnson et al. 1998). Le co-traitement traduit donc à la fois l'effet de l'atAR sur son propre élément de réponse *via* son récepteur et la modification atAR-dépendante de $_{pSer220}$GR. Pour s'affranchir de l'effet RARE-dépendant et se focaliser exclusivement sur modulation de la réponse post-récepteur des GC par l'atAR, nous avons utilisé les HT22 transfectées par un plasmide couplant le GRE et le gène de la luciférase. L'activité transcriptionnelle de GR est diminuée par le traitement par l'atAR en présence ou non de la Dex.

Ces résultats se rapprochent de ceux développés dans l'article de l'équipe de Kino *et al.* en 2007 : l'augmentation de la phosphorylation $_{pSer220}$GR pourrait être en lien avec une diminution de l'activité transcriptionnelle de GR dans les cellules hippocampiques (Kino et al. 2007). Par ailleurs,

Kino *et al.* ont montré l'importance de CDK5 et de p35/p25 dans la phosphorylation et l'activité transcriptionnelle de GR. Cette kinase, dont le peptide activateur p35 n'est retrouvé que dans les cellules du SNC, est largement impliquée dans les fonctions cognitives *via* une action sur la plasticité neuronale. Cependant, la coupure de p35 en p25 entraîne une hyperactivation de CDK5 responsable de phosphorylations aberrantes délétères pour la cellule neuronale. Or, pour plusieurs auteurs, l'atAR est responsable, au niveau neuronal, de l'activation de la CDK5. Pour étudier l'implication de CDK/p35/p25 dans la phosphorylation de GR en présence d'atAR, nous avons utilisé un inhibiteur de l'activité CDK5, la roscovitine.

Nos résultats montrent que l'atAR augmente la phosphorylation de GR en position Ser 220 par l'intermédiaire d'une activité CDK5-dépendante régulée par p35. Il est cependant important de remarquer que la conséquence d'une augmentation de $_{pSer220}$GR peut se traduire dans notre modèle soit par une augmentation de l'activité transcriptionnelle de GR lorsque les cellules ne sont exposées qu'à la Dex, soit à une diminution de la réponse GC lorsque les cellules sont exposées à la Dex et à l'atAR. Dans les deux cas, la roscovitine inverse les conséquences transcriptionnelles des traitements. Cet effet paradoxal laisse penser que l'implication de CDK5 dans l'effet de l'atAR ne doit pas se limiter à une action sur la phosphorylation Ser 220 de GR. La CDK5, avec une prédominance de p25 par rapport à p35 (condition Dex seule) doit favoriser la phosphorylation et/ou le recrutement de coactivateurs responsables de l'augmentation de l'activité transcriptionnelle de GR. Lors de la prédominance de p35 par rapport à p25 (condition atAR+Dex), la phosphorylation/recrutement de coactivateurs ne se fait plus et l'activité transcriptionnelle de GR diminue.

D'autre part, l'atAR et la Dex présentent également des effets opposés sur la morphologie de la cellule et sur le phénotype du cytosquelette d'actine (PUBLICATION N°7). Alors que l'expression des ARNm de l'actine est inchangée, l'organisation des filaments d'actine est modifiée par les deux agonistes. Les changements de conformations de l'actine peuvent être expliqués par des effets non-génomiques tels que l'activation ou l'inhibition de l'activité de calpaïnes (Carragher and Frame 2002). Les calpaïnes sont des protéases à cystéine calcium-dépendante. Elles sont capables d'accélérer le remodelage de la polymérisation d'actine. Ainsi elles participent à la plasticité de la cellule et à l'émission de prolongements cytoplasmiques. La modulation rapide de leur activité est en lien avec l'activation de voies de kinase en particulier de celle de ERK (Carragher and Frame 2002). L'implication de cette voie dans les conséquences morphologiques de la cellule hippocampique reste encore à démontrer.

Enfin, nous pensons qu'une liaison GR/RARβ est possible dans ces cellules (Figure 12). Elle serait

dépendante de l'état de phosphorylation de GR. Cette liaison doit diminuer l'activité transcriptionnelle de GR. Mais la seule publication décrivant ce phénomène montre qu'une telle liaison augmente la réponse GC (Toth et al. 2011). Nos résultats nécessitent d'être complétés par ceux d'une technique de gel shift pour appuyer cette hypothèse.

