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AVANT-PROPOS

L’adénosine est utilisée en cardiologie depuis des décennies, en particulier dans le domaine de la rythmologie, dans le but d’interrompre les accès de tachycardie jonctionnelle. De nombreuses applications cliniques plus récentes ont suivi, elles seront détaillées plus loin. Quoi qu’il en soit, cette molécule fait partie du quotidien des électrophysiologistes. La connaissance du système adénosinergique endogène est en revanche plus récente. Depuis la première description des récepteurs de l’adénosine, les découvertes scientifiques et les applications médicales ont grandi de manière exponentielle. Le rôle de l’adénosine et de ses récepteurs dans la physiopathologie humaine, en particulier, est devenu un champ de recherche très développé, qu’elle soit fondamentale ou clinique. Les récepteurs de l’adénosine sont impliqués dans de nombreux domaines : neurologie, immunologie, cardiologie, inflammation et cancer. Le développement d’agonistes et d’antagonistes spécifiques des récepteurs de l’adénosine est également en expansion. Notre équipe a été un des premiers laboratoires en Europe, à mettre au point une technique de dosage reproductible de l’adénosine dans les milieux biologiques et à s’intéresser aux rôles de ses récepteurs en physiopathologie humaine.

Concernant le domaine de la cardiologie, la compréhension des implications de l’adénosine et de ses récepteurs restait un champ pratiquement vierge au début des années 2000. Notre objectif au cours de ce travail aura été de mieux comprendre l’implication de l’adénosine endogène et de ses récepteurs chez les patients présentant des syncopes neuro-cardiogéniques et au cours de l’insuffisance cardiaque. L’élucidation des mécanismes de régulation de l’adénosine et des ses récepteurs est une première étape indispensable. Les applications thérapeutiques potentielles des ligands qui ciblent les récepteurs de l’adénosine, en particulier les récepteurs A_1 ou A_{2A}, sont actuellement des voies thérapeutiques prometteuses puisque, à titre d’exemple, certains antagonistes de l’adénosine sont en phase IV d’évaluation pour le traitement de la maladie de Parkinson, tandis que le régadénoson, A_2 sélectif, est utilisé dans l’imagerie coronaire.

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ABRÉVIATIONS

A

A_1R	Récepteur A_1 de l’adénosine
A_2R	Récepteurs A_2 de l’adénosine
$A_{2A}R$	Récepteur A_{2A} de l’adénosine
$A_{2B}R$	Récepteur A_{2B} de l’adénosine
A_3R	Récepteur A_3 de l’adénosine
ADA	Adénosine désaminase
ADP	Adénosine diphosphate
APL	Adenosine plasma level
AMP	Adénosine 5’-monophosphate
AMPc	Adénosine 5’-monophosphate cyclique
ATP	Adénosine triphosphate

E

EC_{50}	Concentration en agoniste conduisant à la moitié des effets biologiques maximaux

G

GMPc	Guanosine monophosphate cyclique
GPCRs	G protein-coupled receptors
GRK	G protein-coupled receptor kinase

H

HUT	Head-up tilt test

I

IC_{50}	Concentration en agoniste conduisant à une inhibition de la moitié des effets biologiques maximaux

K

K_D	Constante de dissociation
K_I	Constante d'inhibition
K_M	Constante de Michaelis

M

mAb	Monoclonal antibody

N

NMS	Neurally mediated syncope

P

PAG	Periaqueductal grey
PBMC	Peripheral blood mononuclear cell
PKA	Protéine kinase A
PKC	Protéine kinase C
PLC	Phospholypase C

T

TCR	T-cell receptor

RÉSUMÉ

L'adénosine est un nucléoside ubiquitaire issu de la déphosphorylation de l'ATP qui est libéré par les cellules endothéliales et les myocytes lors de l'hypoxie, de l'ischémie ou du stress oxydatif. Elle exerce un contrôle puissant sur les systèmes nerveux, immunitaire et cardiovasculaire par l'intermédiaire de quatre récepteurs membranaires : A_1R, $A_{2A}R$, $A_{2B}R$ et A_3R. La compréhension de l'implication du système adénosinergique dans le système cardiovasculaire implique la possibilité technique d'un dosage de l'adénosine endogène et la quantification de l'expression de ses récepteurs.

L'adénosine ayant globalement une action hypotensive (via les récepteurs $A_{2A}R$) et chronotrope négative (via les récepteurs A_1R), nous nous sommes intéressés à son implication chez les patients présentant des syncopes neurocardiogéniques, la bradycardie et l'hypotension étant 2 signes cardinaux dans ce syndrome. Les manifestations cliniques de cette affection peuvent être reproduites par le test d'inclinaison (HUT) et/ou le test à l'ATP. Dans un premier temps nous avons réalisé des dosages d'adénosine plasmatiques chez ces patients au moment d'un test d'inclinaison. Les concentrations en adénosine étaient élevées chez les patients présentant un test positif. Par la suite, nous avons comparé les concentrations en adénosine plasmatiques et l'expression des récepteurs A_{2A} en fonction du résultat du test d'inclinaison et du test à l'ATP. Les patients avec test d'inclinaison positif présentaient une concentration en adénosine élevée et sur-exprimaient les récepteurs A_{2A} ; en revanche, les patients avec test à l'ATP positif présentaient des concentrations en adénosine basses et une expression normale des récepteurs A_{2A}. Ces résultats identifient donc bien 2 populations en fonction de l'adénosinémie et de l'expression des récepteurs A_{2A}. Dans l'étape suivante, grâce à l'utilisation d'Adonis, un anticorps monoclonal, fabriqué au laboratoire, et dirigé contre la deuxième boucle extracellulaire du récepteur A_{2A} et ayant des propriétés agoniste, nous avons pu montrer que chez les patients vagaux, malgré une surexpression, les récepteurs A_{2A} restaient fonctionnels puisque la production d'AMPc était conservée. Par ailleurs dans quelques cas, la valeur de l'EC_{50} était nettement plus basse que la valeur du K_D pour Adonis, suggérant l'existence de récepteurs A_{2A} de réserve.

Toujours dans le domaine de la rythmologie, nous avons collaboré à un travail initié par les anesthésistes en montrant qu'au décours d'une chirurgie cardiaque avec circulation extracorporelle, des concentrations en adénosine élevées en pré-opératoire prédisposaient à l'apparition de fibrillation atriale en post-opératoire. L'élévation de l'adénosinémie est en relation avec l'hypoxémie préopératoire. Ce dernier résultat ouvre des perspectives thérapeutiques préventives par utilisation d'antagonistes des récepteurs A_{2A} en particulier.

Notre travail a également concerné la compréhension de l'adaptation du système adénosinergique lors d'hypoxie tissulaire en relation avec une chute du débit sanguin (hypoxémie). Chez l'insuffisant cardiaque sévère, avec chute de la fraction d'éjection et du débit cardiaque, les concentrations en adénosine plasmatique sont élevées, avec un gradient de concentration en faveur de la périphérie par rapport au sinus coronaire. L'origine de la libération d'adénosine est donc indépendante d'une souffrance myocardique intrinsèque mais prend son origine dans l’hypoxie tissulaire et le stress oxydatif périphérique lié à la chute du débit sanguin. De plus, les concentrations en adénosine plasmatique étaient corrélées aux taux d’albumine modifiée par l’ischémie (IMA), un marqueur sensible du stress oxydatif et de l’hypoxie tissulaire.

Dans une autre étape, nous avons utilisé un modèle permettant d’étudier l’influence de l’hypoxie sur le système adénosinergique sur une lignée lymphocytaire T, et nous avons montré qu’en réponse à l’hypoxie, on observait une augmentation rapide de la production d’adénosine, ainsi qu’une up-régulation des récepteurs A_{2A} lymphocytaires (ie surexpression et augmentation de la production d’AMPc). Il existe donc une auto-activation de la régulation adénosinergique lymphocytaire en réponse à l’hypoxie. Ce mécanisme pourrait être le lien entre hypoxie, réponse inflammatoire et système adénosinergique

Mots-clés : Adénosine, Récepteurs de l’adénosine, Syncope neurocardiogénique, Fibrillation atriale, Hypoxie.

ABSTRACT

Adenosine is a ubiquitous nucleoside that comes from the dephosphorylation of ATP and which is released during hypoxia or oxidative stress, by endothelial cells and myocytes. Adenosine interacts on its cell surface receptors, namely A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R, to exert physiological effects on target tissues. Our knowledge about the adenosinergic system was improved because of our ability to measure adenosine plasma levels and to quantify its receptors expression. Because adenosine, via A_1 or A_{2A} receptor activation leads to bradycardia and hypotension, we first tried to understand the implication of the adenosinergic system in patients with neurocardiogenic syncope (NMS for neutrally mediated syncope). Indeed, this syndrome is characterized by relative or absolute bradycardia associated with a drop in blood pressure and a loss of consciousness. The symptomatology can be reproduced by the tilt test (HUT) or by the intravenous administration of ATP (ATP test). First, we measured adenosine plasma levels in patients with NMS just before and during HUT. We found that adenosine plasma levels were higher in patients with a positive HUT. Then, we compared adenosine plasma levels and the expression of A_{2A} receptors in patients with NMS depending on the result of HUT and ATP-test. We found that elevated adenosine plasma levels and A_{2A} receptors overexpression were associated with positive HUT. On the opposite, low adenosine plasma levels and normal expression of A_{2A} receptor were associated with positive ATP test. These results suggest that laboratory tests and adenosine plasma measurement permit to distinguish two different types of population according to their positive ATP test or tilt test. The next step was to determine the function of A_{2A} receptors in patients with NMS. Using Adonis, a monoclonal antibody against extracellular part of A_{2A} receptor, which as agonist properties, we found, using western blotting, that despite a chronic exposure to elevated adenosine plasma levels and an overexpression of A_{2A} receptors, their function was preserved, since the cAMP production by lymphocytes was the same than for healthy subjects. We found, however, that in some cases, the EC_{50} value was lower than the K_D, suggesting the presence of spare receptors in these NMS patients.

In the field of heart rhythm disorders, we participated in a study initiated by anesthesiologists and which demonstrated that in patients that experienced a cardiac surgery with cardiovascular bypass, elevated adenosine plasma levels before the surgery could predict atrial fibrillation after the surgery. The high adenosine plasma level was likely due to hypoxia since basal PaO_2 was lower in susceptible patients.

Another part of our work concerned the adaptation of the adenosinergic system in case of tissue hypoxemia. Patients with heart failure have elevated adenosine plasma levels. However the source of adenosine release remained unknown. We compared the adenosine level in the coronary sinus and in a brachial vein. We found that adenosine level was significantly higher in the brachial vein than in the coronary sinus.

Thus we concluded that the origin of adenosine release was a consequence of tissue hypoxemia and oxidative stress at the peripheral level and that the myocardium itself was not the source of adenosine release.

In a next step, using a cultured human lymphoblastoid T-cell line, we demonstrated that response acute hypoxia a rapid increase in adenosine production and a simultaneous decrease in adenosine deaminase activity. Moreover, A_{2A} receptor expression increased, and we observed an accumulation of intracellular AMPc. We concluded that there is a rapid autoactivation of the adenosinergic system of T-cells in condition of acute hypoxia. These results underlight the relationship between hypoxemia, inflammation and the purinergic response.

Key words: Adenosine, Adenosine receptor, Neurocardiogenic syncope, Atrial fibrillation, Hypoxia

Chapitre un

INTRODUCTION BIBLIOGRAPHIQUE

I. Dérivés puriques

Les dérivés puriques sont des molécules azotées hétérocycliques constituées d'un cycle pyrimidine fusionné à un cycle imidazole, constituent un groupe comprenant les nucléosides, dont le chef de file est l'adénosine, et les nucléotides, dont le principal est l'adénosine triphosphate (ATP). Ces molécules exercent leurs effets cellulaires par l'intermédiaire de leurs récepteurs membranaires spécifiques et participent à de nombreux processus biologiques (Ralevic and Burnstock, 1998).

1. Nucléosides et nucléotides

Un nucléoside est une glycosamine, résultant de la combinaison d'un ose (ribose ou 2-désoxyribose) et d'une base (purique ou pyrimidique), unis par une liaison N-glycosidique. Dans les cellules, les nucléosides peuvent être phosphorylés par des kinases spécifiques pour produire des nucléotides qui sont les constituants moléculaires des acides ribonucléiques (ARN) et désoxyribonucléique (ADN), éléments fondamentaux de toutes les cellules vivantes. Les acides nucléiques comprennent deux bases puriques (adénine et guanine) et trois bases pyrimidiques (cytosine, thymine et uracile), ce qui correspond respectivement à deux nucléosides puriques (adénosine et guanosine) et trois nucléosides pyrimidiques (cytidine, thymidine et uridine). Suivant l'orientation de la liaison N-glycosidique, un nucléoside peut se présenter sous deux formes distinctes (*syn* et *anti*) mais c'est la conformation *anti* qui prédomine dans sa forme naturelle (figure 1).

Les nucléotides sont, non seulement, les unités élémentaires des acides nucléiques, mais leurs dérivés peuvent également avoir des fonctions cellulaires qui leur sont propres : ils interviennent dans de nombreux métabolismes comme transporteur d'énergie (ATP) ; ils interviennent dans la structure ou le métabolisme des oses (UDP-glucose), des lipides (CDP-choline), des acides aminés (S-adénosyl-méthionine), des vitamines (B12 ou cobalamine). De plus, les dérivés puriques font partie de la structure de co-enzymes très divers (NAD, NADP, FAD) et interviennent dans la transduction du signal intra-cellulaire sous formes cycliques (AMPc, GMPc).

2. Origine des dérivés puriques

Les dérivés puriques et pyrimidiques sont synthétisés principalement dans le foie d’où ils sont distribués dans tout l’organisme par les érythrocytes. Ils proviennent, d’une part, de la synthèse de *novo* à partir des éléments intermédiaires du métabolisme énergétique des sucres et des acides aminés et, d’autre part, de la voie de recyclage à partir des produits de dégradations lors du catabolisme des nucléotides ou des nucléosides. Le coût énergétique de la biosynthèse du noyau purique justifie l’activité importante des enzymes de récupération de ces noyaux dans les tissus. L’énergie est fournie par l’ATP synthétisé principalement par phosphorylation oxydative mitochondriale (Schwiebert and Zsembery, 2003). L’alimentation fournit des bases puriques en faible quantité car les enzymes digestives dégradent ces nucléotides en produit final, l’acide urique, insoluble et excrété dans les urines sous forme de cristaux d’urate de sodium. L'ATP est produit à partir de l'adénosine diphosphate (ADP), principalement par phosphorylation oxydative dans la mitochondrie dont il passe la membrane externe par les porines et la membrane interne par des antiports ATP/ADP (Schwiebert, 2001; Schwiebert and Zsembery, 2003). La concentration cytosolique de l’ATP se situe entre 3 et 10 mM, ce qui constitue un stock énergétique pour de nombreuses réactions métaboliques et enzymatiques, tandis que son taux extra-cellulaire est environ 10 nM, ce qui traduit un très fort gradient de concentration. Il faut noter que moins de 1% de l’ATP intra-cellulaire libérée, suffit pour activer les récepteurs purinergiques à la surface cellulaire (Schwiebert and Zsembery, 2003).

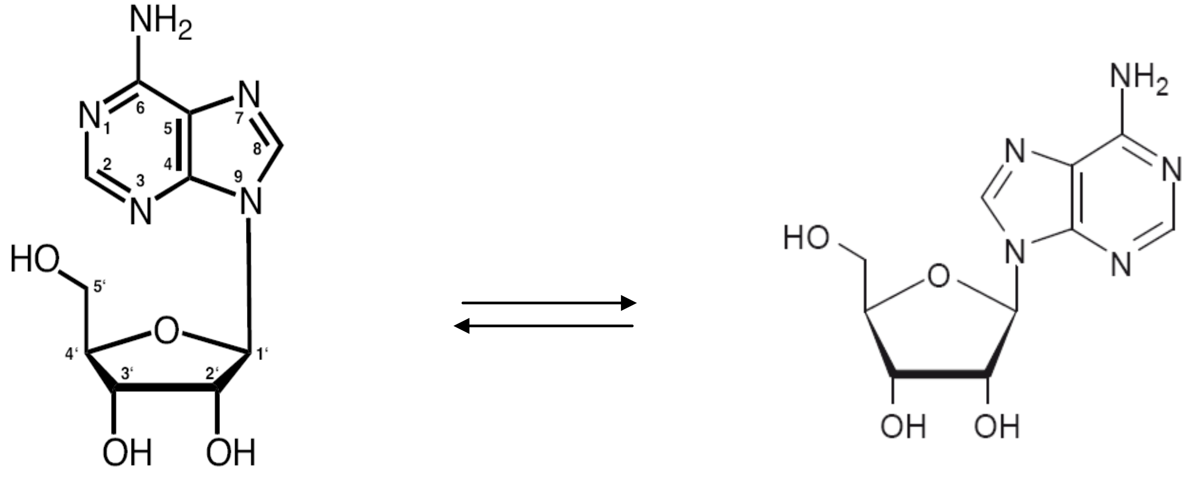

Figure 1. Structure de l’adénosine (*syn*, à gauche et *anti*, à droite)

II. Adénosine

1. Métabolisme

L'adénosine est un nucléoside purique formé d'une base adénine attachée à un noyau ribose via une liaison β-glycosidique (figure 1). Elle est présente dans toutes les cellules de l'organisme. L'adénosine est synthétisée de façon continue aussi bien en milieu intra-cellulaire qu'en milieu extra-cellulaire.

Dans le milieu intra-cellulaire, la formation d'adénosine est assurée, d'une part, par une 5'nucléotidase intra cellulaire, qui déphosphoryle l'adénosine monophosphate (AMP) (Schubert et al. 1979; Zimmermann et al. 1998), et d'autre part, par l'hydrolyse de la S-adénosylhomocystéine (SAH) par la SAH hydrolase (Broch and Ueland, 1980). L'adénosine, générée dans la cellule, est transportée dans les espaces extra-cellulaires principalement par des transporteurs spécifiques bi-directionnels via un système de diffusion facilitée qui maintient l'équilibre des concentrations en adénosine entre milieu intra et extra cellulaire (Figure 2). Dans certains tissus, par exemple les reins ou les cellules mononucléées, il existe des transporteurs de nucléosides, dits concentratifs, capables de maintenir des concentrations intra cellulaires élevées d'adénosine. Ces protéines de transport ont été clonées et nommées ENT1 et ENT2 (pour Equilibrative Nucleoside Transport protein) et CNT1 et CNT2 (pour Concentrative Nucleoside Transport protein) (Anderson et al. 1996; Baldwin et al. 1999). En cas de diminution de l'activité des transporteurs, à cause de certaines drogues ou par baisse de la température, le taux d'adénosine extra-cellulaire augmente (Dunwiddie and Diao, 2000).

Dans le milieu extra-cellulaire, la formation d'adénosine est sous la dépendance de CD39, une ecto-apyrase qui transforme l'ATP en AMP et de CD73, une ecto-5'-nucléotidase qui convertit l'AMP en adénosine (Eltzschig et al. 2006). Ces enzymes sont essentielles pour la production d'adénosine à partir de l'ATP neuronale en conditions physiologiques (Dunwiddie et al. 1997; Zimmermann et al. 1998). Ces réactions s'opèrent en quelques centaines de millisecondes, et l'étape limitante est la déphosphorylation de l'AMP en adénosine par l'ecto-5'-nucléotidase (Dunwiddie et al. 1997). Il existe également une forme soluble de 5'-nucléotidase, qui est libérée avec l'ATP par des terminaisons nerveuses sympathiques stimulées et qui participe à l'hydrolyse extra-cellulaire de l'ATP en adénosine (Todorov et al. 1997). Néanmoins, il a été clairement montré que la synthèse d'adénosine intra-cellulaire est, quantitativement, aussi importante que celle faite à partir des nucléotides extra-cellulaires (Lloyd and Fredholm, 1995; Lloyd et al. 1993). Cette synthèse intra-cellulaire peut même être prédominante grâce à l'activité des 5'-nucléotidases intra-cellulaire dont deux formes, cN-I et cN-II, ont été clonées (Sala-Newby et al. 1999). Le cN-I catalyse la transformation de l'AMP en adénosine, tandis que le cN-II catalyse, respectivement, la transformation de de l'IMP et GMP en inosine et guanosine (Sala-Newby et al. 2000).

Si la concentration en adénosine augmente dans le milieu extra-cellulaire, l'adénosine est transportée dans les cellules par l'intermédiaire d'un système de diffusion facilitée (Plagemann, 1986). Ensuite, elle est phosphorylée en AMP par l'adénosine kinase (K_M = 100 nM) (Spychala et al. 1996) ou dégradée en inosine par l'adénosine désaminase (ADA) de moindre d'affinité (K_M entre 20 et 100 µM) (Lloyd and Fredholm, 1995). Dans le cœur et divers tissus, l'inhibition de l'ADA, induite par l'hypoxie, favorise la formation d'adénosine à partir de l'AMP et conduit à une augmentation majeure de la concentration en adénosine (Decking et al. 1997). L'adénosine kinase étant quasiment absente du milieu extra-cellulaire, l'adénosine ne peut être rephosphorylée en AMP ou ATP (Lloyd and Fredholm, 1995).

L'adénosine peut être aussi libérée dans le compartiment extra-cellulaire sous l'influence de certains neuro-transmetteurs comme l'acide N-méthyl-D-aspartique (NMDA) et le kaïnate qui augmentent la libération d'adénosine d'une manière dose-dépendante (Carswell et al. 1997; Delaney et al. 1998). L'activation des récepteurs de NMDA semble libérer l'adénosine elle-même plutôt qu'un précurseur (Harvey and Lacey, 1997; Manzoni et al. 1994). Une autre source d'adénosine extra-cellulaire est l'AMPc qui peut être libérée par des neurones et convertie, successivement, par des phosphodiestérases extra-cellulaires, en AMP puis, par une ecto 5'-nucléotidase, en adénosine (Jackson et al. 2007; Jackson and Raghvendra, 2004).

2. Régulation

La production d'adénosine dépend de l'état des cellules. En l'absence de stress oxydatif, l'hydrolyse de la SAH produit environ 30% de l'adénosine, le reste est obtenu par déphosphorylation d'AMP (figure 3). Dans ces circonstances, la concentration intra-cellulaire en adénosine est sous contrôle enzymatique et reste faible. En cas de stress cellulaire, la synthèse se fait essentiellement par dégradation d'ATP et la concentration en adénosine extra-cellulaire augmente et peut atteindre jusqu'à 100 fois les valeurs basales (Fredholm et al. 2001; Ralevic and Burnstock, 1998). Dans ces conditions, les cellules en stress captent moins bien l’adénosine extra-cellulaire, ce qui contribue à l’augmentation de sa concentration locale. En plus, quand la concentration intra-cellulaire en ATP s’effondre, suite à l’utilisation excessive d’énergie, celle en adénosine croît considérablement. L’adénosine est ensuite transportée dans l’espace extra-cellulaire via des transporteurs mentionnés ci-dessous (Fredholm et al. 1994). Ainsi en cas d’hypoxie ou d’ischémie, il existe une augmentation des concentrations en adénosine extra-cellulaire qui constitue donc un marqueur très sensible de cet état physiopathologique (Sollevi, 1986). Par ailleurs, la libération directe de l’ATP, qui est ensuite converti dans le compartiment extra-cellulaire en adénosine, fait qu’il existe une relation entre l’ATP et ses récepteurs et entre l’adénosine et ses récepteurs dans le sens où chacune de ces molécules va agir successivement sur son récepteur spécifique et l’ATP peut alors être considéré comme une pro-drogue de l’adénosine (Matsuoka and Ohkubo, 2004).

La régulation de la concentration extra-cellulaire d’adénosine est un processus très dynamique avec un taux de renouvellement considérable. Les concentrations en adénosine dans le milieu extra-cellulaire varient selon les différents tissus et se situe généralement entre 10^{-8} et 10^{-7} M (Headrick, 1996; Siragy and Linden, 1996). Dans le plasma les concentrations chez le sujet normal varie entre 10^{-7} et $7X10^{-7}$ (Guieu et al 1994 et 1999, Saadjian et al. 2002). La dégradation et la captation rapide de l’adénosine par les cellules explique que sa demi-vie plasmatique soit très courte, de l’ordre de quelques secondes (Dawicki et al. 1986; Klabunde and Althouse, 1981; Dussol et al 2004). Cette demi-vie courte conduit à considérer l’implication de l’adénosine dans le processus autocrine et paracrine mais pas endocrine.

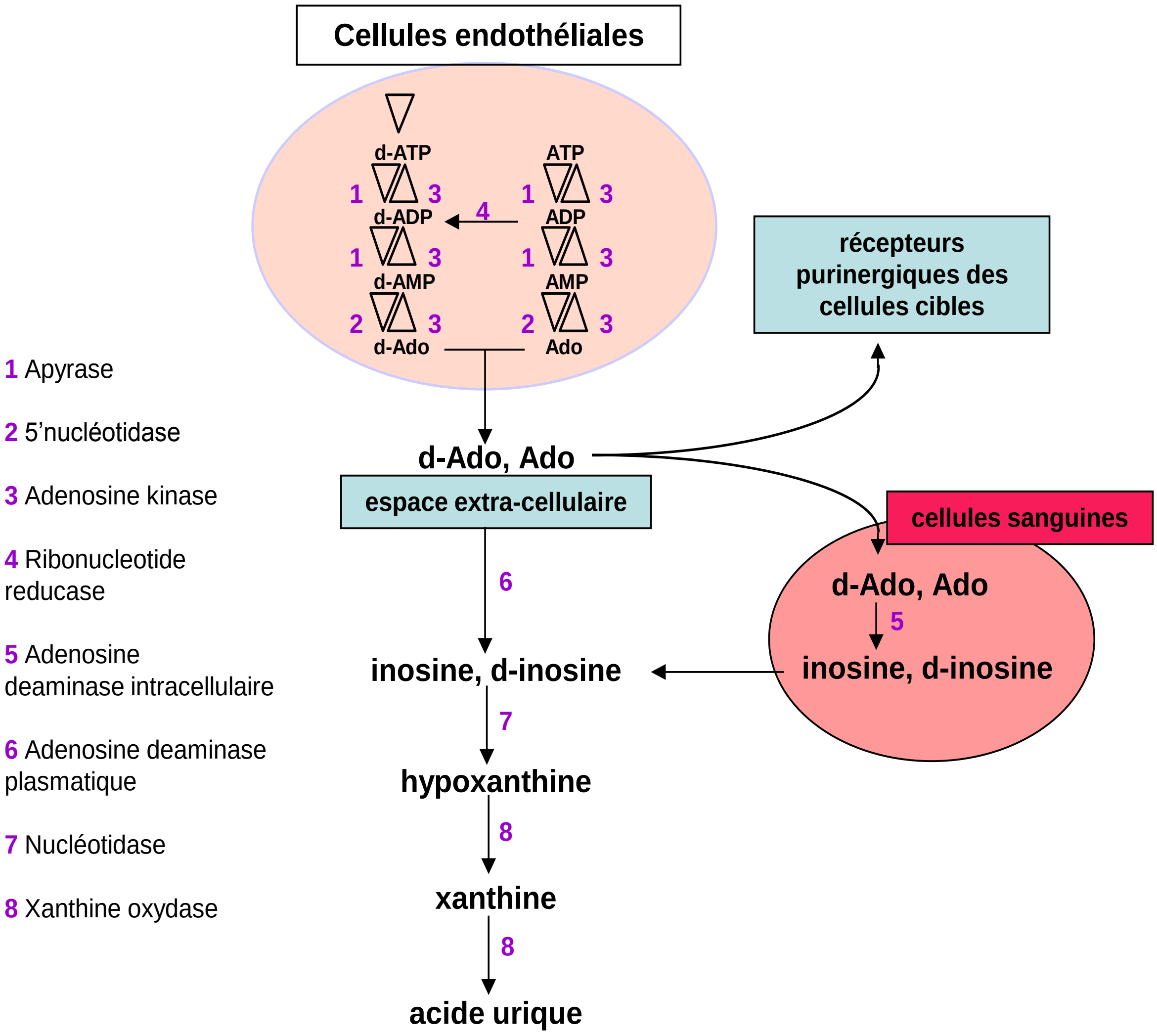
Cellules endothéliales
d-ATP
ATP
1
3
d-ADP
4
ADP
1
3
d-AMP
AMP
2
3
d-Ado
Ado
récepteurs purinergiques des cellules cibles
d-Ado, Ado
espace extra-cellulaire
cellules sanguines
d-Ado, Ado
5
inosine, d-inosine
6
inosine, d-inosine
7
hypoxanthine
8
xanthine
8
acide urique
1 Apyrase
2 5’nucléotidase
3 Adenosine kinase
4 Ribonucleotide reducase
5 Adenosine deaminase intracellulaire
6 Adenosine deaminase plasmatique
7 Nucléotidase
8 Xanthine oxydase

Figure 2. Devenir de l’adénosine extra-cellulaire

D’après Sampol J et al JASN, 2001 modifié

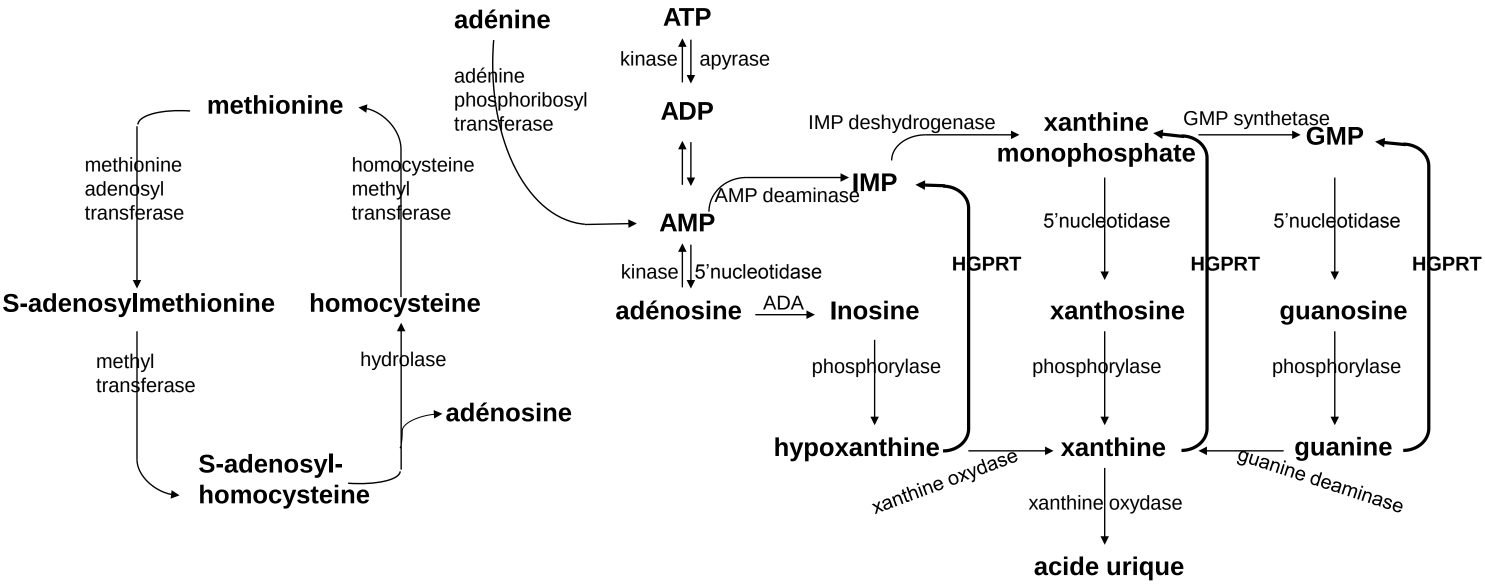
adénine
ATP
kinase
apyrase
adénine phosphoribosyl transferase
methionine
ADP
IMP deshydrogenase
xanthine monophosphate
GMP synthetase
GMP
methionine adenosyl transferase
homocysteine methyl transferase
AMP deaminase
IMP
AMP
5'nucleotidase
5'nucleotidase
kinase
5'nucleotidase
HGPRT
HGPRT
HGPRT
S-adenosylmethionine
homocysteine
adénosine
ADA
Inosine
xanthosine
guanosine
methyl transferase
hydrolase
phosphorylase
phosphorylase
phosphorylase
adénosine
hypoxanthine
xanthine
guanine
S-adenosyl-homocysteine
xanthine oxydase
guanine deaminase
xanthine oxydase
acide urique

Figure 3. Sources principales de l'adénosine.

ADA : adénosine déaminase, HGPRT : hypoxanthine guanine phosphoribosyl transférase.

III. Récepteurs purinergiques

Les nucléotides puriques ou pyrimidiques exercent des effets physiologiques par l'activation de récepteurs cellulaires dont le clonage et les effets pharmacologiques ont permis de distinguer deux grands groupes de récepteurs : les récepteurs pour l'adénosine et les récepteurs pour les nucléotides puriques ou pyrimidiques. Les récepteurs pour l'adénosine (P1) font tous partie de la superfamille de récepteurs couplés à une protéine G tandis que les récepteurs pour les nucléotides se distinguent en récepteurs ionotropiques (type canaux ioniques ; P2X) et en récepteurs métabotropiques couplés à une protéine G (P2Y) (Burnstock, 2007). Seuls les récepteurs de l'adénosine, en particulier les sous-types A_1 et A_{2A}, seront décrits dans ce manuscrit.

IV. Récepteurs de l'adénosine

Les récepteurs de l'adénosine font partie de la superfamille des récepteurs couplés aux protéines G. Ils ont été classés en quatre types, les A_1R, $A_{2A}R$, $A_{2B}R$ et A_3R, en fonction de leur séquence primaire et de leurs propriétés pharmacologiques (Ralevic and Burnstock, 1998; Fredholm et al. 2000 et 2001; Sebastiao and Ribeiro, 2009). Ces récepteurs ont été clonés à partir d'une grande variété de types cellulaires, dans plusieurs espèces, et ont pu être exprimés dans divers modèles cellulaires. Avant les années 80, on utilisait surtout des tests fonctionnels pour étudier l'effet des ligands sur l'animal ou la genèse de seconds messagers. Actuellement, on utilise des lignées cellulaires transfectées ou des animaux génétiquement modifiés pour l'étude de l'expression ou l'étude fonctionnelle de ces récepteurs (Yaar et al. 2005).

Structure

Les récepteurs de l'adénosine sont caractérisés par une structure formée d'une seule chaine polypeptidique comprenant sept hélices transmembranaires, avec une partie amino (N)-terminale extra-cellulaire et une partie carboxy (C)-terminale cytosolique (Costanzi S et al. 2007). Ces hélices, constituées de 25 à 30 résidus d'acides aminés, sont connectées par six boucles comprenant trois boucles intra-cellulaires et trois boucles extra-cellulaires. Les régions extra-cellulaires contiennent des sites pour les modifications post-traductionnelles comme la glycosylation. Les A_1R et A_3R contiennent également des sites pour la palmitoylation dans le domaine C-terminal. Les $A_{2A}R$ possèdent un long segment C-terminal de plus de 120 acides aminés qui ne sont pas indispensables pour le couplage à la protéine G mais qui peuvent intervenir pour la fixation pour des protéines de régulation (Zezula and Freissmuth, 2008). Les homologies de séquence pour les récepteurs humains de l'adénosine sont de 49 % entre les A_1R et A_3R, et de 59 % entre les $A_{2A}R$ et $A_{2B}R$. Dans les A_3R, le résidu Histidine dans l'hélice 6 est absent mais un autre résidu Histidine est présent, à un autre emplacement, dans l'hélice 3. L'implication de ces résidus Histidine dans la fonction de reconnaissance et/ou l'activation du récepteur a été démontrée par mutagenèse dirigée (Costanzi S et al. 2007; Kim et al. 2003). Ces Histidines établissent des liaisons hydrogène avec les ligands.

Tous les récepteurs humains de l'adénosine ont une structure génomique similaire (Fredholm et al. 2000; Olah and Stiles, 2000). Le gène de l'A_1R a été le plus étudié. Il comporte six exons et cinq introns et il est situé sur le chromosome 1q32.1 (Townsend-Nicholson et al. 1995). Ce gène est à l'origine de deux transcrits qui sont sous la dépendance de deux promoteurs, le promoteur A du côté proximal et B du coté distal. Le transcrit contenant les exons 4, 5 et 6 est présent dans tous les tissus exprimant le récepteur tandis que le transcrit contenant les exons 3, 5 et 6 se trouve dans les tissus à fort niveau d'expression tels que le cerveau, les testicules et les reins. Le gène de l'$A_{2A}R$ humain est localisé sur le chromosome 22q11.2 et possède une structure à deux exons (Fredholm et al. 2000) similaire à celle rapportée chez le rat (Chu et al. 1996).

L'$A_{2A}R$ présente un transcrit hybride dans la plupart des tissus examinés (Maenhaut et al. 1990; Peterfreund et al. 1996; Stehle et al. 1992). L'examen d'ARN isolés de cellules PC12 suggère deux sites différents d'initiation de la transcription (Chu et al. 1996). L'expression de l'$A_{2A}R$ peut être stimulée par les protéines kinases C (PKC) (Peterfreund et al. 1997) et par l'hypoxie (Kobayashi et al. 1998), bien qu'on ne connaisse pas précisément les facteurs de transcription impliqués. Il faut noter que l'$A_{2A}R$ est polymorphe chez l'homme (Fredholm et al. 2000). Il existe un polymorphisme (Single Nucleotide Polymormphism, SNP) de population, en particulier, le SNP T1083C est plus représenté chez les caucasiens que chez les asiatiques (Deckert et al. 1996; Soma et al. 1998). Ce polymorphisme, bien que muet, serait associé à certaines pathologies comme les syncopes neurocardiogéniques (Saadjian et al. 2009) ou les attaques de panique (Hamilton et al. 2004).

Le gène de l'$A_{2B}R$ est localisé sur le chromosome 17p11.2 (Jacobson et al. 1995) et présente une structure similaire à celle des gènes des autres sous-types de récepteurs. Chez le rat, l'$A_{2B}R$ présente deux transcrits hybrides de 1.8 et 2.2 kb, ce dernier étant le plus représenté (Stehle et al. 1992). Le gène de l'A_3R humain est situé sur le chromosome 1p13.3. Il présente deux transcrits, dont le plus et le moins représenté sont, respectivement, de 2 kb et 5 kb (Atkinson et al. 1997).

SIGNALISATION

De nombreuses évidences supportent l'idée que les récepteurs de l'adénosine régulent la fonction cellulaire par couplage aux protéines G, bien que certains effets indépendants des protéines G aient été également rapportés (Fredholm et al. 2007). L'effecteur principal des quatre sous-types de récepteurs de l'adénosine est l'adénylcyclase dont l'activité est soit stimulée, soit inhibée suivant le sous-type de récepteur présent sur la cellule. Ainsi les A_1R et A_3R sont couplés aux protéines G_i induisant une diminution de la concentration d'AMPc, tandis que les $A_{2A}R$ et $A_{2B}R$ stimulent l'adénylcyclase par activation des protéines G_s avec, par conséquent, une augmentation d'AMPc. Par ailleurs, la signalisation peut être transmise par d'autres protéines G telles que la G_q ou la G_{olf}. Il a été montré que les $A_{2A}R$ pouvaient se coupler aux différentes protéines G dans les différents tissus (Kull et al. 2000). La protéine G_s est associée aux $A_{2A}R$ dans les systèmes périphériques mais pas dans le striatum où la densité des $A_{2A}R$ est plus élevée. Ainsi, la signalisation des $A_{2A}R$ du striatum passe essentiellement via la G_{olf}. D'ailleurs, un récepteur de l'adénosine peut aussi se coupler à plusieurs protéines G après transfection. Par exemple, les $A_{2B}R$ humains transfectés dans les cellules de mastocytes humains (HEK 293) ou de mastocytes canins (HMC-1) sont couplés à la fois aux G_s et G_q (Auchampach et al. 1997; Linden et al. 1999).

Après l'activation des protéines G, la voie de signalisation affecte les enzymes et les canaux ioniques. L'activation des A_1R induit une inhibition de l'adénylcyclase, une activation de certains types des canaux K^+ (en particulier les KIR, via les sous-unités β,γ de la protéine G), une inactivation des canaux Ca^{2+} de types N, P et Q, une activation de la phospholipase Cβ et une activation des MAP-kinases. Cette voie de signalisation semble être identique pour les A_3R (Englert et al. 2002). Les $A_{2A}R$ et $A_{2B}R$ peuvent aussi induire une augmentation intra-cellulaire de Ca^{2+} selon le type de cellule. La voie de signalisation par les MAP-kinases a été aussi démontrée pour les $A_{2A}R$ et $A_{2B}R$ (Schulte and Fredholm, 2000).

1. Récepteurs A_1 de l'adénosine

L'A_1R a été cloné chez de nombreuses espèces telles que la souris, le rat, le lapin, le chien, les bovins et l'homme. Son polymorphisme présente jusqu'à 10% de variation entre les espèces. Ceci peut expliquer les variations d'affinité (Tucker et al. 1992) et certaines différences dans les processus de régulation (Palmer and Stiles, 1997) ainsi que dans le couplage aux protéines G (Jockers et al. 1994). Il a un poids moléculaire d'environ 36 kDa et une forte affinité pour l'adénosine.

1.1 Distribution

La répartition des A_1R est large et leur transcription est bien corrélée à leurs expressions dans les tissus. On trouve une forte expression dans certaines parties du cerveau (cortex, cervelet, hippocampe, thalamus et tronc cérébral), les cornes postérieures de la moelle, les yeux, les surrénales et le cœur. L'expression des A_1R est moyenne dans le reste du cerveau, les muscles squelettiques, le foie, les reins, les adipocytes, les glandes salivaires, l'œsophage, l'estomac, le colon et les testicules. L'expression est faible dans les poumons, le pancréas et l'intestin grêle (tableau 1).

Tableau 1 : Expression des sous-types de récepteurs de l'adénosine dans différents tissus

	Expression forte	**Expression intermédiaire**	**Expression faible**
A_1R	Cerveau (cortex, cervelet, hippocampe) cornes dorsales de la moelle, yeux, cœur, surrénales	Autres régions du cerveau, muscle squelettique Foie, reins, tissus adipeux, glandes salivaires, œsophage, colon, testicules	Poumons, pancréas
$A_{2A}R$	Rate, thymus, lymphocytes, plaquettes, striatum, bulbes olfactifs	Cœur, poumons, vaisseaux sanguins	Autres régions du cerveau
$A_{2B}R$	Cæcum, colon	Poumon, vaisseaux, yeux, éminence médiane, Mastocytes	Tissus adipeux, surrénales, cerveau, reins, foie, ovaires
A_3R	Testicules Mastocytes	Cervelet, hippocampe Poumons, rate, hypophyse	Thyroïde, cerveau, cœur, intestin, surrénales, foie

1.2 Récepteurs A_1 dans le système cardiovasculaire

L'activation des A_1R induit de nombreux effets dans le système cardiovasculaire tels qu'une diminution du rythme et de la contractilité cardiaque ainsi qu'une atténuation de l'action stimulatrice des catécholamines sur le cœur (Fraser et al. 2003; Zablocki et al. 2004) ; pour revue voir (Shryock and Belardinelli, 1997). Les effets chronotropes (fréquence cardiaque), dromotropes (vitesse de conduction myocardique) et inotropes (contractilité myocardique) négatifs de l'A_1R induits par l'adénosine intraveineuse (Olsson and Pearson, 1990) ont été utilisés dans le traitement des tachycardies supra-ventriculaires. L'activation des A_1R conduit à une modulation indirecte du tonus vasculaire par inhibition de la libération des neurotransmetteurs au niveau pré-jonctionnel dans les fibres sympathiques péri-vasculaires (Goncalves and Queiroz, 1996) et dans les afférences sensitives, sensibles à la capsaïcine (8-méthyle *N*-vanillyle 6-nonénamide) qui est le composé actif du piment *Capsicum* (Rubino et al. 1993). Suivant le tissu étudié, les effets peuvent être opposés. Les A_1R participent avec les A_3R, aux effets protecteurs de l'adénosine dans les accidents d'ischémie-reperfusion myocardiques (Stambaugh et al. 1997), pulmonaires (Neely and Keith, 1995) et cérébraux (Heurteaux et al. 1995). Les canaux potassium ATP-sensible et les PKC semblent être les voies principalement impliquées dans ces phénomènes. Récemment il a été mentionné que les récepteurs A1 pouvaient moduler l'angiotensine II et les entrées de sodium, ouvrant de nouvelles pistes thérapeutiques contre l'Hypertension (Lee DL et al. 2012). Enfin les récepteurs A1 moduleraient les entrées de calcium dans les cellules atriales et ventriculaires embryonnaires via une phospholipase C. Ces effets expliqueraient en partie les effets de l'activation de ces récepteurs sur le rythme cardiaque (Sabourin J et al. 2012).

2. Récepteurs A_{2A} de l'adénosine

L'$A_{2A}R$ a été cloné dans de nombreuses espèces. Son poids moléculaire est de 45 kDa et sa structure cristallographique a été déterminée aux rayons X (Jaakola et al. 2008). Par rapport aux A_1R, les $A_{2A}R$ ont une affinité plus faible pour l'adénosine.

2.1 Distribution

Les $A_{2A}R$ sont principalement exprimés dans le cerveau (en particulier les bulbes olfactifs, le noyau accumbens et le striatum), dans les cellules du système immunitaire, dans les plaquettes et dans les fibres musculaires lisses des vaisseaux et de l'endothélium (voir tableau 1). Il semble que les $A_{2A}R$ soient les récepteurs majoritaires de l'adénosine dans les lymphocytes et les plaquettes. Dans les neurones du striatum, les $A_{2A}R$ sont co-localisés avec les récepteurs D_2 de la dopamine surtout dans les neurones GABA-ergiques striato-pallidaux (Weaver, 1993). D'ailleurs, l'expression des $A_{2A}R$ est faible dans les autres régions du cerveau telles que le cortex et l'hippocampe. L'expression est intermédiaire dans le cœur, les poumons et les vaisseaux.

2.2 Récepteurs A_{2A} dans le système cardiovasculaire

L'activation des $A_{2A}R$ conduit à un relâchement des fibres musculaires des parois vasculaires et à une vasodilatation (Conti et al. 1993; Iwamoto et al. 1994; Belardinelli et al. 1998; Monahan et al. 2000) ; pour revue voir (Shryock and Belardinelli, 1997). Ainsi les souris KO déficientes pour l'$A_{2A}R$ sont spontanément hypertensives (Ledent et al. 1997).