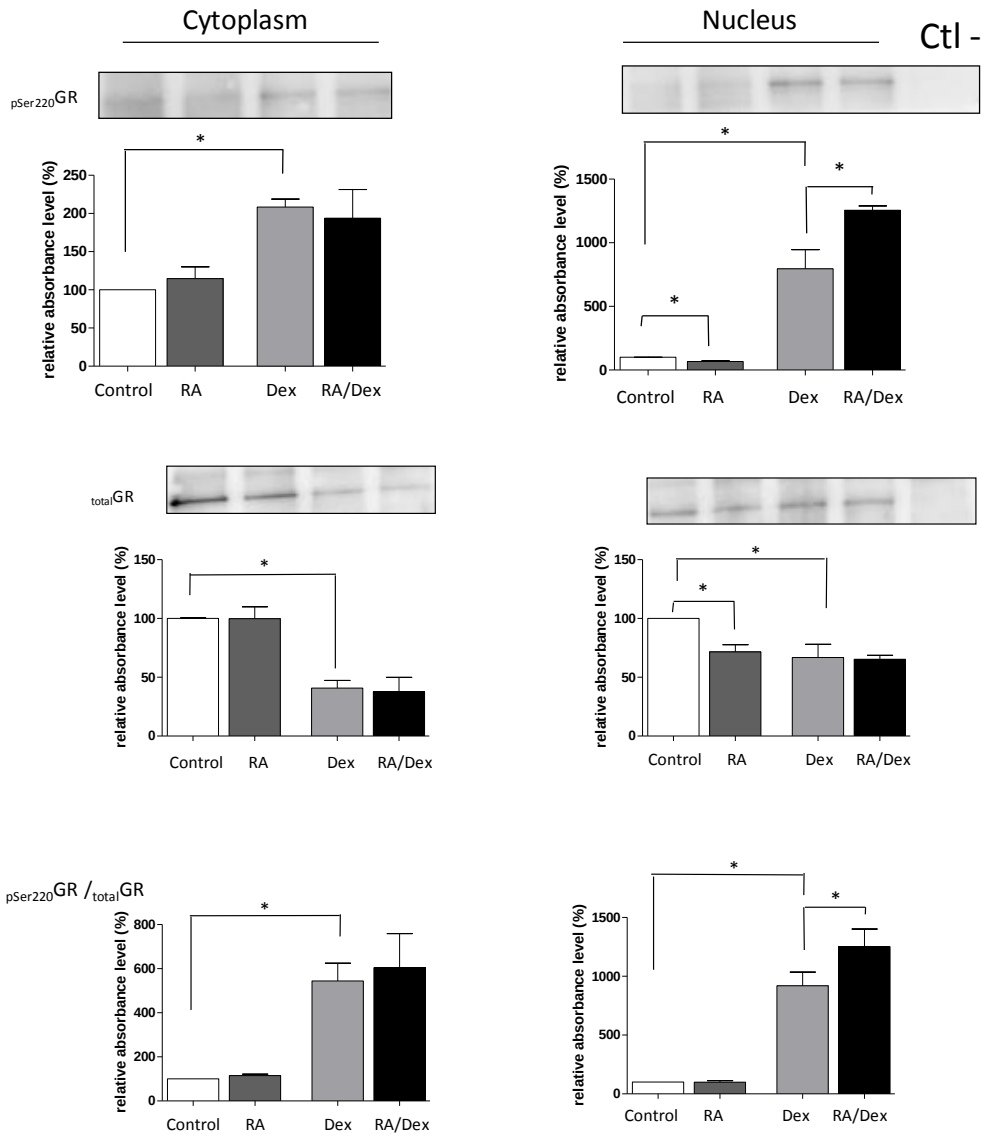

Figure 12 : Résultats de l'immuno-précipitation RARβ/GR.

Après traitement des cellules HT22 pendant quatre jours par un véhicule (Control), l'atAR à 10^{-6} M (RA), la Dex à 10^{-6} M ou un co-traitement à l'atAR et à la Dex (AR/Dex) (10^{-6} M), l'immuno-précipitation du RARβ est réalisée grâce à un anticorps anti-RARβ fixé sur des billes magnétiques Dynebeads Protein G (life technologies, Van Allen Way Carlsbad, CA, USA). La condition Ctl- correspond à l'utilisation d'une immunoglobuline G ne reconnaissant pas RARβ. Puis les protéines retenues sont séparées sur un gel d'électrophorèse. Après transfère, la membrane de nitrocellulose est incubée avec un anti-$_{pSer220}$GR ou un anti-$_{total}$GR. Les résultats représentent la moyenne ± l'écart à la moyenne de trois expérimentations indépendantes. * résultat significativement de la condition contrôle ($p<0,05$).

Importance de la nutrition dans la régulation de l'exposition pré et post-récepteur des glucocorticoïdes au niveau du système niveau central : perspectives et conclusion

De manière plus générale, ces travaux montrent l'importance de l'interaction des voies de métabolisation et de signalisation des GC avec des facteurs métaboliques endogènes (comme les hormones) ou exogènes (comme l'alimentation). Ces interactions sont la base d'une régulation des effets des GC. La Figure 13 synthétise les principaux éléments de notre réflexion.