Dans les artères coronaires où les $A_{2A}R$ sont prédominants par rapport aux autres sous-types, l'effet de la vasodilatation passe principalement par la voie de l'AMPc (Hussain and Mustafa, 1993; Rekik and Mustafa, 2003). Par ailleurs, ces effets sur la vasodilatation sont, en partie, secondaires à la modulation de canaux ioniques, en particulier à l'inhibition de canaux calcium voltage-dépendant, mais également à la modulation de canaux potassium (rectifiant rentrant, K_{ATP}) (Bryan and Marshall, 1999; Tang et al. 1999). Toujours au niveau coronarien, il a été mentionné l'existence de récepteurs de réserve (spare receptors), qui contrôleraient le flux coronaire (Shryock et al. 1998). L'existence de « spare receptors » est objectivée chaque fois que les effets métaboliques cellulaires sont au maximum (par exemple la production d'AMPc), alors même que tous les récepteurs ne sont pas occupés par le ligand, autrement dit chaque fois que la valeur de l'EC_{50} est inférieure au K_D. L'existence de ces récepteurs de réserve au niveau coronarien permet d'expliquer la vasodilatation coronaire en l'absence d'effets systémiques en particulier sur la pression artérielle (Shryock et al. 1998). Il a été suggéré que l'activation des $A_{2A}R$ au niveau central induisait une tachycardie, tandis que l'activation des $A_{2A}R$ périphériques était responsable d'un effet hypotensif (Schindler et al. 2005). A la frontière entre système cardiovasculaire et système nerveux, il a été montré que les $A_{2A}R$ sont très exprimés dans une région du tronc cérébral qui contrôle la fréquence cardiaque et la pression artérielle (Thomas et al. 2000).

A coté du rôle de protection contre l'ischémie assuré par les A_1R, il a été démontré chez la souris que l'activation des $A_{2A}R$ avait une action protectrice contre les atteintes secondaires au phénomène d'ischémie-reperfusion, et que cet effet protecteur était dû, en partie, à une action anti-inflammatoire via les $A_{2A}R$ des lymphocytes (Yang et al. 2005). Dans les plaquettes, l'activation des $A_{2A}R$ induit une inhibition de l'agrégation plaquettaire en augmentant la concentration intra-cellulaire en AMPc (Varani et al. 2000).

3. Récepteurs A_{2B} de l'adénosine

L'$A_{2B}R$ est le sous-type le moins étudié de la famille des récepteurs de l'adénosine. Le gène de l'$A_{2B}R$, se trouve sur le chromosome humain (17p11.2), montre la présence d'un pseudogène sur le chromosome 1q32 ayant 79% d'homologie avec la séquence codante de l'ADNc de l'$A_{2B}R$ (Jacobson et al. 1995). Les $A_{2B}R$ ont une faible affinité pour l'adénosine.

Distribution et rôles biologiques

Les études des ARNm montrent que les A_{2B}R sont présents principalement dans le caecum, le colon et la vessie, ainsi qu'au niveau des noyaux gris de la base du crâne où, à coté des A_{2A}R, ils participeraient au contrôle de la motricité. Ils sont exprimés également, quoiqu'à un faible niveau, dans les poumons, les vaisseaux sanguins, les yeux et les mastocytes (voir tableau 1) (Fredholm et al. 2001). L'affinité de ces récepteurs pour l'adénosine reste faible et leur activation nécessite de forte concentration d'adénosine. L'activation des A_{2B}R induit une dégranulation des mastocytes, impliqués dans les réactions allergiques et inflammatoires (Feoktistov and Biaggioni, 1997), et conduit à une vasodilatation comme l'activation des A_{2A}R (Rubino et al. 1995). Récemment, il a été montré grâce à l'utilisation de souris transgénique (double KO A_{2A}/A_{2B}) que les deux types de récepteurs participaient à la régulation du flux coronaire via les canaux potassium ATP sensible (Sanjani MS et al. 2011). L'activation des A_{2B}R entraîne aussi une inhibition de la prolifération des fibres musculaires lisses vasculaires (Dubey et al. 1996) favorisant leur apoptose et participant vraisemblablement au processus d'athérosclérose (Peyot et al. 2000).

Récemment le rôle des A_{2B} R a été démontré dans les processus de protection contre l'ischémie-reperfusion dans le rein (Weinberg JM et al. 2012). Le rôle de l'A_{2B}R a été mentionné dans le transport des ions chlorures au niveau des cellules épithéliales respiratoires et digestives (Bucheimer and Linden, 2004).

4. Récepteurs A_3 de l'adénosine

Le récepteur A_3 a été cloné dans diverses espèces et son gène est localisé chez l'homme sur le chromosome 1p13.3 (Monitto et al. 1995). Il est le seul récepteur sur lequel l'inosine a un effet en provoquant la dégranulation des mastocytes (Jin et al. 1997). Comme les A_{2B}R, les A_3R ont une faible affinité pour l'adénosine, ce qui implique des concentrations élevées d'adénosine pour leur activation.

Distribution et rôles biologiques

Des études sur les ARNm ont montré un haut niveau d'expression des A_3R dans les testicules et les mastocytes de rat, une expression plus faible dans le cerveau, le cœur, les reins, la rate, le placenta, l'utérus, et l'intestin (Fredholm et al. 2001).

L'activation des A_3R induit une dégranulation des mastocytes, favorisant ainsi l'inflammation (Ramkumar et al. 1993), et conduit à une diminution de l'activité motrice chez la souris (Jacobson et al. 1993). Les A_3R semblent être impliqués dans l'effet cardio-protecteur et neuro-protecteur de l'adénosine comme les A_1R (Tracey et al. 1998). Dans les cellules polynucléées, l'endothélium, les cardiomyocytes et les fibres musculaires lisses, l'activation des A_3R stimule le système anti-oxydatif par augmentation de l'activité de la superoxyde dismutase, de la catalase et de la glutathion réductase (Maggirwar et al. 1994). Enfin, récemment il a été suggéré que les récepteurs A_3 R exerçaient un effet neuroprotecteur dans le SNC via l'inhibition des voies glutamatergiques (Rosito M et al. 2012).

V. Utilisation de l'adénosine en cardiologie

1. Utilisation de l'adénosine en rythmologie

L'adénosine est utilisée au quotidien dans le domaine de la rythmologie clinique depuis des dizaines d'années, que ce soit dans les services d'urgence ou de cardiologie.

Dans le domaine des tachycardies, son injection intraveineuse a un intérêt majeur (Blomström-Lundqvist et al. 2003). D'une part, grâce à son effet inhibiteur de la conduction nodale (récepteur A1), l'adénosine permet la réduction des très banales crises de tachycardie jonctionnelle. De plus, toujours par son effet sur le nœud auriculo-ventriculaire, elle permet l'obtention de diastoles longues chez les patients en crise de tachycardie, dévoilant ainsi l'activité atriale, et permettant un diagnostic plus précis du mécanisme de l'arythmie. Par ailleurs, son injection intraveineuse est une aide au diagnostic des tachycardies à QRS larges, permettant le plus souvent de différencier une tachycardie supra-ventriculaire (dont la cadence sera au moins ralentie) d'une tachycardie ventriculaire (dont le cycle restera inchangé le plus souvent).

Dans le domaine des syncopes, son injection a été proposée (Flammang et al. 2005) en tant que test diagnostique d'une forme particulière de syncope neurocardiogénique, la syncope adénosine-sensible. Ce test à l'ATP consiste en l'injection intraveineuse de 20 mg d'ATP avec enregistrement continu d'un ECG. On considère que ce test est positif si la pause cardiaque induite par cette injection dépasse 6 secondes (Flammang et al. 2005). Ce résultat oriente le clinicien vers le diagnostic de syncope adénosine-sensible. On confirmera ce diagnostic par l'enregistrement d'un ECG au moment de la syncope (grâce à un enregistreur externe d'évènements, ou un holter implantable). Typiquement, le patient présente alors un bloc auriculo-ventriculaire complet et brutal per-critique, dont le mécanisme est extrinsèque (Moya et al. 2009). Le traitement consistera en la mise en place d'un stimulateur cardiaque. Ce test est réalisé au quotidien dans notre unité de prise en charge des syncopes.

Plus récemment, l'injection d'adénosine a été proposée au cours des procédures d'ablation de la fibrillation atriale (Arentz et al. 2005). Le traitement ablatif de la fibrillation atriale, qui est l'arythmie cardiaque la plus fréquemment rencontrée, repose essentiellement sur la déconnection électrique des veines pulmonaires. Cette procédure percutanée est réalisée en routine dans un grand nombre de centres. La déconnection électrique est obtenue dans près de 100 % des cas. Toutefois, des reconnections peuvent survenir de manière différée, a distance de la procédure. Ce faisant, le risque de récidive de fibrillation atriale est majeur. Il a été démontré que l'injection d'adénosine per-procédure, par son action sur le potentiel membranaire (Datino et al. 2010), permettait de mettre en évidence des « conductions dormantes » des veines pulmonaires. De nombreux opérateurs utilisent donc l'adénosine en fin de procédure pour mettre en évidence d'éventuelles conductions dormantes. Elles devront être supprimées afin d'optimiser le taux de succès de l'intervention.

Enfin, des études plus fondamentales ont montré que l'adénosine exogène pouvait avoir aussi bien des effets anti-arythmiques que pro-arythmiques. On sait que l'injection intraveineuse d'adénosine chez l'animal (Kabell et al. 1994) et chez l'homme (Nunain et al. 1992; Botteron et al. 1994) peut induire une FA. Chez les patients présentant une FA paroxystique ou persistante, l'adénosine accélère les zones pérennisant l'arythmie, et augmente sa fréquence dominante (Atienza et al. 2006). Par ses effets sur les canaux potassiques rectifiants rentrants, l'adénosine raccourci la durée du potentiel d'action et sa période réfractaire, ce qui augmente la vulnérabilité aux arythmies atriales. (Kabell et al. 1994; Belardinelli et al. 1995).

2. Utilisation de l'adénosine en condition d'hypoxie myocardique

On sait que l'adénosine peut-être libérée sous l'effet d'un stress, en particulier hypoxique. Ses effets protecteurs dans des conditions d'ischémie tissulaire ont conduit à son utilisation dans différentes situations.

L'adénosine peut-être utilisée en **cardioplégie au cours d'interventions de chirurgie cardiaque** sous circulation extracorporelle. De manière très intéressante, son usage, en plus d'être aussi efficace que la stratégie classique, diminue le risque de FA post-opératoire (Jakobsen et al. 2012).

Au cours de l'infarctus du myocarde avec élévation du segment ST, une procédure de revascularisation coronaire est indiquée en urgence. Si possible, elle consiste en une angioplastie coronaire percutanée. Cette procédure permet dans la grande majorité des cas une désobstruction de l'artère. Toutefois, malgré une réouverture de l'artère, il peut subsister une obstruction de la micro-vascularisation coronaire, phénomène de « no reflow », de très mauvais pronostic. Dans ce contexte, il est recommandé par la société européenne de cardiologie de réaliser des injections intra-coronaires d'adénosine, visant à désobstruer la microcirculation, et limiter ainsi la taille de l'infarctus. Plusieurs études montrent l'efficacité de cette approche (Steg et al. 2012).

3. L'adénosine endogène et ses récepteurs.

Les données exposées ci-dessus portent sur les effets de l'adénosine exogène, le plus souvent suite à une injection intra-vasculaire. Elles soulignent une fois de plus le caractère très polymorphe de ses effets, y compris dans un seul domaine, celui de la cardiologie. Les effets de l'administration d'adénosine ont été étudiés de manière exhaustive. En revanche, il n'y a encore qu'une dizaine d'années, aucune donnée sur l'implication de l'adénosine endogène et de ses récepteurs en cardiologie n'était publiée. Ce manque de données est lié à la difficulté technique du dosage de l'adénosine endogène, ou de la quantification de l'expression et de la fonction de ses différents récepteurs. Notre équipe a été pionnière dans ces domaines. Ont été mis au point de manière successive une technique fiable de dosage de l'adénosine plasmatique en routine (Guieu et al. 1994 ; Guieu et al. 1999) ainsi qu'une technique de quantification de l'expression des A_{2A}R (Carrega et al. 2007) sur cellules mononuclées circulantes (reflet de leur expression au niveau cardiaque). Plus récemment, un anticorps monoclonal spécifique du A_{2A}R, mis au point dans notre laboratoire, présentant une activité agoniste a pu être obtenu et caractérisé (By et al. 2009), élargissant encore le champ des travaux de recherche réalisables.

Compte-tenu des nombreux effets cliniques de l'administration d'adénosine, et de la capacité de notre unité de recherche à analyser le système adénosinergique endogène, nous nous sommes intéressés aux implications de l’adénosine endogène et de ses récepteurs dans le domaine de la cardiologie, plus précisément ceux des syncopes neurocardiogéniques et de l’insuffisance cardiaque.

Chapitre deux

Travaux originaux

PRESENTATION DES TRAVAUX

A. Implication du système adénosinergique en rythmologie.

1. L'adénosine endogène au cours des syncopes neuro-cardiogéniques
2. Adénosine et expression des $A_{2A}R$ dans les différentes formes de syncopes neuro-cardiogéniques
3. Fonction des récepteurs A_{2A} R chez les patients présentant des syncopes neuro-cardiogéniques
4. Recherche de récepteurs A_{2A} R de réserve chez les lymphocytes humains circulants
5. Implication du système adénosinergique chez une enfant présentant des syncopes en rapport avec un bloc-AV peri-prandial paroxystique
6. Implication de l'adénosine endogène pour l'induction de fibrillation atriale en post-opératoire de chirurgie cardiaque

B. L'adénosine dans les phénomènes d'hypoxie tissulaire

1. Origine de la sécrétion d'adénosine chez l'insuffisant cardiaque
2. Régulation adénosinergique d'une lignée de lymphocytes T humains dans des conditions d'hypoxie in-vitro

A. Implication du système adénosinergique en rythmologie

1. L'adénosine endogène au cours des syncopes neuro-cardiogéniques

Les syncopes sont des pertes de connaissance brèves en rapport avec une diminution du débit sanguin cérébral (Flammang et al. 2005). Elles sont fréquentes puisqu'elles représentent 1 à 5% des hospitalisations dans des services d'urgences (Goldschlager et al. 2003). Leur étiologie est diverse et parfois d'origine cardiaque, consécutive à des troubles du rythme ou de la conduction et potentiellement mortels. Le plus souvent il s'agit de syncopes neuro-cardiogéniques (ou vagales) dont le caractère bénin est bien connu (Brignole 2005 ; Moya et al. 2009). Leur diagnostic est, le plus souvent, cliniquement évident. Elles surviennent chez un patient sans cardiopathie sous-jacente. En fin de repas ou suite à un facteur déclenchant, comme un stress ou la station debout dans une atmosphère confinée, en position debout ou assise, le patient décrit une sensation de lipothymie avec des prodromes à type de bouffées de chaleur, sueurs froides, nausées (le cortège vagal). Les observateurs notent une pâleur prononcée. Va suivre une perte de connaissance brève, parfois traumatique. Le retour à la conscience est rapide, sans confusion post-critique mais avec une asthénie qui pourra durer le reste de la journée. Au moment de la syncope, on note un collapsus avec chute de la pression artérielle associé ou non à une bradycardie. Cette syncope est due à une activation excessive de la 10ème paire de nerfs crâniens, le nerf vague. C'est le nerf vague qui est responsable d'une vasodilatation artérielle périphérique plus ou moins associée à une bradycardie, qui induit la chute de la pression artérielle. Si le caractère bénin de ces syncopes est, comme nous l'avons dit, clairement établi, elles peuvent se reproduire fréquemment et représenter un handicap fonctionnel majeur voire une entrave à la vie sociale. Par ailleurs, leur physiopathologie est complexe et partiellement inexpliquée. Le traitement comprend l'explication de la pathologie et la réassurance. On demandera aux patients de s'allonger, jambes surélevées en cas de prodromes. On recommandera par ailleurs une bonne hydratation pour augmenter la volémie et lutter contre la baisse de la pression artérielle, proscrivant les traitements vasodilatateurs ou diurétiques. Toutefois ce traitement reste peu efficace.

Si le diagnostic n'est pas évident et doit être confirmé, un examen permet de reproduire la symptomatologie : le test d'inclinaison (Moya et al. 2009; Sutton et al. 1999). Le patient est allongé sur une table. On suit la pression artérielle et électrocardiogramme en continu grâce à un dispositif digital (finapress). La table de tilt est ensuite inclinée à 70 degrés pendant 45 minutes au maximum. Durant cette phase d'inclinaison, une syncope vagale peut survenir associée à un collapsus tensionnel. Cet examen a une sensibilité médiocre mais une très bonne spécificité.

Les phénomènes induisant une syncope vagale sont une vasodilatation artérielle périphérique plus ou moins associée à une bradycardie. Or, l'adénosine peut induire une vasodilatation artérielle périphérique par l'intermédiaire de ses $A_{2A}R$ et une bradycardie par l'intermédiaire de ses A_1R. Nous avons donc émis l'hypothèse que l'adénosine pouvait-être impliquée dans la physiopathologie des syncopes vagales. Pour le confirmer, nous avons réalisé des prélèvements veineux pour le dosage du taux d'adénosine plasmatique au cours d'un test d'inclinaison chez des patients présentant des syncopes neurocardiogéniques.

Ce travail est détaillé dans l'article : **"Role of endogenous adenosine as a modulator of syncope induced during tilt-testing"** ci dessous.

Publication 1

“Role of endogenous adenosine as a modulator of syncope induced during tilt-testing”

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Role of Endogenous Adenosine as a Modulator of Syncope Induced During Tilt Testing

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Background—Previous reports that used head-up tilt testing and adenosine administration have suggested that adenosine may be an important endogenous mediator that may trigger a vasovagal response in susceptible patients. However, little is known regarding endogenous adenosine plasma levels (APLs) during vasovagal syncope provoked by tilt testing. The aim of this study was to determine whether APLs differ in patients with a positive head-up tilt test compared with those with a negative test and whether APLs are modified during tilt-induced vasovagal syncope.

Methods and Results—APLs (mean±SEM) were measured during head-up tilt test in 26 patients who presented with unexplained syncope. In the 15 patients with a negative test, APLs were 0.39±0.03 μmol/L at baseline, 0.22±0.03 μmol/L immediately after tilting, and 0.44±0.03 μmol/L after 45 minutes. APLs were significantly higher in the 11 patients with a positive test (2.66±0.67 μmol/L at baseline and 3.22±0.85 μmol/L immediately after tilting) than in those with a negative test. During tilt testing–induced syncope, APLs increased to reach 4.03±0.66 μmol/L (ie, a 52% increase compared with baseline levels; $P<0.02$). Furthermore, we observed that the higher the APL during syncope, the shorter the time to appearance of symptoms.

Conclusions—This study showed that APLs were higher in patients with a positive tilt test than in patients with a negative test and that they increased during tilt testing–induced syncope. These observations suggest that adenosine release may be involved in the triggering mechanism of syncope induced during tilt testing. **(*Circulation.* 2002;106:569-574.)**

Key Words: adenosine ■ syncope ■ tests

Neurally mediated syncope is a common clinical problem that may alter the quality of life of affected patients. The syndrome is characterized by arterial vasodilatation associated with relative or absolute bradycardia. The pathophysiological mechanisms responsible for the genesis of neurally mediated syncope have not been elucidated. Defective baroreflex response to an increase in sympathetic activity in a setting of ventricular hypovolemia has been advocated. Other mechanisms such as alterations in neurohumoral mechanisms may play a role.[1]

Tilt testing is an established tool for the evaluation of patients with unexplained syncope.[2] Several reports[3–5] have shown that exogenous adenosine or ATP is an effective agent for the provocation of neurally mediated syncope in susceptible patients, with a diagnostic yield comparable to that of tilt testing. Therefore, it appeared likely that adenosine might be an endogenous modulator that may play a role in triggering vasovagal syncope in predisposed patients. However, to the best of our knowledge, there is no information regarding endogenous adenosine plasma level changes during neurally mediated syncope induced by tilt testing. We evaluated endogenous adenosine plasma levels in patients with unexplained syncope and compared patients with a positive head-up tilt test to those with a negative test at baseline and during vasovagal syncope or presyncope.

Methods

Patient Selection

In this prospective study, we measured adenosine plasma levels during head-up tilt testing in 26 patients referred to our institution for unexplained syncope who fulfilled the inclusion criteria. Patients had to experience at least 2 episodes of syncope within the year preceding inclusion in the study. The cause of syncope was unexplained in all patients despite complete medical and neurological evaluation, including routine laboratory tests, 12-lead ECG, 24-hour ECG ambulatory monitoring, chest radiograph, M-mode and 2D echocardiography, carotid sinus massage, and further evaluation of any clinical or historical findings indicative of a possible cause of the syncope. Patients thought to have vasovagal syncope and with a noncontributory above-mentioned workup were included in the study. All patients underwent head-up tilt testing for the first time. Patients with orthostatic hypotension, anemia, and endocrine abnormalities such as diabetes, hypoglycemia, or thyroid dysfunction were excluded from the study. The following patients were also excluded from the study: those with ECG abnormalities (sinus bradycardia <50 bpm or bundle-branch block) or with an abnormal electrophysiological study that showed sinus node dysfunction, altered AV

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TABLE 1. Clinical Characteristics of the Patients

Variables	Population (26 Patients)	Negative Tilt Tests (15 Patients)	Positive Tilt Tests (11 Patients)	*P*
Age, y	52±3.3	56.9±3.4	45.3±5.9	NS
Sex, M/F	15/11	9/6	6/5	
Weight, kg	65±3.1	68.5±4.5	60.3±3.8	NS
Height, cm	166±2	168±3	165±3	NS
No. patients with heart disease	2	2	0	
Hypertension	1	1	0	
Mild mitral regurgitation	1	1	0	
None	24	13	11	
History of syncopal episodes				
Time since first episode, mo	4 (2–6)	4 (3–7.5)	3 (2–4.75)	NS
Syncopes and presyncopes per month	1.2 (0.5–2)	0.7 (0.4–1.2)	2 (1.6–2.2)	<0.05
Syncopal episodes per patient	3 (2–4)	2 (2–3)	4 (3–6)	<0.05
Presyncopal episodes per patient	1.5 (0–3)	0 (0–2)	3 (0.25–3.75)	<0.05
No. patients with presyncopal episodes	14	6	8	
No. patients with vasovagal symptoms	11	3	8	

Values are given as number of patients, mean±SEM, or median (interquartile range). NS indicates not significant.

conduction, or significant ventricular arrhythmias during programmed ventricular stimulation; patients with implanted pacemakers or with syncope related to aortic stenosis, hypertrophic cardiomyopathy, pulmonary hypertension, or subclavian steal syndrome; and those with drug-induced syncope. No patient in the present study had an adenosine or ATP test before inclusion in the study. Patients included in the study were drug free and were instructed to avoid coffee and tea for the 24 hours preceding the study. None of them was a regular tea or cola consumer. Usual daily consumption of coffee was low or moderate (≤3 cups of coffee), with an estimated consumption of caffeine $<3\ \mathrm{mg \cdot kg^{-1} \cdot d^{-1}}$ in all patients. No patient was treated with adenosine antagonists. Patients were studied in a fasting state after informed written consent was obtained. Clinical characteristics of patients are summarized in Table 1.

Study Protocol

Head-Up Tilt Test

Tilt testing was performed in a quiet and comfortable room maintained at 21°C with dimmed lights and equipped for cardiopulmonary resuscitation. Patients were allowed to lie on an electrically controlled tilt table (Siemens) for 30 minutes. During this time, an intravenous line containing 5% dextrose was inserted into one arm and an intravenous catheter was inserted into the other arm to facilitate blood sampling. A 6-lead ECG and an external blood pressure recorder were attached and regularly activated to familiarize the patient with their operation. The footboard support and chest and knee belts were adjusted and secured. The table was then smoothly tilted within 20 seconds to the 60° position for 45 minutes. The heart rate was monitored continuously and blood pressure measured manually by the same operator every 2 minutes. The table was returned quickly to the supine position when symptoms occurred or the test ended. Three blood samples were collected during every tilt test: (1) just before the table was tilted, (2) immediately after the table was tilted, and (3) either during syncope, immediately after the table was reset in the horizontal position (positive test), or after 45 minutes of tilting (negative test).

Definitions

Presyncope was defined as the induction of symptoms of imminent syncope, and syncope was defined as complete and transient loss of consciousness. A positive test was defined by the development of syncope or presyncope in association with relative bradycardia (≥20% decrease in heart rate compared with baseline) or hypotension (systolic blood pressure <80 mm Hg).

Adenosine Plasma Levels

Samples were collected and treated as described previously.[6,7] Venous blood (3 mL) was withdrawn under vacuum together with a stop solution in Vacutainer tubes (Becton Dickinson). This method allowed blood samples to be mixed rapidly with 4 mL of stop solution, which prevents adenosine degradation and uptake. The stop solution was composed of dipyridamole 0.2 mmol/L, 4.2 mmol/L ethylene-diamine-tetraacetic acid disodium (Na_2 EDTA), erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) 5 mmol/L, α,β-methyleneadenosine-5′-diphosphate (AMPCP) 79 mmol/L, heparin sulfate 1 IU/mL, deoxycoformycin 1 μg/mL, and 0.9% NaCl. The sample with the stop solution was centrifuged at 2500g for 10 minutes, and the supernatant was deproteinized by addition of 2 mL of 70% perchloric acid before a second centrifugation. The supernatant was lyophilized and redissolved in 1 mL of 50 mmol/L sodium phosphate buffer (pH 4).

Samples were analyzed by high-performance liquid chromatography (Waters) as described previously.[6,7] Briefly, a modular system with a UV detector was used. Lyophilized samples were dissolved in 1 mL of phosphate buffer (pH 4) and eluted with a methanol gradient on a Merck LIChrospher C18 column. Adenosine was identified by its elution time and after incubation with adenosine deaminase, which increases the inosine peak and decreases the adenosine peak. Adenosine was quantified by comparison of these peak areas with those given by predefined quantities of adenosine. In these conditions, the sensitivity threshold was 10 pmol/L injected in 1 mL of plasma matrix. The intra-assay and interassay coefficients of variation for nucleosides ranged from 1% to 3%. The absence of xanthine derivatives was checked on each chromatogram. Sample treatment and adenosine assay were performed by a technician who was blinded to the study.

Reagents and Other Agents

Adenosine (crystallized, 99% pure), adenosine deaminase, and dipyridamole were obtained from Boehringer Mannheim; inosine (99% pure), AMPCP, EDTA, and deoxycoformycin from Sigma; and EHNA from Burroughs Welcome.

Statistical Methods

Age, weight, height, left ventricular ejection fraction, heart rate, blood pressure, adenosine levels, and time to tilt symptoms are

TABLE 2. Patients With Negative Test: Individual Parameters and Mean Values

Patients	Sex, M/F	Age, y	Heart Rate, bpm			Arterial Pressure, mm Hg			Adenosine, μmol/L		
			Baseline	Tilt	45th min	Baseline	Tilt	45th min	Baseline	Tilt	45th min
1	M	60	58	68	82	120/60	140/70	130/80	0.40	0.30	0.50
2	F	60	84	88	92	120/70	130/70	120/80	0.40	0.45	0.50
3	M	64	68	74	62	140/70	150/80	150/80	0.35	0.14	0.50
4	F	64	62	64	65	125/65	120/65	115/70	0.60	0.35	0.65
5	M	37	64	70	84	115/70	110/70	110/70	0.45	0.10	0.30
6	M	70	67	76	80	140/75	130/70	110/75	0.55	0.20	0.50
7	M	66	63	73	64	130/70	130/60	130/60	0.35	0.15	0.70
8	F	73	78	86	85	150/80	145/80	120/80	0.20	0.30	0.45
9	M	62	87	97	98	155/80	190/95	175/95	0.45	0.25	0.25
10	M	36	56	67	60	120/70	130/80	130/80	0.30	0.15	0.35
11	F	70	80	89	96	145/85	160/80	165/90	0.30	0.07	0.40
12	M	45	60	66	70	140/75	130/70	140/70	0.35	0.20	0.40
13	F	51	80	84	76	140/80	140/90	130/85	0.25	0.20	0.40
14	M	62	65	88	100	140/80	130/80	145/90	0.50	0.25	0.40
15	F	33	62	96	98	110/65	105/75	110/80	0.45	0.15	0.25
Mean			68.9	79.1	80.8	137/73	136/76	133/79	0.39	0.22	0.44
SEM			2.6	2.9	3.7	3.5/2.0	5.3/2.5	5.0/2.4	0.03	0.03	0.03

Tilt indicates immediately after tilting; 45th min, 45th minute of tilting, ie, the end of the test.

reported as mean±SEM. Number of syncope and presyncope episodes and time since first episode are expressed as median and interquartile range, with comparisons made by Mann-Whitney *U* test.

Intragroup and intergroup variations were compared by 1- and 2-way ANOVA followed by a paired bilateral *t* test. We used the Wilcoxon test for intragroup comparison with each patient as his or her own control. A probability value <0.05 was considered significant.

Results

Demographics and Symptoms

Twenty-six patients referred for evaluation of syncope fulfilled the inclusion criteria. The mean age was 52±3.3 years (range, 15 to 73 years), as shown in Table 1. In all patients, syncope was unexplained on the basis of the clinical workup and was suspected to be vasovagal in nature, and a head-up tilt test was indicated. No significant difference was observed between the group of patients with a positive tilt test and the group with a negative tilt test with respect to sex, age, weight, height, baseline heart rate, and blood pressure. All had a normal left ventricular ejection fraction (68±6%). Regarding the history of syncopal episodes, the time since first episode was comparable between the 2 groups, but the frequency of syncope and presyncope events per month was significantly higher in the patients whose test was positive (2.4±0.6 versus 0.9±0.2 in those whose test was negative; $P<0.05$; Table 1).

Head-Up Tilt Test

The head-up tilt test was positive in 11 patients and negative in 15. In patients with a negative test, no significant modification of blood pressure was noted during the test. The heart rate increased significantly ($P<0.005$) immediately after tilting and 45 minutes later (15% and 17%, respectively; Table 2). Two patients with a negative tilt test underwent a diagnostic electrophysiological study that was noncontributory. In the 11 patients with a positive test, syncope occurred in 9 and presyncope in 2 (Table 3). Heart rate increased significantly immediately after tilting (18%; $P<0.0001$). Symptoms occurred after 22.4±3 minutes (range, 2 to 38 minutes). On syncope, systolic blood pressure dropped by an average of 59% ($P<0.0001$) and heart rate by 38% ($P<0.0001$). All patients recovered rapidly from their symptoms as soon as the table was reset to the horizontal position.

Adenosine Plasma Levels

In patients with a negative tilt test, adenosine plasma levels were 0.39±0.03 μmol/L at baseline, 0.22±0.03 μmol/L immediately after tilting, and 0.44±0.03 μmol/L after 45 minutes (Table 2). In contrast, patients with a positive test exhibited significantly higher ($P<0.0001$) adenosine plasma levels (2.66±0.67 μmol/L) in the supine position (ie, 7 times greater than in the negative group), 3.22±0.85 μmol/L immediately after tilting (ie, ≈14 times greater than in the negative group), and 4.03±0.66 μmol/L during syncope, which was ≈52% higher on average ($P<0.005$) than baseline values (Figure 1). There was no overlap between the 2 groups. None of the 15 patients with a negative tilt test had a baseline adenosine plasma level >0.6 μmol/L, and none of the 11 patients with a positive test had a level <0.9 μmol/L (Tables 2 and 3). Linear relationships have been established at the moment of syncope between adenosine plasma levels and (1) time to tilt-induced symptoms and (2) heart rate; the higher the adenosine plasma level, the shorter the time to tilt symptoms and the lower the heart rate (Figure 2).

Discussion

The main findings of this study were 2-fold. (1) Patients with a positive tilt test had much higher baseline adenosine plasma

TABLE 3. Patients With Positive Test: Individual Parameters and Mean Values

Patients	Sex, M/F	Age, y	Symptoms	Time to Symptoms, min	Heart Rate, bpm			Arterial Pressure, mm Hg			Adenosine, μmol/L		
					Baseline	Tilt	Syncope	Baseline	Tilt	Syncope	Baseline	Tilt	Syncope
1	M	70	Syncope	20	78	98	52	120/70	120/80	50	3	6.5	5
2	M	65	Syncope	2	70	80	38	130/80	140/80	<50	9	9.19	8.74
3	F	16	Syncope	15	82	91	41	110/60	115/80	<50	3	2	5
4	F	33	Syncope	19	75	80	40	120/60	120/60	<50	2.45	5.5	5.25
5	M	68	Syncope	20	96	100	49	140/70	130/70	<50	1.85	0.94	5.85
6	F	45	Syncope	38	74	80	58	120/80	110/80	60	0.9	0.5	1.2
7	M	52	Presyncope	25	60	75	48	115/60	105/70	60	0.9	1.3	2
8	F	66	Presyncope	34	80	100	55	120/80	125/70	60	2	1	2.2
9	F	42	Syncope	31	55	55	45	140/65	135/70	50	1.5	1.25	2.5
10	M	58	Syncope	22	66	74	53	110/60	105/60	65	2.9	4.2	3.14
11	M	15	Syncope	20	55	85	40	120/70	100/70	<50	1.75	3	3.5
Mean				22.4	71.9	85.3	44.9	122/69	119/72	49.5	2.66	3.22	4.03
SEM				3.0	2.9	3.1	2.3	3.1/2.5	4/2.6	3.05	0.67	0.85	0.66

Tilt indicates immediately after tilting.

levels than patients with a negative tilt test, with no overlap between the 2 groups. Patients with a negative head-up tilt test had baseline adenosine plasma levels that were in the same range as those reported in the venous blood of normal healthy volunteers.[6,8] (2) During syncope, adenosine levels increased by an average of 52% compared with baseline levels. The higher the adenosine plasma level, the earlier the symptoms appeared and the greater the slowing of heart rate.

Several investigators have hypothesized that adenosine is an important modulator that can trigger vasovagal syncope in susceptible patients.[3–5,9] Indeed, the injection of exogenous adenosine or ATP during head-up tilt testing provoked a vasovagal response in those patients with syncope, with a yield comparable to that of isoproterenol.[3–5,10] Adenosine-sensitive syncope has been identified as a cause of syncope in some patients with an abnormal response to an ATP test.[9] More recently, Brignole et al[10] suggested that ATP and head-up tilt tests may identify different patient populations with syncope. They also noted that the 2 syndromes do overlap. The clinical features of their patients with both a positive head-up tilt test and a positive ATP test differed from those of patients with tilt-induced syncope alone or with adenosine-sensitive syncope alone. They concluded that numerous mechanisms may be responsible for neurally mediated syncope and that syncopal attacks may be caused by a vasovagal mechanism and/or by an adenosine-mediated mechanism.[10] One may argue that the high adenosine levels observed in patients with a positive tilt test could be explained by the circulatory disorders associated with vasovagal symptoms (severe bradycardia, hypotension, and low blood flow). This is unlikely, because high adenosine levels were present at baseline, before tilting, in patients with a positive test. Our results support the hypothesis that endogenous adenosine is involved in the mechanisms that trigger a vasovagal response during tilt testing in susceptible patients, as suggested by Shen et al.[3] Although several mechanisms may be implicated in the pathogenesis of neurally mediated syncope in these patients, the end result could be an increase in adenosine plasma levels.

Endogenous Adenosine and Syncope

Adenosine acts on blood vessel tone and on the sinoatrial node via activation of 4 subtypes of P1 purinergic receptors: A1, A2A, A2B, and A3.[11] The activation of A1 receptors mediates cardiac depression through negative chronotropic, dromotropic, and inotropic effects[12] and diminishes blood vessel tone via the prejunctional inhibition of neurotransmitter release on the perivascular sympathetic[13] and capsaicin-

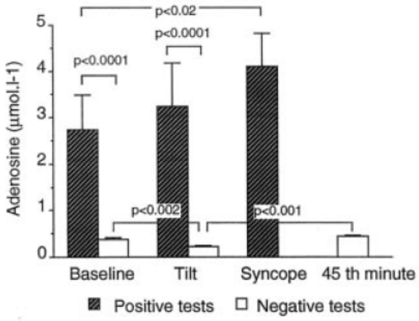

Figure 1. Adenosine plasma levels: intergroup and intragroup comparisons. Tilt indicates immediately after tilting; 45th min, 45th minute of tilting, ie, end of test in nonresponders.

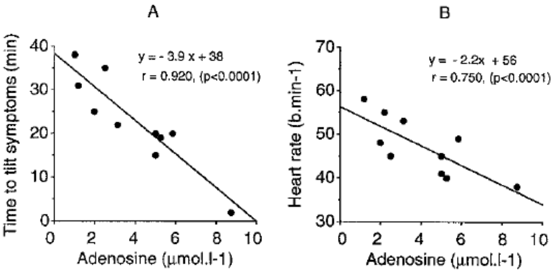

Figure 2. Relationship at moment of syncope between adenosine plasma levels and time to tilt-induced symptoms (A) and heart rate (B).

sensitive sensory afferents.[14] A2 receptors are subdivided into A2A (high affinity) and A2B (low affinity) subtypes.[15] The activation of these 2 subtypes mediates artery relaxation.[16,17] The affinity of these receptors for adenosine depends on the receptor subtype in the following rank order: A1>A2A>A2B. Therefore, the tissue repartition of adenosine receptor subtypes, which is submitted to interindividual variations, and local adenosine concentrations have crucial biological effects. At high concentrations (≈ 1 μmol/L and more), all receptors are activated, inducing synergistic effects on cardiac depression and vasodilatation.[8] Thus, the high adenosine plasma levels observed in patients who developed symptoms during the head-up tilt test may have intervened in the triggering of a vasovagal syncope in these susceptible patients. The fact that the time to symptoms was shorter and the heart rate lower in patients with high adenosine plasma levels also supports this hypothesis. These findings may also explain why adenosine receptor antagonists are effective in the treatment of patients with vasovagal syncope.[18]

Desensitization and Downregulation

In the present study, syncope burden was higher in patients with a positive head-up tilt test and with high adenosine levels. However, these patients had no repeated syncopal episodes. This observation suggests receptor desensitization in patients with high adenosine plasma levels. For example, the number of A1 receptors in the AV node decreased by nearly 50% during chronic administration of agonists.[19,20] These 2 mechanisms (desensitization and downregulation) may differ from one patient to another because of their individual sensitivity. Furthermore, expression and function of purinergic receptors can differ from one receptor subtype to the other.[11]

On the other hand, nonspecific adenosine antagonists, such as xanthine derivatives, upregulate adenosine receptors[21] and increase adenosine plasma levels.[22] Consequently, subjects with heavy consumption of methylxanthines may have a potential compensatory increase in adenosine levels. Attention was paid to not include in the present study any patients with heavy caffeine consumption. Caffeine withdrawal, with resultant changes, may increase susceptibility to syncope development and a positive response to tilt testing or an increase in adenosine plasma levels. This is unlikely to have occurred in the present study, because caffeine consumption was low, and no xanthine derivatives were detected on the chromatograms during adenosine assay. The observation of high adenosine levels in patients with a positive head-up tilt test suggests that none of them was in a recent caffeine withdrawal period.[22]

During syncope, adenosine plasma concentration showed an additional increase in 10 of 11 patients. This observation may be explained by the recruitment of a greater number of receptors ("spare receptors"), as has been reported in the coronary circulation.[23] Such increments in adenosine plasma levels, even small ones (as in patient 8, who developed a delayed presyncope), may be sufficient to induce symptoms in patients with high receptor sensitivity. In a single patient (patient 2), adenosine plasma levels did not increase at the time of syncope. This patient had very high adenosine plasma levels and developed syncope quickly, within 2 minutes after tilting. The rapid circulating hypovolemia that results from venous pooling could have been the combined factor that precipitated syncope in this patient.

Adenosine and Baroreflex Function

Defective baroreflex function has been advocated as a potential mechanism to account for the development of neurally mediated syncope.[1] It was also reported that adenosine and its antagonists could modulate baroreflex activation[24] and that acute administration of caffeine could inhibit baroreflex sensitivity in humans.[25] Thus, it is possible that high adenosine plasma levels could contribute to the baroreflex dysfunction that occurs in some patients with neurally mediated syncope.

Source of Adenosine Release

One may speculate on the possible mechanism responsible for high adenosine plasma levels in patients with a positive tilt test. Adenosine is released by endothelial cells and vascular myocytes, particularly during ischemia.[8] Nevertheless, ATP and adenosine are also released by sympathetic fibers[26,27] and by poorly myelinated or nonmyelinated fibers, including C fibers.[28] The activation of myocardial mechanoreceptors, which are C fibers, is thought to be one of the mechanisms underlying vasodilatation and hypotension-induced syncope.[1,28,29] Thus, we hypothesize that the activation of these fibers and increased sympathetic tone may be involved in adenosine release into the extracellular space. Even if there is no objective evidence for an increased sympathetic activation in patients with higher adenosine baseline levels (eg, similar heart rate and blood pressure), it can be hypothesized that sympathetic activation could be counterbalanced and masked by the effects of high adenosine concentration, which decreased heart rate and blood pressure. This hypothesis is supported by the observation that the lower the heart rate, the higher the adenosine plasma levels.

Our results suggest that adenosine plasma levels may allow pretest identification of patients who will have a positive head-up tilt test. However, additional studies in patients with neurally mediated syncope are needed to extend these initial observations.

Study Limitations

Although the present study included enough patients to reach statistical significance and to draw meaningful conclusions, additional studies including a larger group of patients are desirable. It is well known that neurally mediated syncope is not a uniform syndrome, and neurohumoral mechanisms are not the only mechanisms at play. In the present series, we mainly observed patients with a mixed response, ie, with both vasodepressive and cardioinhibitory properties. This obviously does not represent all varieties of patients with neurally mediated syncope.

The suggestion that adenosine may be a mediator of vasovagal syncope finds support in studies that showed that exogenous adenosine infusion was able to induce syncope in susceptible patients and in those demonstrating that adenosine antagonists are able to prevent tilt-induced syncope.[3–5,10]

Furthermore, the present study suggests that these patients can be identified, and if further studies could show that syncope is reproduced by exogenous adenosine infusion in these patients, this will favor a causal mechanism of elevated adenosine plasma levels.

Conclusions

The present study evaluated adenosine plasma levels at baseline and during tilt testing in patients with unexplained syncope and showed that baseline levels of adenosine plasma were higher in patients with a positive tilt test than in patients with a negative tilt test, with no overlap between the 2 groups. A further increase in adenosine plasma levels was noted in most patients during tilt-induced syncope. The higher the adenosine plasma level, the earlier is the occurrence of syncope and the greater the decrease in heart rate.

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Conclusion

Nous avons montré que les concentrations en adénosine endogène étaient plus élevés chez les patients dont le test d’inclinaison allait être positif. Par ailleurs, plus les taux de base étaient élevés, plus la syncope survenait rapidement dans le test, et plus la fréquence cardiaque était basse au moment de la syncope. Cette étude est la première à démontrer l’implication de l’adénosine endogène dans la physiopathologie des syncopes neuro-cardiogéniques.

Toutefois, de nombreuses questions restent en suspens : comment sont exprimés les différents récepteurs de l’adénosine ? Quelle est leur affinité pour les agonistes ? Ou bien encore quelle est la fonction de ces récepteurs ?

2. Adénosine et expression des récepteurs A2A dans les différentes formes de syncopes neuro-cardiogéniques

Introduction

Un autre test peut-être utilisé dans le diagnostic des syncopes neuro-cardiogéniques : le test à l'ATP. On devrait d'ailleurs parler plutôt de test à l'adénosine car l'ATP ayant une durée de vie très brève dans l'organisme, celle ci n'pas le temps d'atteindre sa cible, et c'est l'adénosine qui agira sur la conduction auriculo-ventriculaire. Ce test consiste en une injection intraveineuse de 20 mg d'ATP (striadyne®) ou de 10mg d'adénosine. Le test sera positif si l'on enregistre une pause cardiaque de plus de 6 secondes. Il semble que ce test permette de mettre en évidence un type particulier et plus rare de syncope neuro-cardiogénique : les syncopes adénosine-sensible. Ces syncopes se caractérisent par la survenue brutale d'un bloc auriculo-ventriculaire complet responsable d'une pause cardiaque. Le mécanisme n'est pas une anomalie organique des voies de conduction, mais il est au contraire extrinsèque, et donc neuro-cardiogénique. Si l'on sait que les patients présentant des syncopes neuro-cardiogéniques et un test d'inclinaison positif ont des taux d'adénosine plasmatiques élevés, sur-expriment les récepteurs A_{2A} R et présentent un polymorphisme particulier pour le gène du récepteur A_{2A}R, aucune donnée n'était publiée concernant les patients avec un test à l'ATP positif. Le but de cette étude était de comparer les concentrations en adénosine plasmatique et l'expression des récepteurs A_{2A} chez les patients présentant des syncopes neuro-cardiogéniques, en fonction des résultats obtenus lors du test d'inclinaison et du test à l'ATP.

Ce travail est détaillé dans l'article : **"Adenosine plasma level and A2A adenosine receptor expression: correlation with laboratory tests in patients with neurally mediated syncope"** ci dessous.

Publication 2

“Adenosine plasma level and A2A adenosine receptor expression: correlation with laboratory tests in patients with neurally mediated syncope”

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ORIGINAL ARTICLE

Adenosine plasma level and A_{2A} adenosine receptor expression: correlation with laboratory tests in patients with neurally mediated syncope

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ABSTRACT

Objectives The purpose of this study was to investigate the hypothesis that responses to the ATP test and head-up tilt test (HUT) may be correlated with different purinergic profiles.

Design and setting The ATP and HUT identify distinct subsets of patients with neurally mediated syncope (NMS). Adenosine and its A_{2A} receptors (A_{2A}R) may be implicated in the pathophysiology of NMS in patients with positive HUT. Nothing is known about the purinergic profile of patients with positive ATP.

Patients and measures This prospective study includes a consecutive series of patients with suspected NMS. All patients underwent both HUT and ATP. Before testing, samples were collected for measurement of baseline adenosine plasma level (APL) and expression.

Results A total of 46 patients (25 men and 21 women) with a mean age of 57±18 years were enrolled. The HUT test was positive in 27 patients and the ATP test in 20. Both tests were positive in 9 and negative in 8. High APL was associated with high probability of positive HUT while low APL was associated with high probability of positive ATP. Expression of A_{2A}R was lower in patients with positive ATP than in those with positive HUT.

Conclusion These findings indicate that patients with NMS present different purinergic profiles and that responses to HUT and ATP are correlated with these profiles.