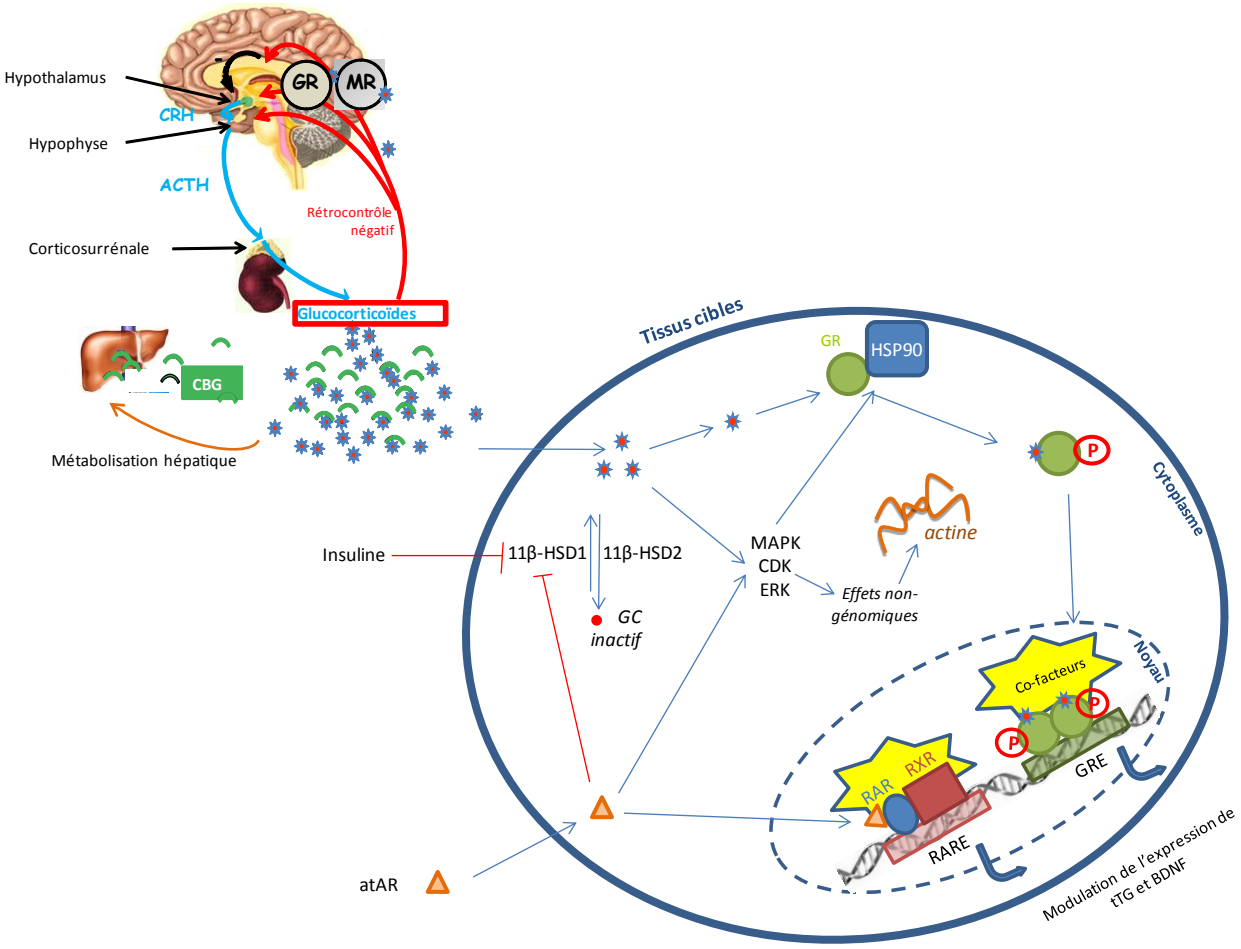

Figure 13 : Synthèse de l'effet modulateur de l'insuline et de l'atAR sur l'exposition aux GC.

CRH : *corticotropin releasing hormone*, ACTH : *adrenocorticotropic hormone*, GR : Glucocorticoid receptor, MR : Mineralocorticoid receptor, CBG : *Corticosteroid Binding Globulin*, 11β-HSD1 et 2: 11 beta-hydroxystéroïde-déshydrogénase de type 1 et 2, MAPK : , CDK : Cyclin dependent kinase, ERK : *extracellular signal-regulated protein kinase*, HSP90 : *Heat shock protein*, RAR : *retinoic acid receptor*, RXR : *retinoic X receptor*, RARE : *retinoic acid-responsive element*, GR : Glucocorticoid receptor, GRE : *glucocorticoid-responsive element*, tTG : tissue transglutaminase, BDNF : brain-derived neurotrophic factor, atAR : acide rétinoïque all-trans,

La suite de nos recherches visera à approfondir la compréhension des mécanismes impliqués et des conséquences de ces modulations de la réponse GC sur l'organisme. L'importance de la régulation pré et post-récepteur des GR semble capitale pour expliquer la survenue des effets délétères des GC

au niveau du SNC. Nous nous intéresserons donc plus particulièrement aux conséquences cognitives et mnésiques de ces régulations.