INTRODUCTION

The head-up tilt test (HUT) and ATP test are used for diagnosis of suspected neurally-mediated syncope (NMS).[1–7] However, the intra-patient correlation between the two tests is weak, suggesting that they may identify distinct patient subsets. It is well established that endogenous adenosine acts on the cardiovascular system through its protein G-coupled receptors, namely A_1R, A_{2A}R, A_{2B}R and A_3R.[8] Adenosine and its A_{2A} receptors (A_{2A}R) may be implicated in the pathophysiology of NMS in patients with positive HUT. In this regard, previous patient-control studies have shown that positive HUT results are correlated with high adenosine plasma levels (APL),[9] high A_{2A}R expression[10] and presence of a single nucleotide polymorphism in the A_{2A}R gene.[11] However, no previous study has compared APL levels and A_{2A}R expression in the function of ATP and HUT results in the same patients with NMS. The purpose of this study was to investigate the hypothesis that responses to HUT and ATP are correlated with different baseline purinergic profiles.

METHODS

Patient selection

A total of 46 consecutive patients with suspected NMS were enrolled. Suspicion of NMS was based on the occurrence of syncope consistent with NMS and absence of (1) structural heart disease, ECG abnormalities, or rhythm disturbances which may explain the syncope; (2) orthostatic hypotension; and (3) non-syncopal causes of transient loss of consciousness. Patient selection criteria also included occurrence of one or more episodes of syncope within the preceding year, absence of drug use for 3 months, and first-time HUT and ATP. Patients with carotid sinus syndrome were not included.

To compare APL in patients with NMS and the general population, a matched control group of 14 healthy volunteers was recruited consecutively from the medical and technical staff working at our institution during the study period. None of the controls had a history of syncope.

This study was conducted in compliance with the principles of the Declaration of Helsinki and approved by the Ethics Committee for Human Research of our university-affiliated hospital. All patients and controls provided written informed consent to participate.

ATP and HUT

The ATP and HUT tests were performed in that order during the same session. A sufficient interval for complete recovery was allowed between the two tests. Test protocols were performed in accordance with current recommendations.[2] The ATP test was performed by rapid injection (<2 s) of 20 mg of ATP (Striadyne®, Genopharm, Saint Thibault des Vignes, France) via a brachial vein followed by a 20 ml 5% glucose flush. Positive outcome was defined as complete AV block or sinus pause with ventricular asystole >6 s.[12]

The HUT test was performed after overnight fasting in a quiet room equipped for cardiopulmonary resuscitation. To facilitate blood sampling, an

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intravenous catheter was inserted in one arm at least 30 min prior to testing. Patients were strapped to the tilt table. After 10 min in the supine position, the table was tilted to the 60° position for a maximum of 45 min. Surface ECG and finger blood pressure were monitored beat by beat (Task Force Monitor, CNSystems, Graz, Austria). Positive outcome was defined as development of syncope or presysncope in association with significant arterial hypotension. To avoid a false positive response, no pharmacological provocation was used.

Collection of blood samples

Baseline blood samples were collected and processed as described previously[9–11 13] using laboratory-prepared tubes containing 3 ml of cold-stop solution under vacuum. This method allows whole blood to mix quickly with the cold-stop solution so as to prevent degradation or red blood cell uptake of adenosine.[13] Samples were collected a few minutes before ATP administration. Patients and controls were placed in the supine position during sample collection. After collection, samples were immediately placed and maintained on ice until centrifugation.

Adenosine plasma level

APL was determined as previously described.[13] After deproteinisation, adenosine was quantified using high pressure liquid chromatography (ChromSystem, Munchen, Germany). The technicians who performed sample treatment and assays were unaware of the result of HUT and ATP.

Western blot analysis of $A_{2A}R$ expression in peripheral blood mononuclear cells

Western blot analysis of $A_{2A}R$ expression has been previously described.[14] Briefly, peripheral blood mononuclear cells (PBMC) were isolated using Vacutainer tubes (Ficoll-based CPT system; Becton Dickinson, Franklin Lakes, New Jersey, USA) and frozen at −70°C. At the time of analysis, samples were quickly thawed at 37°C and solubilised in a 4% sodium dodecyl sulphate (SDS) aqueous solution by 30 min sonication at 47 kHz. After protein quantification by micro BCA® (Pierce Biotechnology, Rockford, Illinois, USA), 15 mg of PBMC solubilisates in 62.5 mM Tris-HCl buffer, pH 8.3, containing 2% SDS, 10% glycerol, 0.01% bromophenol blue, and 5% mercaptoethanol were submitted to standard electrophoresis in Mini Protean II system (BioRad, Hercules, California, USA). Separated proteins in 12% acrylamide minigel were electrotransferred onto a PVDF membrane that was then placed in the blot holder of the SNAP i.d. protein detection system (Millipore, Billerica, Massachusetts, USA), saturated with non-fat dried milk, and incubated for 20 min with primary antibody, that is, a laboratory-produced anti-$A_{2A}R$ human monoclonal antibody.[14] Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mouse monoclonal antibody (Clone GAPDH-71.1, Sigma) was used as a loading control to normalise blot results. Blots were visualised by horseradish peroxidase-labelled anti-mouse IgG Fab specific antibodies and enhanced chemiluminescence substrate (SuperSignal West Femto, Pierce Biotechnology) using a Kodak system. Bands were measured densitometrically on a Macintosh computer using the public-domain NIH Image software developed at the US National Institutes of Health.

Statistical analysis

Quantitative variables were expressed using median and range values. Correlation between APL and quantitative variables was assessed using the Spearman test. Quantitative variables were compared using the non-parametric Mann−Whitney U test or Kruskal−Wallis test, depending on the number of groups. The non-parametric Wilcoxon test was used to compare APL between the patient groups defined according to outcome of ATP and HUT. An adjusted P-value was used to control the false positive rate.[15] Logistic regression was used to estimate the probability of positive laboratory test (ATP or HUT) according to APL values. All statistical tests were two-sided and p values <0.05 were considered as statistically significant. Analyses were performed with R free software (V.2.8.1).

RESULTS

The clinical characteristics of the 46 patients and 14 controls enrolled in this study are presented in table 1. Women accounted for 46% of the patient population and 60% of positive ATP tests (p=0.09). Positive results were obtained in 27 patients (59%) for HUT and in 20 patients (43%) for ATP. Both tests were positive in nine patients (20%) and negative in eight patients (17%). Using the modified VASIS classification,[16] positive HUT responses were classified type 1 (mixed response, with mild bradycardia) in 10 patients, type 2A (cardio-inhibition with heart rate below 40 bpm, but without asystole) in 5, type 2B (cardio-inhibition with asystole of more than 3 s.) in 4 and type 3 (pure vasodepression) in 8. Positive responses to ATP involved AV block in 18 patients and sinus pause in 2. The mean duration of the maximum RR interval during positive ATP was 12.0±5.7 s.

Adenosine plasma level

Higher APL was observed in patients with positive than negative HUT. Conversely, APL was lower in patients with positive than negative ATP (figure 1A). APL was lowest in patients with positive ATP and negative HUT and highest in patients with positive HUT and negative ATP (figure 1B). Patients in whom both tests were positive or negative had intermediate APL

Table 1 Clinical characteristics of patients: overall and subgroups in function of tests results

	Overall n=46	ATP+ n=20	ATP− n=26	HUT+ n=27	HUT− n=19	Controls n=14
Age (years, mean±SD)	57±18	62±19	53±18	56±17	58±22	54±14
Sex (male/female)	25/21	8/12	17/9	15/12	10/9	8/7
Heart disease, n (%)	1 (2)	0 (0)	1 (2)	0 (0)	1 (2)	–
ECG abnormalities, n (%)	10 (21.7)	5 (10.8)	5 (10.8)	6 (13)	4 (8)	–
Total number of syncopal episodes (mean±SD)	7.4±11.8	6.7±11.0	8±12.7	9.4±14.6	4.6±5.6	–
Symptom duration (months, mean±SD)	59±117	36±88.1	77±134	81±147	27±34	–
Triggering factors, n (%)	19 (41.3)	8 (17.3)	11 (23.9)	12 (26)	7 (15.2)	–

Structural heart disease was stable angina pectoris.

ECG abnormalities included isolated complete right bundle branch block in three patients, left anterior hemiblock in two and atrial fibrillation in five.

n, number of patients.

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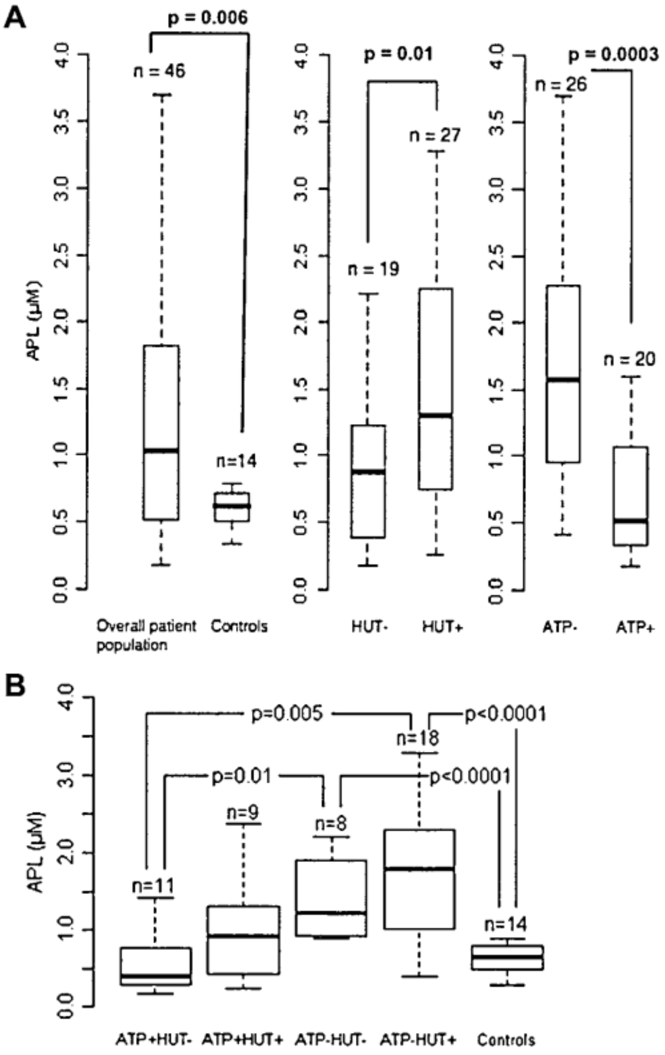


Figure 1 Adenosine plasma levels (APL) in patients and controls. Box plot showing baseline adenosine plasma level (APL) in 46 patients with unexplained syncope and 14 controls. Box plot shows median, quartile, and extreme values. (A) Patients were separated into subgroups, depending on their responses to the ATP test or head-up tilt test, considered independently. (B) Patients were separated into subgroups, depending on their response to both the ATP test and the head-up tilt test.

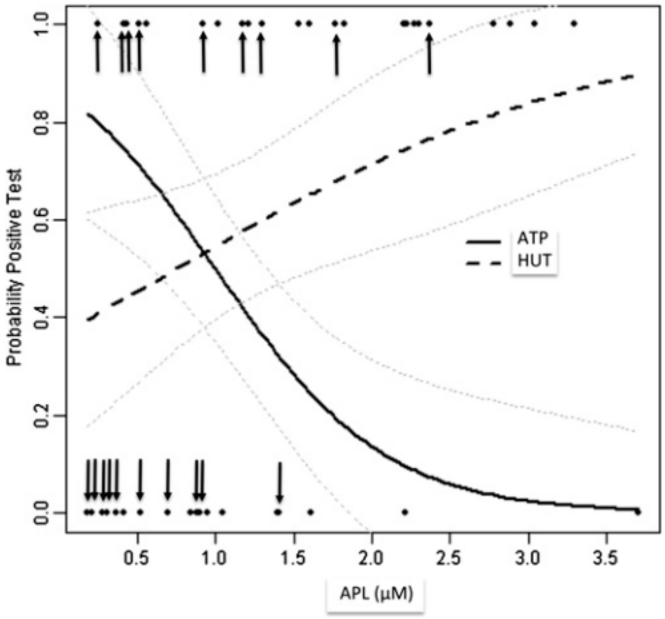


Figure 2 Probability of positive ATP or head-up tilt (HUT) test (with 95% CIs; dotted curves) in function of adenosine plasma level (APL in µM) in 46 patients. Top dots: positive HUT test; bottom dots: negative HUT test. Arrows: positive ATP test.

values. Compared to controls, APL was higher in patients with negative ATP regardless of HUT results (figure 1B). Low APL was associated with higher probability of positive ATP and high APL was associated with higher probability of positive HUT (figure 2).

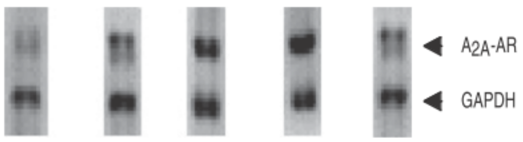
A_{2A}R expression

A_{2A}R expression was lowest in patients with positive ATP and negative HUT and highest in patients with negative ATP and positive HUT (figure 3).

DISCUSSION

The main finding of this study is that positive responses to HUT and ATP in patients with suspected NMS are correlated with high and low APL values respectively. High APL is associated with higher probability of positive HUT while low APL is associated with higher probability of positive ATP. These findings suggest that susceptibility to HUT and ATP in patients with NMS may be correlated with different purinergic profiles.

It is well established that the stimulation of A_1R is associated with bradycardia,[8] while the stimulation of A_{2A}R results mainly in vasodilation.[8 17] Our results suggest the presence of two distinct groups of patients: one with low APL and low A_{2A}R expression and with positive ATP-test, and another with high APL, high A_{2A}R expression and positive HUT. Our hypothesis is that patients in the first group may be sensitive to the small increase in APL that occurs during ATP injection because of the activation of high affinity adenosine expression (mostly A_1R), leading to bradycardia. The patients from the second group are insensitive to ATP administration because most of their high affinity A1R may be desensitised by the chronic exposure to high APL. In addition, the small increase in APL that occurs after ATP administration is probably insufficient to activate low affinity adenosine receptors (mostly A_{2A}R); but these receptors are still activated during HUT because of the strong increase in APL that occurs during this test.[9]



A_{2A}/GAPDH	ATP+/HUT−	ATP+HUT+	ATP-/HUT-	ATP-/HUT+	Controls
Mean±SD	0.71±0.17*	1.19±0.24	1.3±0.3	1.83±0.47**	0.88±0.12
Median	0.73	1.2	1.2	1.8	0.85
range	0.45-1.03	0.88-1.5	0.8-1.7	1.2-2.5	0.75-1.1

Figure 3 A_{2A}R expression evaluated using Western blot and laboratory-produced A_{2A}R monoclonal antibody.[14] Top: typical Western blot obtained in each patient group. Bottom: mean±SD, median and range of A_{2A}R expression in each patient group and in control group. *$p \leq 0.01$ compared with other patient groups; **$p < 0.05$ compared with other patient groups and controls. NB: Quantification of A_{2A}R was performed using the A_{2A}R/GAPDH ratio.

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However, because A_1R expression has not been evaluated (they are not expressed on peripheral blood cells), this last point is only speculative. Conversely, we observed that patients with high APL and $A_{2A}R$ expression are insensitive to the ATP test. Our explanation for this finding is that chronic exposure to high APL level desensitises A_1R. In this group, that is, insensitive to the ATP test, recruitment of low affinity adenosine receptors (mostly $A_{2A}R$) may result from the strong increase in APL occurring during HUT[9] and lead to vasodilation.

Central or peripheral baroreceptor reflex abnormalities or alterations in neurohumoral mechanisms could play a pivotal role in the genesis of NMS.[18] Despite differences in their receptors, adenosine and the neurotransmitter acetylcholine have remarkably similar effects on cardiac function.[19,20] A possible explanation is the similarity of their receptor-effector coupling systems. In addition to having direct effects, acetylcholine and adenosine act synergistically against the stimulatory action of the sympathetic neurotransmitters norepinephrine and epinephrine on adenyl cyclase. Thus, excitatory and inhibitory effects of the adrenergic, cholinergic and purinergic outflows are integrated at the level of the receptor-effector coupling system, resulting in the final cardiac effect.

It is likely that NMS syndrome is a shared clinical manifestation of several different conditions that have not yet been characterised. Thus, HUT and ATP may identify different subsets of patients with 'so-called' NMS. This hypothesis is consistent with two clinical findings in patients with syncope. First, though some overlap exists, HUT tends to be positive in younger patients of both genders and ATP in older women.[6,7,16,21] Second, positive HUT and ATP are rarely observed in the same patient.[6,7]

Syncope due to extrinsic (or functional) mechanisms in patients with a normal heart probably involves a wide spectrum of pathogenic pathways in addition to adenosine. A well-known aspect of the spectrum involves the neural mechanisms underlying typical vasovagal and tilt-induced syncope. Another aspect recently described by our group is 'idiopathic paroxysmal AV block'. In this regard, we recently described cases characterised by low APL and positive ATP test suggesting that enhanced susceptibility of the AV node to adenosine was the cause of the block.[22] The latter study involved a selected population of patients with a long history of syncope associated with documented paroxysmal AV block characterised by sudden onset without change in PP interval. Long-term follow-up showed no sign of progression to permanent AV block. Median APL value in these patients was also fourfold lower than that in patients with syncope due to classical reflex sinus arrest.

Study limitations

This study has three main limitations. The first involves population size. While the population was large enough to reach statistical significance for APL and $A_{2A}R$ expression differences, further study in a larger group of patients will be needed especially for detailed analysis of the clinical characteristics of NMS patients with normal or low APL. The second limitation is that APL was evaluated only at baseline so that its role as a marker of susceptibility for laboratory test response can only be assumed. Though implication of the adenosine pathway in responses to HUT and ATP is plausible, our data are insufficient to prove a causal relationship. Further study will be needed not only to demonstrate this point but also to evaluate the role of different adenosine receptor subtypes in NMS. Controls were selected based on syncope-free history. However, we cannot rule out the possibility that some of them might have had positive HUT and/or ATP tests. However, the probability of positive tests in healthy controls is very low: 0–9% for HUT[23,24] and <5% for ATP.[12]

CONCLUSION

The findings of this study indicate that positive responses to HUT and ATP in patients with NMS correspond to different purinergic profiles. While the direct clinical value of these findings is unclear, they provide a sound basis for future prospective clinical follow-up studies to correlate the purinergic profile with outcome of NMS.

Contributors JCD, FF, MB, JR, RG: design of the study and writing the paper. AM, EP, and SP: patient selection, criteria of inclusion. YB and JC: biological analysis. RG: statistical analysis.

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Competing interests None declared.

Patient consent Obtained.

Ethics approval Ethics approval was obtained from the Ethics Committee for Human Research of Timone University Hospital.

Provenance and peer review Not commissioned; internally peer reviewed.

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Conclusion

Notre étude semble confirmer que le test à l'ATP et le test d'inclinaison individualisent 2 populations différentes de patients. Le test à l'ATP sera plus souvent positif chez les patientes âgées. Par ailleurs, on constate chez ces patients un taux d'adénosine plasmatique bas et une faible expression des récepteurs A_{2A} comparativement aux patients dont le test d'inclinaison est positif, chez lesquels nous confirmons que les taux d'adénosine ainsi que l'expression des récepteurs A_{2A} sont élevés. Un résultat intéressant est qu'une forte concentration en adénosine en basal est un indice prédictif de positivité du tilt test (avec un cut-off aux environs de 0,9µM), tandis qu'une concentration basse en adénosine laisse présager d'un ATP test positif.

Ces résultats ouvrent la voie à de nouvelles études sur les profils purinergiques des patients lors de syncopes neuro-cardiogéniques.

3. Fonction des récepteurs A_{2A} chez les patients présentant des syncopes neuro-cardiogéniques

Introduction

Les différents travaux de notre équipe ont permis de montrer que les patients présentant des syncopes vagales, ont des concentrations en adénosine plasmatique élevés, sur-expriment les récepteurs A_{2A}, et présentent un polymorphisme particulier du gène du récepteur A_{2A}. Toutefois, un élément important n'avait jamais été étudié jusqu'ici: la fonction des récepteurs A_{2A}. En effet, on peut émettre l'hypothèse que la concentration en adénosine étant élevée et les récepteurs surexprimés, ces derniers soient désensibilisés, et que son activation ne conduise plus à la production d'AMPc (par découplage de la protéine G par exemple). Dans ce cas, l'implication de l'adénosine dans la physiopathologie des syncopes vagales aurait pu être remise en question. Le but du travail décrit ci-après était donc d'étudier la fonction des récepteurs A_{2A} chez les patients présentant des syncopes neuro-cardiogéniques. Pour ce faire, nous avons étudié 16 patients et 8 témoins. En utilisant Adonis, un anticorps monoclonal anti-récepteur A_{2A} ayant des propriétés agonistes développé au sein de notre laboratoire, nous avons recherché pour chaque sujet les valeurs de K_D et EC_{50}. Le K_D représente la concentration d'anticorps pour laquelle la moitié des récepteurs sont occupés. L'EC_{50} représente la concentration d'anticorps pour laquelle la moitié de l'effet biologique maximal (production d'AMPc) est obtenu.

Ce travail est détaillé dans l'article : **"A2A adenosine receptor function in patients with neurally mediated syncope"** ci dessous.

Publication 3

“A2A adenosine receptor function in patients with neurally mediated syncope”

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Submitted manuscript.

A_{2A} adenosine receptor function in patients with neurally mediated syncope

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Abstract

Aims:

Adenosine is a possible mediator in neurally mediated syncope (NMS) via the activation of its receptors. High expression of adenosine A_{2A} receptors (A_{2A} R) has been reported in NMS. The function of these over-expressed receptors in this population has never been evaluated.

Methods and Results

We used Adonis, a specific made antibody with A_{2A}R agonist properties, to evaluate binding parameters (ie dissociation constant K_D) and cAMP production (ie EC_{50}) by peripheral blood mononuclear cells (PBMC) of 16 NMS patients. Eight healthy volunteers served as controls.

A_{2A} R expression was higher in patient than controls; mean: 11.5± 1.2 vs 7.7±0.8 AU, $p=0.04$. Also, K_D values were higher in patients than controls: $2.1\pm0.02\ X10^{-7}$ M vs $5\pm1X10^{-8}$ M, $p<0.01$In controls, K_D values were lower than EC_{50} ($5\pm1.7\ X\ 10^{-8}$ M vs $2.8\pm0.4\ 10^{-7}$ M, $p<0.01$), but in patients, K_D values did not differ from EC_{50}: $2.\pm0.2X10^{-7}$ M vs $2.5\pm0.4X10^{-7}$ M, $p>0.05$. However, four patients had lower EC_{50} ($3.5\pm0.3X10^{-8}$ M) than K_D ($2.9\pm1.2X10^{-7}$ M; $K_D/EC_{50}=9.6$), suggesting the presence of spare receptors.

Conclusion

The function of A_{2A} receptors of patients with NMS was preserved since their stimulation by Adonis led to cAMP production with an EC_{50} comparable to those in controls. However, their affinity was lower than those of controls. Results from some patients gave evidence of the presence of spare receptors. The role of these spare receptors in the physiopathology of NMS remains to be determined. Our results strongly suggest that A_{2A} R are implicated in the physiopathology of NMS.

Adonis resolves into heavy and light chains and the light chain is more clearly visualized. At the time of analysis, frozen PBMC pellets were quickly thawed at 37°C and solubilized in 62.5 mM Tris-HCl buffer, pH 8.3, containing 2% SDS, 10% glycerol, 0.01% bromophenol blue, and 5% mercaptoethanol. After 3 min sonication at 47 kHz, samples were submitted to standard electrophoresis in Mini Protean II system (BioRad, Hercules, CA). Separated proteins in 12% acrylamide minigel were electrotransferred onto a PVDF membrane that was then placed in the blot holder of the SNAP i.d. protein detection system (Millipore, Billerica, MA), saturated with non-fat dried milk, and incubated 20 min with Adonis [9]. Blots were visualized by horseradish peroxidase-labeled anti-mouse IgG Fab-specific antibodies and enhanced chemiluminescence substrate (SuperSignal West Femto, Pierce Biotechnology, Rockford, IL) using a Kodak system. Bands (45 kDa for $A_{2A}R$ expression and 25 kDa for Adonis binding) were measured densitometrically on a Macintosh computer using the public-domain NIH Image software developed at the U.S. National Institutes of Health.

Dissociation constant (K_D values for Adonis bindingwere estimated using five increasing concentrations of Adonis and linear regression analysis (threeparameter logistic equation) with a sigmoidal dose-response binding equation, using Prism software (GraphPad Software, Inc La Jolla CA). K_D was defined as the concentration in Adonis at which binding sites are 50% occupied. K_D was expressed in M [10].

cAMP assay

Adonis has agonist properties. These properties allow the use of Adonis as specific ligands for cAMP production [9]. otal cellular cAMP measurement was achieved on PBMC pellets using the Amersham Biotrak Kit (GE healthcare Bio-Sciences, Upsala, Sweden). Stimulation was stopped by adding dodecyltrimethylammonium bromide acetate buffer. Tests were in triplicate and results were expressed as percent of the maximal cAMP production obtained with the highest concentration of Adonis. EC_{50} was defined as the concentration in Adonis that leads to half of the maximal cAMP production by PBMC.

Statistical Analysis

Quantitative variables were expressed using means and standard deviations (SD). Variance analysis (two-way ANOVA) was used to compare APC, K_D, and EC_{50} values. All statistical tests were two-sided and P-values less than 0.05 were considered statistically significant. Analyses were performed with Prism® software.

Introduction

Neurally mediated syncope (NMS) is a common clinical problem that can worsen the quality of life of affected patients. Symptomatology is caused by arterial vasodilation and/or bradycardia. The pathophysiology of this syndrome is incompletely understood. Previous studies supported the notion that endogenous adenosine may be a mediator [1]. Adenosine acts blood vessel tone and sinoatrial node via activation of four subtypes of membrane receptors (A_1, A_{2A}, A_{2B}, and A_3 [2]). Stimulation of A_1 receptors (A_1 R) leads mainly to bradycardia while activation of A_{2A} (A_{2A} R) leads mainly to vasodilatation [2,3]. Higher baseline adenosine plasma concentrations (APC) have been observed during positive head-up tilt test (HUT) along with a positive correlation between APC rise and the onset of HUT-induced syncope [1]. Overexpression of A_{2A} R has also been observed in patients with unexplained syncope and positive HUT [4,5]. A_{2A} R are positively coupled with adenylcyclase, and the activation of these receptors induces cAMP production in target cells. Whereas most biological effects (vasodilation) are secondary to the increase in cAMP production in target cells (ie myocytes of arterial vessels [3]), nothing is known about the cAMP production induced by the activation of these overexpressed receptors. The most frequent adaptation of target cell in response to high ligand concentration is the down regulation and or desensitization of its membrane receptors. It is possible that A_{2A} R of NMS patients could be desensitized due to chronic exposure to high APC.

The aim of this study was to evaluate the expression and function of A_{2A} R in peripheral blood mononuclear cells (PBMC) in a population known to overexpress these receptors [4,5], taking into account that the behavior of PBMC A_{2A} R mirrors the behavior of A_{2A} R of cardiovascular tissues[6].

Methods

Patient selection

A total of 16 consecutive patients with suspected NMS were enrolled. Suspicion of NMS was based on absence of (i) structural heart disease, ECG abnormalities, or rhythm disturbances that may explain the syncope; (ii) orthostatic hypotension; and (iii) non-syncopal causes of transient loss of consciousness. Patient selection criteria also included occurrence of one or more episodes of syncope within the preceeding year, absence of drug use for 3 months, and first-time HUT. Patients with carotid sinus syndrome were not included.

A matched control group of 8 healthy volunteers was recruited consecutively from the medical and technical staff working at our institution during the study period, and served as controls for biological parameters. None of the controls had a history of syncope. All were drug free at the time of the study.

This study was conducted in compliance with the principles of the Declaration of Helsinki and approved by the Ethics Committee for Human Research of our university-affiliated hospital. All patients and controls provided written informed consent to participate.

Collection of blood samples

Baseline blood samples were collected and processed as described previously [1,7] using laboratory-prepared tubes containing 3 ml of cold-stop solution under vacuum. This method allows whole blood to mix quickly with the cold-stop solution so as to prevent degradation or red blood cell uptake of adenosine [8]. Patients and controls were placed in the supine position during sample collection. For patients, samples were collected a few minutes before tilting the table. After collection, samples were immediately placed and maintained on ice until centrifugation.

Adenosine plasma concentration (APC)

APC was determined as previously described[1,7,8]. After deproteinization, adenosine was quantified using high pressure liquid chromatography (ChromSystem, Munchen Germany).

Incubation of Adonis with PBMC

Blood samples from patients and controls were collected from brachial vein into tubes containing ficoll (CPT Beckton Dickinson, Franklin Lakes, NJ). PBMC were prepared according to the manufacturer's instructions and 0.25 x 10^6 cells was mixed with various doses of Adonis, a homemade monoclonal antibody with high specificity for A_{2A} R[9] in 1.5 ml Eppendorf tubes containing 1 ml of RPMI 1640 medium with 10% heat-inactivated fetal calf serum, 2 mmol/l L-glutamine, and 100 IU/ml penicillin-streptomycin. After 90 min incubation at room temperature under shaking, PBMC were rinsed twice with PBS by 10 min centrifugation at 200 g. Cell pellets were kept at -20°C until use.

Western blot analysis of Adonis binding to A_{2A} R of PBMC

Western blot for A_{2A} R expression has been previously described [9]. This method serves to visualize and quantify the antibody bound to A_{2A}R. After running in electrophoresis conditions,

Results

Clinical characteristics of the patients are given in Table 1. Among the 16 patients, 7 (43%) had negative HUT and 9 (56%) had positive HUT. APC was higher in patients than controls (figure 1A). A_{2A} R expression was higher in patients than controls; means: 11.5 ±1.2 AU vs 7.8 ±0.8 AU p=0.04 (figure 1B).

Means K_D values were higher in patients than controls: 2.1 ± 0.2 X10^{-7} M vs 5 ± 1X10^{-8} M, p <0.01 (figure 2), while mean EC_{50} values of patients did not differ from those of controls: 2.5 ± 0.6 X10^{-7}vs 2.8 ± 0.4 X10^{-7}, p>0.05 (figure 3). Means K_D values of controls were lower than means EC_{50} (5 ± 1.7 X 10^{-8}M vs 2.8 ± 0.4 10^{-7} M, p<0.01) but means K_D values of patients did not differ significantly from their mean EC_{50}: 2.1 ± 0.2X10^{-7} M vs 2.5 ± 0.4X10^{-7} M, p>0.05. However, 4 patients had lower EC_{50} (3.5 ± 0.3X10^{-8} M) compared with K_D (2.9 ± 1.2X10^{-7} M) with a K_D/EC_{50} ratio = 9.6 (figure 4).

Discussion

The main finding of this study is that A_{2A} Rs of patients with NMS were not desensitized since their stimulation by agonist (Adonis) led to cAMP production by PBMC, withEC_{50} values similar to those in PBMC of controls. However, in patients, the affinity of A_{2A} R for Adonis was lower than that in the healthy control subjects. The latter result may be due to the chronic exposure to high APC.

Previous reports showed that patients with NMS and positive HUT had high APC and overexpressed A_{2A} R [4,5,7]. Because of the presence of high APC levels in such patients, it could be hypothesized that A_{2A} Rs were desensitized and/or down regulated. Here, we found that the function of A_{2A} Rs of patients was preserved.

In some cases, the EC_{50} values were lower than the K_D, suggesting the existence of receptors of reserve (spare receptors). Indeed, the presence of spare receptors is suspected when the maximal biological effects (ie cAMP production) occurs while not all the receptors are occupied by the agonist (here Adonis). In this case, the biological response caused by the increase in ligand concentration is proportionally greater than the increment in receptor occupancy.

The EC_{50}/K_D ratio we found is in the range of that in previous reports using CCPA (cyclopentyl adenosine) as organic ligand [11]. The absence of spare receptors in healthy subjects raises the question of the role of these spare receptors in the pathophysiology of NMS.

Spare receptors have been observed in the coronary system of guinea pigs [11]. Spare receptors in the cardiovascular system may help explain the various actions of endogenous or exogenous adenosine depending on its relative concentration. As an example, the presence of spare receptors in the coronary circulation explains that vasodilation occurs at lower adenosine concentration than for AV sinusal node effects [11].

Here, the presence of spare receptors may have therapeutic consequences. Because adenosine and its receptors are involved in NMS, the use of adenosine receptor antagonists could logically be used [12]. However, the presence of a great number of spare receptors may explain the weak effects of adenosine receptor antagonists [13]. Indeed, if the maximal biological effects (ie bradycardia and/or vasodilation) occur when only a small number of adenosine receptors are occupied and activated by adenosine, a very high concentration of adenosine receptor antagonist would be necessary to displace adenosine from its receptors and to have therapeutic effects.

Conclusion

We found that A_{2A} receptors of patients with NMS were preserved since their stimulation led to cAMP production. Our results strongly suggest that A_{2A} R are implicated in the physiopathology of NMS. We also found that some patients had spare receptors.

Study limitation

The relatively small number of patients included is sufficient to confirm the function of $A_{2A}R$ was preserved but does not permit the evaluation of a possible relationship between the presence of spare receptors and clinical manifestations. The role of these spare receptors in the physiopathology of NMS needs further investigations.

Legend figures

Figure 1A

Adenosine plasma concentration (APC) evaluated in patients (n=16) suffering from neurally mediated syncope and in control subjects (n=8). * $p < 0.05$ compared with controls.

Figure 1B

Example of A_{2A} R expression evaluated on PBMC of healthy subjects (1) and of patients (2). Western blot was evaluated from 0.25 $X10^6$ cells (see methods).

Figure 2

Saturation curve of A_{2A} adenosine receptor binding, using Adonis, a monoclonal antibody as specific ligand. Data are expressed as means and SD. Quantification of binding was evaluated by Western blot (WB). White disks controls (n=8). Black disks: patients (n=16). A: example of WB in a control subject; B example of WB in a patient. Blots represent light chain (25 kDa) of Adonis bound to A_{2A}R after incubation of PBMC with increasing concentrations of Adonis.

Figure 3

Production of cAMP in peripheral blood mononuclear cells from patients (dark circles) or controls (white circles). cAMP production was obtained using Adonis as a specific agonist, at increasing concentration. Data are expressed as means and SD.

Figure 4

Saturation curve of A_{2A} R binding (A), using Adonis as specific ligand, compared with cAMP production curve (B) for four patients. Data are expressed as means and SD.

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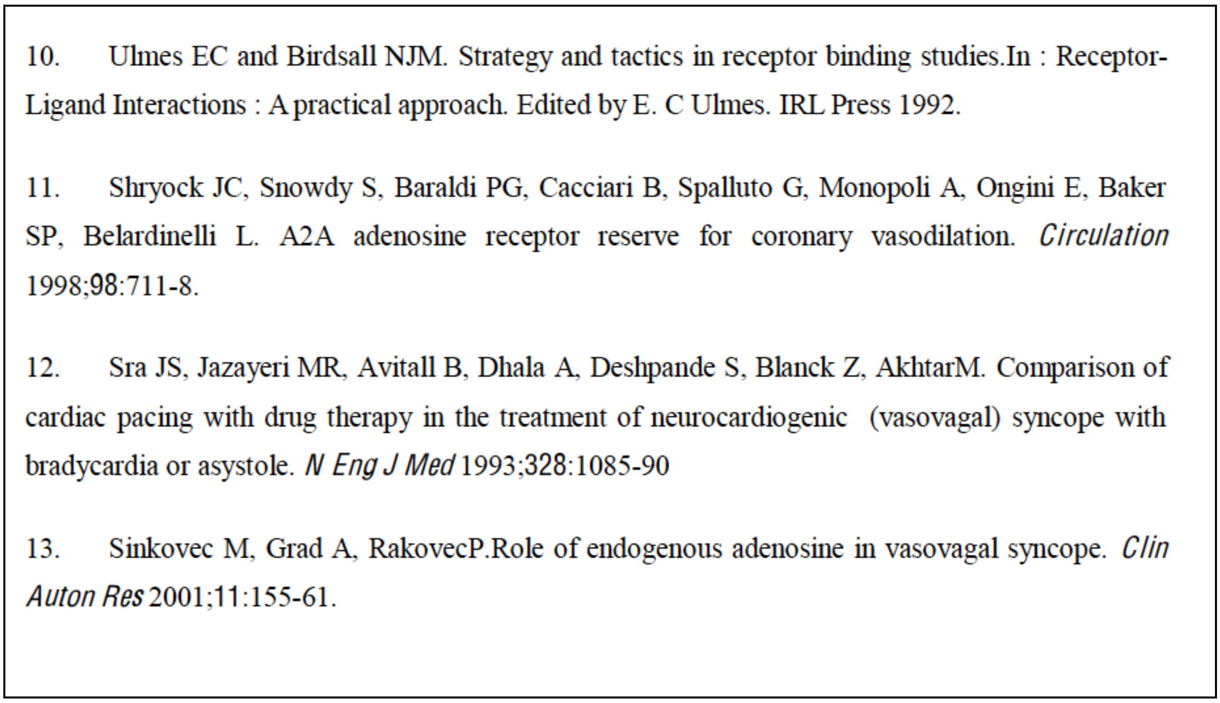

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Table

Patient number	1	2*	3	4*	5	6*	7	8	9	10	11	12	13	14	15	16*	Mean	SD
Age (years)	38	71	49	62	42	23	74	49	39	18	52	65	54	58	28	55	48.6	16.4
Gender	H	H	F	H	F	H	F	F	F	F	H	F	F	F	H	F	-	-
HTA	N	N	N	N	N	N	Y	N	N	N	N	N	N	N	N	Y	-	-
LVEF (%)	60	70	65	65	70	70	65	70	65	70	60	65	65	70	65	65	66.25	3.4
Total of syncope	30	3	2	4	30	50	4	10	4	1	1	20	2	22	15	20	13.63	14.2
Time since 1st syncope (months)	20	30	10	0.2	11	10	13	35	20	0.1	0.2	0.8	12	0.3	1	3	15	10.5
Syncope last 12 months	3	1	1	4	7	5	2	1	1	1	1	2	2	6	2	4	2?7	2
BP baseline (mmHg)	123/83	115/77	111/77	124/78	102/76	108/72	124/88	109/79	107/72	115/82	133/91	155/102	119/70	116/75	124/72	150/92	120.9/80.4	14.7/8.9
HR baseline (/min)	67	57	76	87	66	61	64	76	67	66	57	65	48	58	64	75	65.8	9.3

Clinical characteristics of patients.

M: male; F: female; LVEF: left ventricular ejection fraction; Y: yes; N: no;BP: blood pressure; HR: heart rate; Neg: negative; Pos: positive.

*** presence of spare receptors**

Figure 1

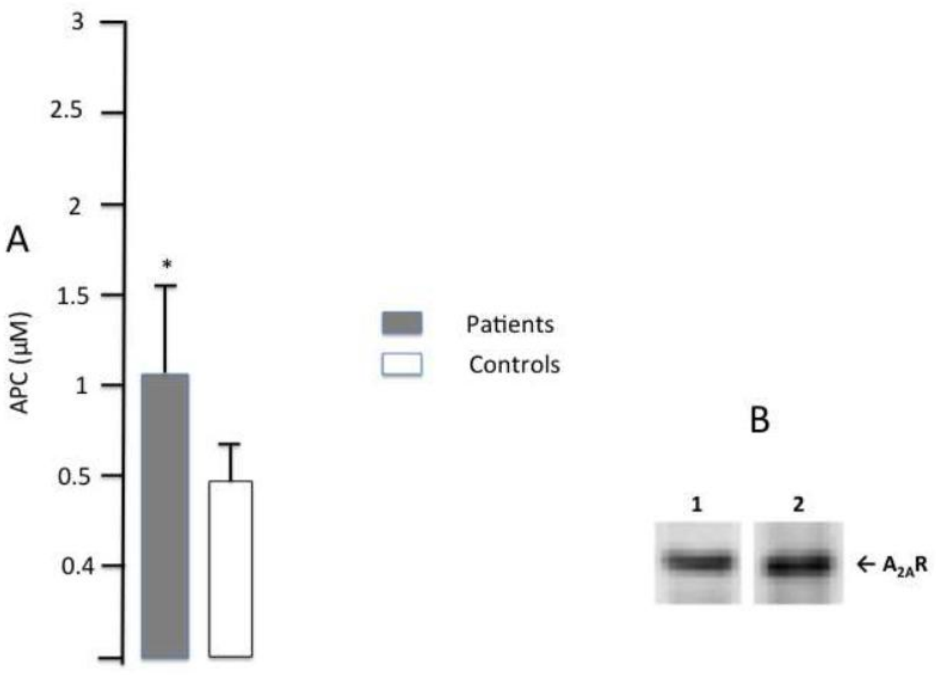


Figure 2

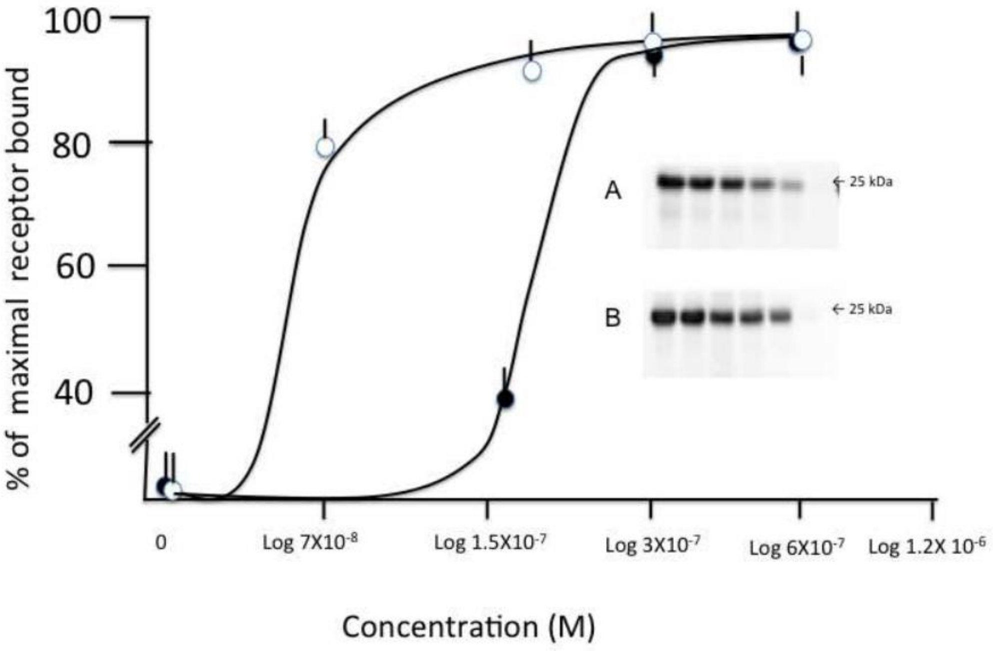


Figure 3

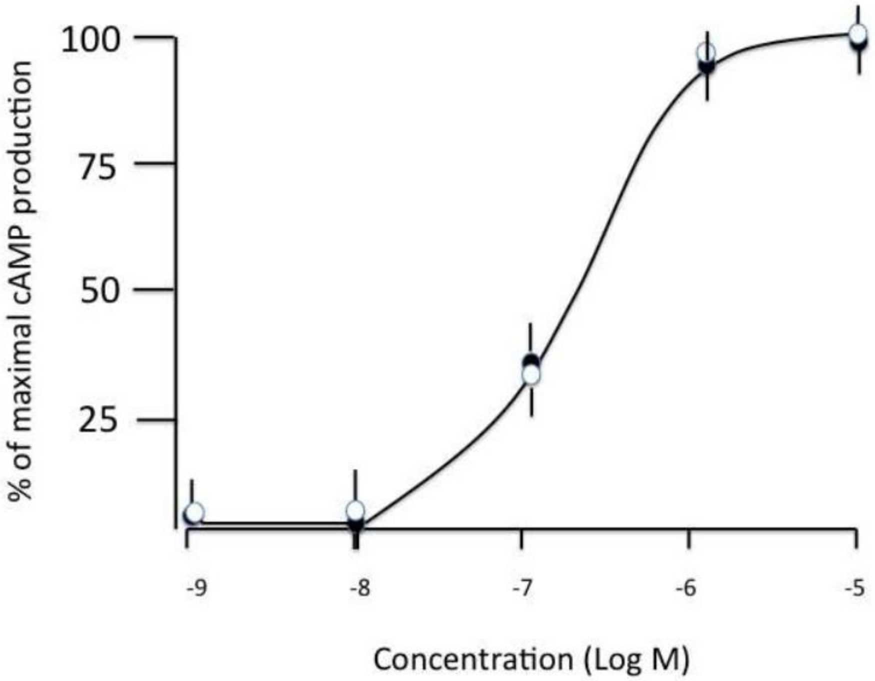
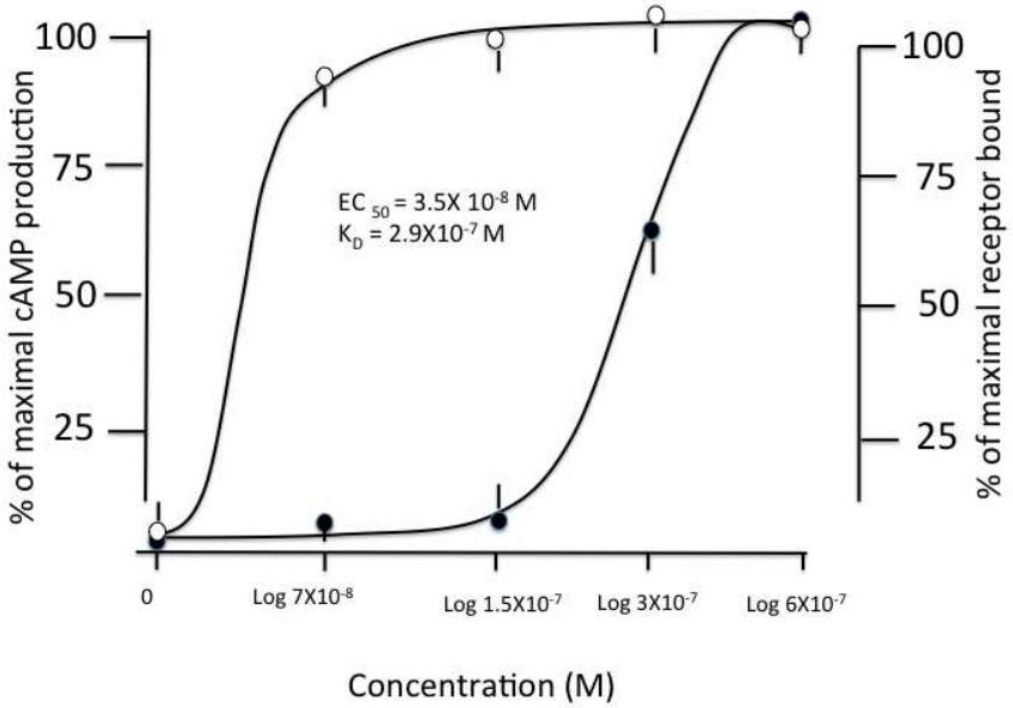


Figure 4



Conclusion

Globalement nous retrouvons chez les patients une surexpression des récepteurs A_{2A} lymphocytaires comme cela avait été décrit précédemment. La fonction des récepteurs A_{2A} lymphocytaire est préservée chez les patients, puisque la production d'AMPc suite à la fixation d'Adonis est comparable à la production trouvée avec les lymphocytes des sujets sains. Cependant, l'affinité des récepteurs pour Adonis est diminuée par comparaison avec les sujets sains. Globalement, chez les patients, comme chez les témoins, la valeur du K_D différant peu de celle de l'EC_{50}. Cependant, pour 4 patients sur 16, nous avons observé une valeur d'EC_{50} très nettement plus faible que celle du K_D (9.6 fois en moyenne), suggérant la présence de récepteurs de réserve. En effet, on parle de récepteur de réserve quand l'effet biologiques est maximal alors que seule une fraction des récepteurs est occupée par l’agoniste. La conséquence clinique de la présence de ces récepteurs de réserve chez les patients présentant des syncopes vagales reste à déterminer. La préservation de la fonction des récepteurs A_{2A} confirme l’implication de l’adénosine et de ses récepteurs dans la physiopathologie des syncopes neurocardiogéniques.