Concernant l'étude *in vitro* des mécanismes de réponse aux GC, plusieurs aspects doivent être développés :

- Le mécanisme de modification du rapport p35/p25 par l'atAR doit être précisé. Nous pensons que cet effet doit passer par une modification de l'activité calpaïne puisque l'enzyme est responsable de la coupure de p35 en p25. De plus, nous devons confirmer la liaison entre le récepteur de l'atAR, et des GC puis en étudier les conséquences.

- Le modèle des cellules HT22 pourra être utilisé pour approfondir les interactions qui existent entre les récepteurs nucléaires. L'importance du *ratio* MR/GR au niveau de l'activité neuronale hippocampique pose la question des conséquences des effets non-génomiques des GC et des minéralocorticoïdes sur l'activité transcriptionnelle des MR et des GR. Il nous semble important d'introduire dans notre modèle les effets liés aux MR. D'autant qu'une étude récente montre que la phosphorylation des MR au niveau neuronal implique également CDK5 avec des effets GR- et MR-dépendants opposés sur l'expression de neurotrophines (Kino et al. 2010).

- L'expression de la 11β-HSD1 et 2 étant très faible dans notre modèle, nous souhaiterions mettre en place un modèle de co-culture avec des lignées de cellule gliale comme les BV2 pour étudier l'impact de l'environnement neuronale sur ces voies non-génomiques. Ce type de modèle impliquerait alors d'intégrer l'effet de facteurs pro-inflammatoires comme des interleukines ou les produits avancés de la glycation (AGEs) et leur récepteur dans notre schéma d'étude. L'étude de l'impact des AGE dans notre modèle serait particulièrement intéressante au regard de la thématique du laboratoire puisqu'il s'agit de résidus moléculaires très réactifs issus de la glycation non enzymatiques des protéines. Des AGEs sont en particulier trouvés en excès dans le plasma et les tissus des mammifères au cours du vieillissement et du diabète. Leurs effets pro-inflammatoires participent aux complications de ces situations physiopathologiques (Luevano-Contreras and Chapman-Novakofski 2010; Yaffe et al. 2011).

Les conséquences de la modulation pré- ou post-récepteur des GC obtenu *in vitro* et *in vivo* au cours de ce travail doivent être confirmées et approfondies par d'autres études cliniques.

Les conséquences de la modification de phosphorylation des GR, associées aux travaux montrant une diminution de l'activité de la 11β-HSD1, seront évaluées dans une étude clinique en cours

(partenariat public-privé Nutrimémo, mené par V. Pallet). Les effets bénéfiques de la prise de vitamine A et d'acide gras insaturés par une population de personnes âgées pendant deux ans seront évalués en termes de performances cognitives et mnésiques. L'activité cortisolique sera appréciée par des dosages de cortisol salivaire et de ses métabolites urinaires. Cette étude devrait permettre de confirmer le lien entre le métabolisme de la vitamine A, la régulation de la signalisation des GC et les conséquences au niveau central.

Enfin, concernant le rôle du diabète dans la dysrégulation de l'axe corticotrope, une étude (PHRC Corticodiab, mené par P. Barat), incluant un nombre d'enfants plus importants et comprenant une exploration cognitive et une exploration morphologique et fonctionnelle hippocampique par IRM, est en cours. Elle devrait permettre de confirmer ces résultats, d'avancer dans la compréhension du processus et d'appréhender les conséquences cognitives d'une telle modification de l'exposition cortisolique. Les concentrations urinaires du cortisol et de ses métabolites ainsi que du cortisol salivaire seront relevés pour explorer l'axe corticotrope. Il y sera ainsi approfondit i) le lien entre dysfonctionnement de l'axe corticotrope, diabète et cognition et ii) l'importance du facteur environnemental alimentaire au travers de l'étude des 30 paires diabétique/non diabétique appartenant à la même fratrie puisqu'ils suivront *a priori* le même régime alimentaire.

Une meilleure connaissance des systèmes régulant l'exposition et la réponse cellulaire des GC devrait permettre de sensibiliser la communauté scientifique et médicale à l'importance de l'évaluation des retentissements délétères des GC endogènes ou médicamenteux. Ces retentissements se manifestent en effet dans de nombreuses situations physiologiques (vieillissement) ou pathologiques (causes endogènes ou exogènes d'augmentation de l'imprégnation GC). Arriver à prévenir ou à réduire les effets délétères des GC par des traitements simples tels que des modifications diététiques serait un avantage avec un excellent rapport bénéfice risque.

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