4. Recherche de récepteurs A2A de réserve chez des lymphocytes humains circulants

Introduction

L'adénosine régule de nombreux processus physiologiques chez le lymphocyte humain. Les récepteurs A_{2A} ont notamment un effet immunosuppresseur sur les lymphocytes via la production d'AMPc. L'étude de la fonction et de l'expression de ces récepteurs lymphocytaires n'avait jamais pu être réalisée chez l'homme faute de matériels (récepteurs) en quantité suffisante et de réactifs (ligands agonistes) satisfaisants. En effet, pour pouvoir évaluer la présence ou non de récepteurs de réserve, il faut pouvoir disposer d'un ligand « suicide », c'est à dire d'un ligand qui se fixe au récepteur de manière quasi irréversible, au moins le temps de l'expérimentation. Adonis est un anticorps monoclonal fabriqué au laboratoire qui peut être considéré comme un tel ligand au moins sur une courte période nécessaire à l'étude.

Plusieurs modèles d'interactions ligand-récepteur ont été décrits, et parmi eux, le concept de récepteur de réserve. Il existe des récepteurs de réserve quand l'effet biologique est maximal alors que seule une fraction des récepteurs est occupée. Nous avons étudié l'expression et la fonction des récepteurs A_{2A} chez des lymphocytes circulants provenant de patients atteints de syncopes neurocardiogéniques, connus pour exprimer fortement les $A_{2A}R$, et nous avons recherché la présence de récepteurs de réserve.

Ce travail est détaillé dans l'article : **"Search for adenosine A2A spare receptors on peripheral human lymphocytes"** ci dessous.

Publication 4

“Search for adenosine A2A spare receptors on peripheral human lymphocytes”

Jacquin L, **Franceschi F**, By Y, Durand JM, Condo J, Deharo JC, Michelet, Fenouillet E, Guieu R and Ruf J.

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Search for adenosine A_{2A} spare receptors on peripheral human lymphocytes

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ABSTRACT

Some ligand–receptor couples involve spare receptors, which are apparent when a maximal response is achieved with only a small fraction of the receptor population occupied. This situation favours cross-reactions with low-affinity ligands, which may be detrimental for cell signaling. In the case of the adenosine A_{2A} receptors ($A_{2A}R$), which have an immunosuppressive effect on lymphocytes through cAMP production, the presence of spare $A_{2A}R$ remains to be established. We examined the situation using patients over-expressing lymphocyte $A_{2A}R$ and an agonist-like mAb to $A_{2A}R$. We found that maximal mAb binding and functional response varied among the patients whereas the dissociation constant and half-maximal effective concentration had similar mean values (0.19 and 0.18 μM, respectively). Lymphocyte $A_{2A}R$ expression was correlated to plasma adenosine level and $A_{2A}R$ occupation but not to $A_{2A}R$ response. These results are consistent with a lack of a reserve of functional $A_{2A}R$ on human lymphocytes as a general rule and suggest that the amount and functional state of the expressed $A_{2A}R$ determine the maximal level of the lymphocyte response to adenosine.

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1. Introduction

Adenosine, an endogenous purine nucleoside, acts through four classes of G protein-coupled receptors (A_1R, $A_{2A}R$, $A_{2B}R$ and A_4R) to exert various physiologic effects [1]. Most of these actions affect cardiovascular, neuronal and immune cells [2–7]. In lymphocytes, adenosine regulates multiple physiologic processes including inflammation [8–14] and congenital defect of adenosine deaminase – an enzyme that degrades the nucleoside and hence regulates its plasma level – associated with aberrant signaling through adenosine receptors contributes to the severe combined immunodeficiency syndrome [15]. $CD4^+$ and $CD8^+$ T-cells express $A_{2A}R$, $A_{2B}R$, and A_3R [16–21]. Numerous in vivo and in vitro studies suggest that $A_{2A}R$ selectively inhibit T-cell receptors of activated T-cells, thereby inhibiting lymphocyte inflammatory activity [18,22–25]. Activation of the $A_{2A}R$ on $CD4^+$ T-cells prevents myocardial ischemia–reperfusion injury by inhibiting the lymphocyte accumulation and activation in the reperfused heart [26]. $A_{2A}R$ can also prevent Th1/Th2 development [27] as well as T-cell apoptosis [28]. Finally, it was reported that adenosine produced by regulatory T-cells mediates immune suppression activity through $A_{2A}R$ [29,30]. Thus, the study of the expression and function of $A_{2A}R$ on lymphocytes appears to be of pivotal importance to evaluate their immuno-regulatory role.

Various ligand–receptor models are available in pharmacology and may be applicable to agonist–$A_{2A}R$ interactions in order to study the role of $A_{2A}R$ in lymphocyte regulation. Among them, the spare receptor theory [31] postulates that a given ligand can exert maximum biological effect while occupying only a small amount of the available receptors. Such a reserve of receptors may allow for a response to low, transient ligand concentrations and low-affinity interactions which are compatible with several T-cells properties, hence supporting the presence of spare receptors for T-cell receptor activation [32]. From a pharmacological point of view, the presence of spare receptors is evidenced if the maximal ligand response (E_{max}) is obtained at less than the maximal occupation of the receptors (B_{max}). This pattern is usually determined by comparing the half-maximal effective concentration (EC_{50}) that refers to the ligand concentration necessary to reach 50% of the maximal effect with the dissociation constant (K_D) that refers to the ligand concentration necessary to reach 50% of the maximal binding. If the EC_{50} is less than the K_D, spare receptors are existing.

Spare $A_{2A}R$ were reported in cardiac tissue of guinea pig using an irreversible $A_{2A}R$ antagonist to block receptor response to various agonists [33]. In other study on mouse T-cells, using gene targeting to generate mice lacking one or two alleles of $A_{2A}R$, the authors showed that the decrease in the number of $A_{2A}R$ in thymocytes from $A_{2A}R^{+/-}$ and $A_{2A}R^{-/-}$ mice versus the $A_{2A}R^{+/+}$ control mice resulted

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Abbreviations: $A_{2A}R$, adenosine A_{2A} receptors; E_{max}, maximal ligand response; B_{max}, maximal occupation of the receptors; EC_{50}, half-maximal effective concentration; K_D, dissociation constant; NMS, neurally-mediated syncope; APC, adenosine plasma concentration; PBMC, peripheral blood mononuclear cells; AU, arbitrary units

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in proportional decrease in the adenosine-induced cAMP and apoptosis responses of their respective T-cells [34]. No investigations were conducted on human lymphocyte $A_{2A}R$ expression, occupation and function because of some experimental constraints, mainly low $A_{2A}R$ expression in normal individuals and lack of efficient ligand to stably bind to the receptor. Here, we took advantage of Adonis, an agonist-like mAb which steadily binds to an extra-cellular part of the $A_{2A}R$ [35], to investigate the presence of a reserve of $A_{2A}R$ in peripheral lymphocytes of patients with neurally-mediated syncope (NMS) who generally display high adenosine plasma concentrations (APC) and increased lymphocyte $A_{2A}R$ expressions [36–38].

2. Materials and methods

2.1. Selection of patients

Ten patients with clinical symptoms of NMS [38] were enrolled. NMS patients were chosen because they frequently exhibit high APC [36,39] associated with high expression levels of $A_{2A}R$ on peripheral lymphocytes [37], which was found to mirror the increase in $A_{2A}R$ expression on disease target organs [40]. This study was conducted in compliance with the principles of the Declaration of Helsinki and approved by the Ethics Committee for Human Research of our university hospital. All patients provided written informed consent to participate.

2.2. Adenosine assay

Blood sampling was processed as described using laboratory-prepared tubes containing 3 ml of cold-stop solution composed of inhibitors of degradation and red blood cell uptake of adenosine [36,39,41]. Samples were then maintained on ice until centrifugation. After deproteinization, adenosine was quantified by HPLC (Chrom-Systems, Munich, Germany), adenosine being identified by its elution time and spectrum [39]. Measurement was made by comparison of peak areas with those obtained using standards. The intra-assay and inter-assay coefficients of variation ranged from 1% to 3%.

2.3. Incubation of Adonis with lymphocytes

Blood samples were collected from brachial vein into 8 ml tubes containing sodium citrate/Ficoll (BD Vacutainer CPT, Beckton Dickinson, Franklin lakes, NJ). Peripheral blood mononuclear cells (PBMC) were prepared according to the manufacturer's instructions. Viable cell recovery was consistently over 98% with more than 95% of lymphocytes and less than 3% granulocytes. 0.25×10^6 cells were mixed with Adonis (0–1.2 μM in 1 ml culture medium), an IgM mAb directed against a linear part of the second external loop of $A_{2A}R$ [35]. After 90 min incubation at room temperature under shaking, PBMC were either washed twice with phosphate-buffered saline, pH 7.3 for binding and $A_{2A}R$ expression tests or only centrifuged without washing for functional test. Cell pellets were kept at −20 °C until use.

2.4. Western blotting

To quantify both Adonis binding to lymphocytes and the lymphocyte $A_{2A}R$ expression, we used the Western blotting procedure previously described [42]. PBMC pellets previously incubated with or without Adonis were lysed by a 3 min sonication treatment at 47 kHz with the SDS–PAGE loading buffer containing 5% mercaptoethanol. Samples were then submitted to 12% SDS–PAGE analysis followed by transfer onto a PVDF membrane. Filters were incubated 20 min with Adonis and then with horseradish peroxidase-labeled anti-mouse IgG Fab specific antibodies prior to chemiluminescent staining with SuperSignal West Femto (Pierce Biotechnology, Rockford, IL).

In SDS–PAGE analysis in reducing condition, Adonis resolved in heavy (65 kDa) and light (25 kDa) IgM chains bands. Quantification of Adonis bound to $A_{2A}R$ was based on the detection of the light chain of the IgM because it is readily detected by the labeled second antibody directed partly to the light chain of Fab portion of mouse IgG shared by mouse IgM. Densitometry analysis used ImageJ 1.42q (National Institutes of Health, USA) and results from duplicates were expressed as arbitrary units (AU) defined as pixels of the light chain band versus blot background.

The lymphocyte $A_{2A}R$ expression was similarly quantified except that AU were defined as pixels of the canonical $A_{2A}R$ band versus blot background. $A_{2A}R$ from PBMC not previously incubated with Adonis migrated at 45 kDa and the corresponding band was detected by Adonis binding during the Western blot procedure.

Adonis binding curves were established using six concentrations of Adonis and non linear regression analysis were performed with a one site specific binding equation $Y = B_{\max} \times X/(K_D + X)$ using Prism 5 (GraphPad Software, Inc La Jolla, CA). $B_{\max}$ expressed using the same units as Y values and K_D expressed using the same units as X values were estimated for Adonis binding to each PBMC preparation.

2.5. cAMP test

The agonist properties of Adonis allow its use as a specific effector for cAMP production [35]. Briefly, cellular cAMP measurement was achieved on PBMC pellets incubated with Adonis by ELISA using the Amersham Biotrak Kit (GE healthcare Bio-Sciences, Upsala, Sweden). Stimulation was stopped by adding dodecyltrimethylammonium bromide acetate buffer. Results of duplicates were expressed as AU defined as cAMP production generated in the presence versus in the absence of Adonis. We examined the results obtained using six concentrations of Adonis with Prism 5 (GraphPad Software) and the sigmoidal dose–response curve: $Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10^{[(\text{Log EC}_{50} - X) \times \text{Hill Slope}]})$. Top ($E_{\max}$ here) and Bottom are plateau values in the units of Y and EC_{50} is the concentration of Adonis that gives a response half way between Top and Bottom values. Hill slope describes the steepness of the family of curves.

2.6. Statistical analysis

Quantitative variables were expressed using means ± SEM. Paired t-test was used to compare K_D and EC_{50} values and correlation tests were made between adenosine, $B_{\max}$ and $E_{\max}$ values. All the tests were two-tailed and P-values less than 0.05 were considered as statistically significant. Analyses were performed with Prism 5 (GraphPad Software).

3. Results

3.1. Adenosine levels

The patients listed in Table 1 (7 women and 3 men; mean age: 52 years) were selected on the basis of NMS clinical symptoms. They had APC ranging from 0.29 to 2.91 μM with a mean value ± SEM of 1.29 ± 0.27 μM. As expected from the literature on NMS, most patients (7 out of 10) had higher APC than control subjects (0.20 to 0.70 μM; data from the Biochemistry Laboratory of the Timone Hospital, Marseille, France).

3.2. Saturation curves and dose–response curves

Adonis binding to PBMC from patients was assessed by Western blotting visualizing the light chain of bound Adonis (Fig. 1(A)). After densitometry analysis of the bands, the resulting Adonis binding curves exhibited various plateau values ranging from 3.05 to 68.13 AU (pixels of the light chain band versus blot background) with a mean

Table 1
List of patients with results.

Patients	Sex	Age	APC (μM)	B_{max} (AU)	K_D (μM)	E_{max} (AU)	EC_{50} (μM)	$A_{2A}R$ (AU)
1	Female	62	1.55	63.14	0.12	22.57	0.48	19.56
2	Female	18	0.99	26.66	0.15	32	0.1	19.53
3	Female	39	2.37	68.13	0.09	16.5	0.08	32.02
4	Female	49	1.82	59.16	0.08	28.29	0.11	12.12
5	Female	74	0.77	27.33	0.33	34.62	0.36	6.41
6	Male	38	2.91	61.49	0.26	41.73	0.23	17.97
7	Male	62	0.29	12.39	0.42	49.89	0.09	8.69
8	Female	49	0.66	8.92	0.23	21.56	0.17	7.29
9	Female	52	1.01	3.05	0.08	31.57	0,11	9.24
10	Male	71	0.5	12.37	0.13	36	0.06	8.61
	Mean	51.7	1.29	34.26	0.19	31.47	0.18	14.14
	± **SEM**	5.43	0.27	8.18	0.04	3.14	0.04	2.56

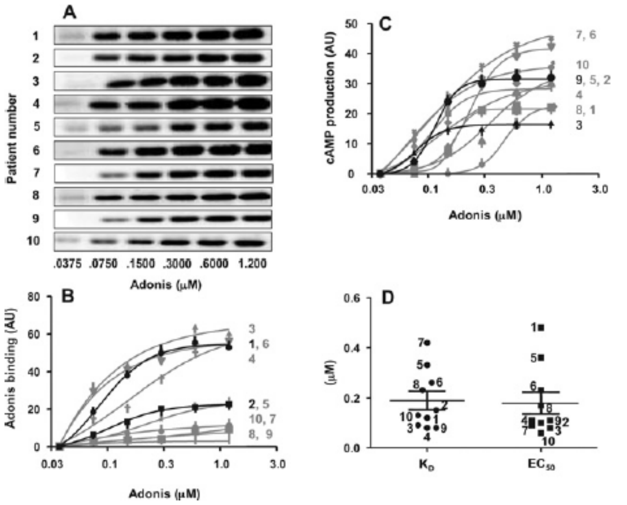

Fig. 1. (A) Western blots of Adonis (25 kDa light chain) binding to PBMC from the 10 patients. For each patient, 6 doses of Adonis were tested in duplicate on separate gels and one representative set of blots is shown. (B) Binding curves giving B_{max} and K_D values. Curves derived from the pixel analysis of the Adonis light chain blots. (C) Dose–response curves giving E_{max} and EC_{50} values for the 10 patients. For each set of curves, 2 representative curves are in black, the 8 other ones are in gray. Patient numbers are at right of the curves. Results expressed in arbitrary units (AU) as defined in Materials and methods are means ± SD of duplicates. (D) Comparison of K_D (black circles) and EC_{50} (black squares) values interpolated from complete binding and dose–response curves with Adonis. Results are expressed in μM. Each symbol with the corresponding patient number represents individual mean value. Horizontal bars are means ± SEM for the 10 patients.

value ± SEM of 34.26 ± 8.18 AU (Fig. 1(B) and Table 1). Representative curves from Patients 1 and 2 were highlighted in black in Fig. 1 (B). Binding reached a plateau value at 63.14 AU of Adonis binding for Patient 1 and at 26.66 AU for Patient 2, both patients exhibiting similar K_D value (0.12 and 0.15 μM, respectively; Table 1).

Adonis response curves for cAMP production also exhibited various plateau values ranging from 16.50 to 49.89 AU (cAMP production generated by Adonis versus basal cAMP level in the absence of Adonis) with a mean value ± SEM of 31.47 ± 3.14 AU (Fig. 1(C) and Table 1). Representative curves from Patients 9 and 3 were highlighted in black in Fig. 1 (C). A maximal effect at 31.57 AU of cAMP production was obtained for Patient 9 whereas only 16.5 AU were obtained for Patient 3, both patients exhibiting similar EC_{50} values (0.11 and 0.08 μM, respectively; Table 1).

Fig. 1(D) shows that, taken as a whole, the distribution of K_D and EC_{50} values extrapolated from the above curves was not statistically different: K_D values ranged from 0.08 to 0.42 μM with a mean value ± SEM of 0.19 ± 0.04 μM and EC_{50} values ranged from 0.06 to 0.48 μM with a mean value ± SEM of 0.18 ± 0.04 μM. We noticed 2 exceptions, Patient 7 displaying the highest K_D (0.42 μM) had a 5-fold lower EC_{50} (0.09 μM) and, conversely, Patient 1 displaying the highest EC_{50} (0.48 μM) had a 4-fold lower K_D (0.12 μM) (Fig. 1(D) and Table 1).

3.3. *Expression of $A_{2A}R$*

We found various lymphocyte $A_{2A}R$ expression levels with values ranging from 6.41 to 32.02 AU (pixels of the $A_{2A}R$ band versus blot background) with a mean value ± SEM of 14.14 ± 2.56 AU. Correlation studies were performed to compare lymphocyte $A_{2A}R$ expressions to APC, B_{max} and E_{max}. Lymphocyte $A_{2A}R$ expressions were found to be correlated with APC (Fig. 2(A)) and B_{max} (Fig. 2(B)) but not E_{max} (Fig. 2(C)) values. Once more, we noticed 2 exceptions: Patient 7 displaying the highest E_{max} (49.89 AU) had low $A_{2A}R$ level (8.69 AU) while Patient 3 displaying the lowest E_{max} (16.5 AU) expressed the highest $A_{2A}R$ level (32.02 AU).

4. Discussion

We found that B_{max} of Adonis to $A_{2A}R$ varied from subject to subject whereas K_D values were similar. Various E_{max} were also found whereas the EC_{50} values were similar. More importantly, the K_D values were found to be statistically not different from the EC_{50} values, suggesting a lack of a reserve of receptors according to the criteria previously defined. In 8 out of the 10 patients tested, the highest cAMP production occurred when all the receptors were occupied by Adonis. Results from 2 patients, however, illustrate opposite situations: Patient 7 exhibited a K_D which was 5-fold higher than EC_{50}, consistent with the situation where a few occupied receptors produce a maximal effect (presence of spare receptors), and Patient 1 exhibited a K_D which was 4-fold lower than EC_{50}, indicating a situation where a low effect is obtained while all receptors are occupied. The latter case suggests that many $A_{2A}R$ of this subject are not functional.

We also performed experiments to address the lymphocyte $A_{2A}R$ expression using Western blotting. As previously reported in patients with intradialytic hypotension during hemodialysis [43] and spontaneous and head-up-tilt-induced syncope [37,38], we found an overexpression of lymphocyte $A_{2A}R$ which correlated with the increase in APC and therefore with the maximal level of Adonis binding. This suggests that adenosine and $A_{2A}R$ are upregulated concomitantly and/or interdependently. More intriguingly, lymphocyte $A_{2A}R$ expression was generally not correlated with E_{max} suggesting a non linear relation between the amount of receptors and their overall efficiency. Therefore, adenosine appeared necessary but not sufficient to modulate its immunosuppressive effect via the $A_{2A}R$. Two patients illustrate opposite situations regarding E_{max}: Patient 7, previously suspected with spare receptors, who expressed a few but highly efficient receptors and, conversely, Patient 3 who expressed high level of receptors exhibiting low overall efficiency. The former, displaying a K_D higher than EC_{50}, had probably some receptors engaged in an hyper-active

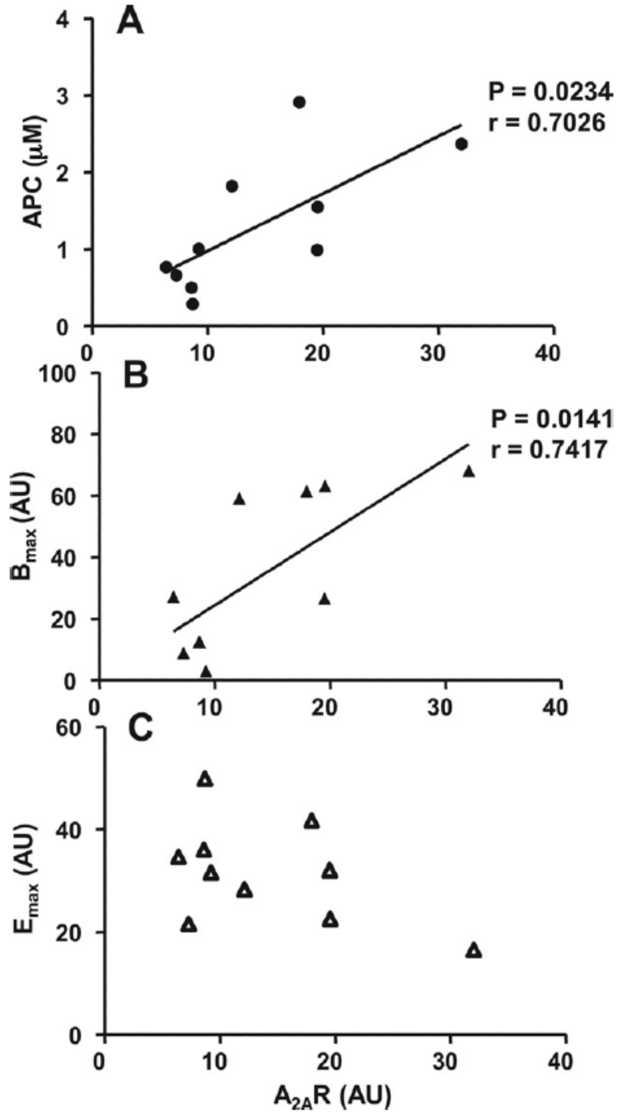

Fig. 2. Correlation study between lymphocyte $A_{2A}R$ expression levels and (A) APC, (B) B_{max} and (C) E_{max}. Results are expressed in μM for adenosine plasma levels and arbitrary units (AU) as defined in Materials and methods for $A_{2A}R$ expression, B_{max} and E_{max} levels. $A_{2A}R$ expressions were measured using Western blotting. Each symbol represents individual mean value. Significant correlation ($P < 0.05$) for the 10 patients is indicated at right.

state with an efficient coupling to the effector (Gs for cAMP production) whereas the latter, displaying a K_D similar to EC_{50}, likely expressed mostly inactive receptors, unmounted to cell-surface and constituting a putative reserve of intra-cellular standby receptors.

T-cells possess a T-cell receptor reserve to increase the flexibility of cell response to ligands of weaker potency [44]. Accordingly, we suggest that a reserve of $A_{2A}R$ would promote cross-reactions with low affinity ligands such as weak and partial agonists/antagonists. Consequently, stimulating or blocking ligands other than adenosine would impede the fine tuning of adenosine-induced immune regulation by occupying more receptors to respond. However, absence of spare receptors does not systematically mean that the amount of expressed $A_{2A}R$ on lymphocytes is the limiting factor to determine the extent of the effector response. Regarding the NMS situation, $A_{2A}R$ are frequently over-expressed in peripheral lymphocytes and some patients having a specific $A_{2A}R$ gene polymorphism express more receptors [45]. However, the resulting efficiency of the $A_{2A}R$ response can also depend on the functional state of the receptors. The multimerization process of G protein-coupled receptors [46] modulates agonist efficiency when occupancy of a single receptor is needed to obtain a full agonist action. For a recent example, TSH receptor signals via activation of Gs to stimulate cAMP production when TSH binds to one protomer of the TSH receptor homodimer [47]. Therefore, the presence of spare receptors may also refer to the functional state of the multimerized receptors.

Together, these data are consistent with a lack of spare $A_{2A}R$ on lymphocytes as a general rule and their putative presence in some patients appeared to be dependent on the functional state of the targeted receptors. Therefore, the adenosinergic signaling efficiency in lymphocytes likely involves many but not a few $A_{2A}R$ expressed on lymphocytes and this is the amount and functional state of these expressed receptors that determine the maximal level of the lymphocyte response to adenosine.

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Conclusion

Chez 10 sujets, les valeurs des K_D et EC_{50} ont pu être comparées. Cependant, les valeurs moyennes sont semblables (0.19 et 0.18 µM, respectivement). De plus, alors que l'expression lymphocytaire des $A_{2A}R$ est statistiquement corrélée au taux d'adénosine plasmatique et au niveau maximal de fixation d'Adonis sur le récepteur, elle n'est pas corrélée à la production d'AMPc. Ces résultats ne plaident pas en faveur de la présence systématique de $A_{2A}R$ de réserve sur les lymphocytes circulants mais pourrait concerner quelques patients (voir travail suivant). D'autre part il est difficile de savoir si dans quelques cas ou l'EC_{50} est plus basse que le K_D, ceci n'est pas en relation avec une multimérisation du récepteur qui peut favoriser la fonction effectrice des $A_{2A}R$ (donc diminuer l'EC_{50}) par rapport à leur capacité de fixation du ligand (K_D), mimant ainsi la présence de récepteurs de réserve.

5. Implication du système adénosinergique chez une enfant présentant des syncopes en rapport avec un bloc-AV peri-prandial paroxystique

Introduction

Les syncopes sont fréquentes chez l'enfant, le plus souvent d'origine vagale. Nous décrivons dans le cas clinique suivant l'histoire d'une enfant âgée de 6 ans, ayant présenté plusieurs épisodes de syncopes. Cette observation est exceptionnelle à plus d'un titre. En effet, un BAV complet paroxystique avait pu être documenté de manière très fréquente sur un simple holter rythmique de 24 heures. Une forte relation avec les repas a pu être mise en évidence : les épisodes de BAV avaient lieu dès le début de chaque repas, et dans l'heure qui suivait. Devant l'absence de cardiopathie sous-jacente, et la preuve qu'il ne s'agissait pas d'un trouble de la conduction organique, l'hypothèse d'un BAV extrinsèque était retenue. Nous avons tenté de comprendre si l'adénosine pouvait être impliquée dans la physiopathologie de ce trouble de la conduction paroxystique.

Ce cas clinique est détaillé dans la publication : **"Peri- and post prandial complete AV block in a child with syncope and adenosine hypersensitivity"** ci-dessous.

Publication 5

“Peri- and post prandial complete AV block in a child with syncope and adenosine hypersensitivity”

Franceschi F, Khairy P, Guieu R and Deharo JC

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Periprandial and postprandial complete AV block in a child with syncope and adenosine hypersensitivity

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Syncope is common in childhood, with the vast majority of episodes considered neuroregulatory and benign, occurring in the absence of cardiovascular disease.[1] Potentially life-threatening cardiogenic syncope is predominantly due to ventricular tachyarrhythmias, with a minority caused by bradycardia. Neurogenic syncope encompasses a variety of subtypes, including vasovagal, carotid sinus hypersensitivity, and situational syncope. Exceptionally, neuroregulatory syncope may be induced by swallowing.[2] Herein, we report the case of a child with syncope and documented periprandial and postprandial complete atrioventricular (AV) block, and offer insights into underlying pathophysiological mechanisms.

Case report

A 7-year-old girl, under investigation for dizzy spells with 4 episodes of syncope over the past 4 years, was referred following 24-hour Holter documentation of transient complete AV block (Figure 1). One hundred and sixty-nine asymptomatic episodes of sudden-onset paroxysmal AV block were recorded, with 18 pauses exceeding 3 seconds. Her past medical history was otherwise unremarkable, with no maternal heart or collagen vascular disease and no family history of syncope or sudden death. Repeated cerebral computed tomography (CT) scans, electroencephalograms, and cerebral magnetic resonance imaging studies were deemed normal. Antiepileptic pharmacological therapy was empirically initiated several months prior and discontinued for lack of effect. The physical examination was normal, with a weight of 24 kg. Her electrocardiogram (ECG) revealed normal sinus rhythm at 100 beats/min, with a normal PR interval, QRS axis, and QRS duration. Negative T-waves were present from V1 to V3, with a QTc interval of 480 ms. Blood tests, including serum electrolytes and calcium levels, were normal. Echocardiography confirmed the absence of structural heart disease.

An electrophysiological study performed under general anesthesia revealed an AH of 56 ms, HV of 38 ms, and H potential duration of 12 ms. The AV nodal effective refractory period was 230 ms (at 400 ms), with a Wenckebach cycle length of 260 ms. No sinus node dysfunction or carotid sinus hypersensitivity was elicited. Given the suspicion of an extrinsic cause for AV block, an adenosine test was performed by administering a weight-adjusted bolus of 10 mg. The response was consistent with hypersensitivity to adenosine,[3] as complete AV block persisted for 10.6 seconds (Figure 2) and was reproduced twice. A tilt-table test (60°) without (45 minutes) and with (20 minutes) 0.2 mg of oral glyceryl trinitrate spray was negative. Cardiac telemetry subsequently revealed clusters of transient complete AV block that seemed to coincide with meals. The suspected association was further elucidated by first administering a cold sparkling beverage with no response. However, nearly 30 seconds after swallowing solid food, paroxysmal complete AV block occurred, persisted during the entire meal, and resolved 1 hour afterward. Further gastrointestinal work-up with gastroesophageal endoscopy, barium swallow, and 24-hour esophageal manometry with pH testing was entirely normal.

Endogenous adenosine plasma levels were tested before a meal and every 10 minutes thereafter until 50 minutes, while logging the onset and duration of AV block.[4] As shown in Figure 3, endogenous adenosine plasma levels were elevated throughout and peaked at 30 minutes. Oral caffeine therapy (5 mg/kg) was attempted for 48 hours under continuous-loop monitoring, given its properties as an adenosine antagonist.[5] A partial response was noted, with 42% fewer asymptomatic episodes of paroxysmal AV block (i.e., 98 events), but no reduction in the number of pauses exceeding 3 seconds (i.e., 23 pauses). An epicardial VVI pacemaker with a bipolar left ventricular lead was therefore implanted and programmed to backup pacing at 40 beats/min. Symptoms immediately resolved. The patient remains asymptomatic at 2 years of follow-up, with 0.4% ventricular pacing.

Discussion

Adenosine-sensitive neurocardiogenically mediated AV block has been previously described, but typically occurs in older individuals with no situational triggers and fewer events than observed in our patient.[6,7] To date, 60 cases of swallow syncope have been reported,[8] including 4 children.[2] It is characteristically associated with underlying

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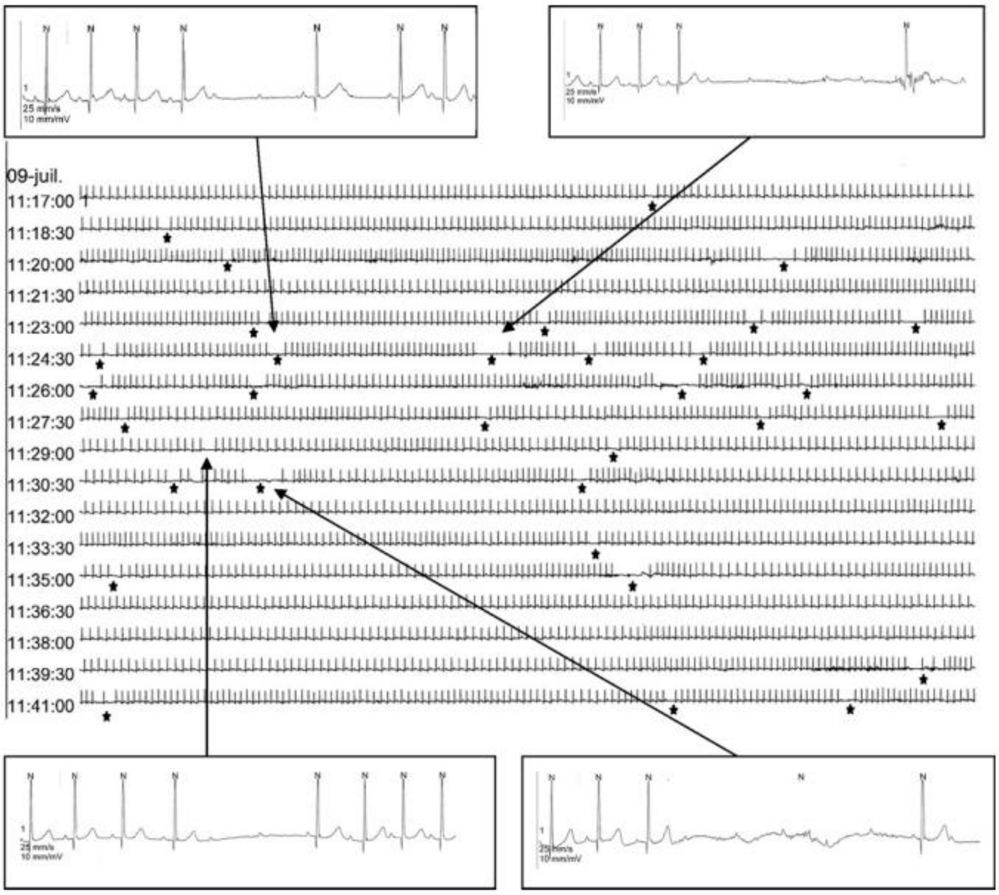

Figure 1 Pattern of AV block. A 24-minute segment of a postprandial Holter recording is shown, notable for clusters of transient AV block (stars). Four enlarged samples are shown.

gastrointestinal, esophageal, thoracic, or cardiac disease and may involve AV block and/or sinus bradycardia, sinoatrial block, or atrial fibrillation.[2] Although the mechanism remains unclear, a neuroregulatory phenomenon involving the vagus nerve is likely implicated. Atropine can abort or prevent associated arrhythmias.[2] However, swallow syncope pathognomonically occurs during swallowing and resolves promptly thereafter. To our knowledge, the delayed and persistent pattern of AV block observed in our patient has not previously been described. Because swallow syncope is thought to reflect an enhanced vagal response to esophageal dilation, it may be speculated that a similar response in the more distal gastrointestinal tract (e.g., hypersensitivity to gastric filling) may be implicated in our patient. Mechanical distention was most likely involved given that AV block occurred after every meal, regardless of the food temperature or body position (i.e., sitting or supine), and that fluid intake alone did not provoke a similar response.

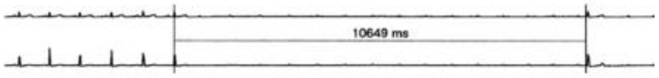

Figure 2 Test for adenosine hypersensitivity. Complete AV block with a 10.6-second ventricular pause was recorded after administering a weight-adjusted 10-mg dose of adenosine triphosphate, consistent with adenosine hypersensitivity.

Higher adenosine plasma levels have been noted in patients with unexplained syncope and positive tilt tests, with further increases during induced syncope.[9] Although transient AV block is expected after an adenosine bolus, our patient's prolonged response (>10 seconds) suggests adenosine hypersensitivity. Because our patient had elevated

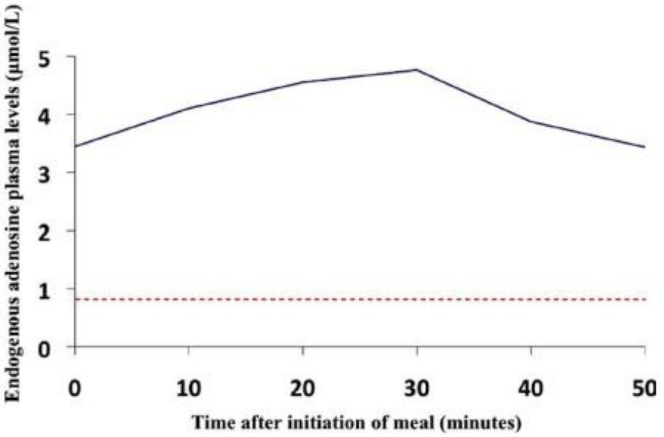

Figure 3 Endogenous adenosine plasma levels before, during, and after a meal. Endogenous serum adenosine levels are shown by the solid blue line. The baseline level (3.4 μmol/l) was drawn just before initiating the meal. The dotted red line represents the upper limit of normal in healthy subjects (≤ 0.8 μmol/l). Adenosine levels peaked at 30 minutes (4.8 μmol/l) and returned to the elevated baseline state by 50 minutes.

endogenous baseline levels that further increased after the meal, with transient AV block persisting for 1 hour, it may be hypothesized that a combination of an enhanced gastrointestinal vagal response and AV nodal hypersensitivity to adenosine were implicated. This remains speculative because adenosine's half-life is only a few seconds and blood samples were collected in a brachial vein, potentially remote from its site of release. Moreover, it cannot be determined whether the further increase in adenosine plasma levels induced by eating was the cause or the consequence of AV block, because tissue ischemia triggers adenosine release. Nevertheless, the time course of events suggests that elevated baseline adenosine levels cannot be explained by AV block. The partial response to caffeine, a competitive adenosine antagonist, further supports this hypothesis.

Conclusion

We report a case of syncope in a 7-year-old girl with documented complete AV block related to meals. The extensive workup excluded primary cardiac electrophysiological, neurological, and gastrointestinal disorders. Given the time course of AV block, elevated endogenous adenosine plasma levels that further increased during a meal, and reproducible abnormal response to an adenosine bolus, we speculate that a combination of an enhanced gastrointestinal vagal response and adenosine hypersensitivity may be implicated. Symptoms were effectively relieved by backup VVI pacing.

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Conclusion

Les différentes explorations ont permis de montrer que le nœud AV de cette enfant présentait une hypersensibilité à l'adénosine (le test à l'ATP induisant un BAV complet avec une pause de plus de 10 secondes). De plus, les taux d'adénosine endogène étaient très supérieurs à la normale, s'élevant encore en post-prandial. Enfin, l'administration d'un antagoniste non spécifique de l'adénosine permettait de diminuer de manière importante le nombre de BAV sur 24h. Il existe donc très probablement un lien entre l'adénosine et le BAV paroxystique syncopal de cette jeune patiente. Dans l'état actuel de l'arsenal thérapeutique, aucune solution médicamenteuse n'a pu lui être proposée. Peut-être un antagoniste spécifique du récepteur A1 aurait-il pu être intéressant. La mise en en place d'un stimulateur cardiaque épicardique a permis de supprimer les syncopes.

6. Implication de l'adénosine endogène pour l'induction de fibrillation atriale en postopératoire de chirurgie cardiaque

Introduction

La fibrillation atriale (FA) est l'arythmie cardiaque la plus fréquemment rencontrée. Son incidence est particulièrement importante chez les patients porteurs de cardiopathies. L'adénosine est connue pour avoir des effets pro-arythmogènes. Par exemple, une fibrillation atriale peut être induite par une injection intraveineuse d'adénosine. La prise en charge thérapeutique de la FA a considérablement évoluée récemment, en particulier avec l'apparition d'un traitement interventionnel: l'ablation de la fibrillation atriale. Certains opérateurs réalisent des injections d'adénosine per-procédure afin de confirmer l'efficacité de la déconnection des veines pulmonaires. Si d'énormes avancées ont vu le jour dernièrement concernant le traitement de cette arythmie, sa physiopathologie reste en partie incomprise. Dans le domaine du post-opératoire de chirurgie cardiaque, la présence de fibrillation atriale est un facteur de morbidité. Dans un travail initié par nos collègues anesthésistes réanimateurs, nous avons voulu savoir si l'adénosine endogène pouvait-être impliquée dans l'apparition de FA en post-opératoire de chirurgie cardiaque. Pour ce faire, ont été réalisé des dosages d'adénosine endogène en pré-opératoire, et évalué la présence ou non de FA en post-opératoire. Ont également été mesurées les concentrations en albumine modifiée par l'ischémie (IMA) qui est un marqueur sensible d'hypoxiemie/ischémie tissulaire au cours de la chirurgie cardiaque (Kerbaul F et al 2007). En effet, l'hypothèse testée était que l'hypoxémie tissulaire peropératoire pouvait favoriser la survenue de FA via la libération d'adénosine. Ma participation dans ce travail a été, en tant que rythmologue, de définir précisément les critères d'inclusion (vérifier l'existence d'une FA), de s'assurer de l'absence de FA dans l'histoire clinique des patients, précédent l'intervention.

Ce travail est détaillé dans l’article : **“High endogenous adenosine plasma concentration is associated with atrial fibrillation during cardiac surgery with cardiopulmonary bypass”** ci dessous.

Publication 6

“High endogenous adenosine plasma concentration is associated with atrial fibrillation during cardiac surgery with cardiopulmonary bypass”

Nee L, **Franceschi F**, Resseguier N, Gravier G, Giorgi R, Gariboldi V, Collart F, Michelet P, Deharo J-C, Guieu R, Kerbaul F

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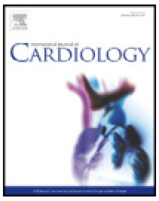

Letter to the editor

High endogenous adenosine plasma concentration is associated with atrial fibrillation during cardiac surgery with cardiopulmonary bypass

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Post-operative atrial fibrillation (AF) is a common complication after cardiac surgery [1], which impacts short and long term mortality [2]. Hypoxia is known to increase AF risk [3]. Adenosine may be implicated in AF [4]. It is a ubiquitous nucleoside that acts on the cardio-vascular system via four membrane receptors namely A_1, A_{2A}, A_{2B}, and A_3 subtypes. Adenosine is a sensitive marker of hypoxia which is strongly released during cardiopulmonary bypass (CBP) [5].

The aim of this clinical prospective study was to measure adenosine plasma concentration (APC) during cardiac surgery with CBP and to determine whether high APC is linked to AF occurrence. We also determined ischemia-modified albumin (IMA), which is a sensitive and early marker of hypoxia and oxidative stress during cardiac surgery [5].

The study was performed in accordance with the principles of Ethical Publishing in the International Journal of Cardiology [6]. Thirty seven adult patients, in sinus rhythm, with no active inflammatory disease or pulmonary hypertension, who were undergoing valvular replacement were prospectively and consecutively included. Patients were observed 6 weeks after the operation. Controls for APC and IMA levels were healthy subjects, without treatment, from the medical staff (n = 15; 8 men and 7 women, mean age 58 ± 7 years). Anesthesia and CBP procedure have been previously described [5].

All patients underwent continuous electrocardiographic monitoring through intensive care unit exit. The time of atrial fibrillation onset was systematically identified by visual inspection of the electrocardiogram. Patients who developed transient atrial fibrillation (arrhythmia duration < 10 min) were not included. In case of atrial fibrillation, amiodarone was administered intravenously during the first 48 h. They were also monitored by pulse oximetry, end-tidal carbon dioxide capnography, systemic arterial line (mean blood pressure, MBP) and either transesophageal echocardiography or pulmonary artery catheterisation in order to assess cardiac output. PaO_2/FiO_2 was also calculated. In patients with abnormal preoperative left

Table 1
Characteristics of patients included (n = 37).

	AF group (n = 12)	Non AF group (n = 25)	p
Age ± SD (years)	74 ± 9	65 ± 13	0.024
Sex ratio (F/M)	6/12	5/20	NS
BMI (kg/m^2)	24 ± 4	25 ± 4	NS
Euroscore	6 ± 2	5 ± 2	0.059
Coronaropathy, n (%)	2 (17)	9 (36)	NS
Tobacco (n%)	3 (25)	10 (40)	NS
Diabetes n (%)	2 (17)	7 (28)	NS
Preoperative PaO_2 (mm Hg)	79 ± 10	87 ± 11	NS
Creatinine clearance (mL/min)	67 ± 32	84 ± 32	0.057
Preoperative LVEF (%)	57 ± 12	57 ± 11	NS
Preoperative Systolic PAP (mm Hg)	38 ± 16	29 ± 8	0.006
CPB duration (min)	86 ± 27	91 ± 31	NS
Aorta clamping duration (min)	60 ± 24	64 ± 24	NS
Need vasopressor drugs; n (%)	3 (25)	6 (24)	NS
Mechanic ventilation duration (h)	7 ± 7	4 ± 1	NS
ICU stay (days)	2.6 ± 1.1	2.5 ± 0.9	NS

Data are expressed as means ± standard deviation.
AF group = group of patients presenting postoperative atrial fibrillation.
Non AF group = group of patients who remained in a sinus rhythm during the postoperative period.
BMI: body mass index (kg/m^2).
LVEF: left ventricular ejection fraction.
PAP: pulmonary arterial pressure.
CPB: cardiopulmonary bypass.

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ventricular ejection fraction, (<50%), norepinephrine was administered if the postoperative mean arterial pressure was low (<50 mm Hg) despite an initial infusion of dobutamine. In patients with normal preoperative left ventricular ejection fraction, norepinephrine was added if postoperative mean arterial pressure was low (<50 mm Hg).

IMA level and adenosine plasma concentration (APC) were measured on blood samples collected from radial artery simultaneously as previously described [5], at the beginning of CBP (T0), at the end of CBP (T1), the morning following surgery at 8:00 am (T2) and 24 h later (T3). Troponin Ic (cTnI) was also measured.

Clinical characteristics of the 37 patients included are shown in Table 1.

Postoperative AF occurred in 12 (32%) patients. In 10 cases, AF occurred before postoperative day 2 and in two cases between postoperative days 2 and 3. Sinus rhythm was obtained after postoperative day 3. PaO_2 was significantly lower at TO in patients with AF (AF

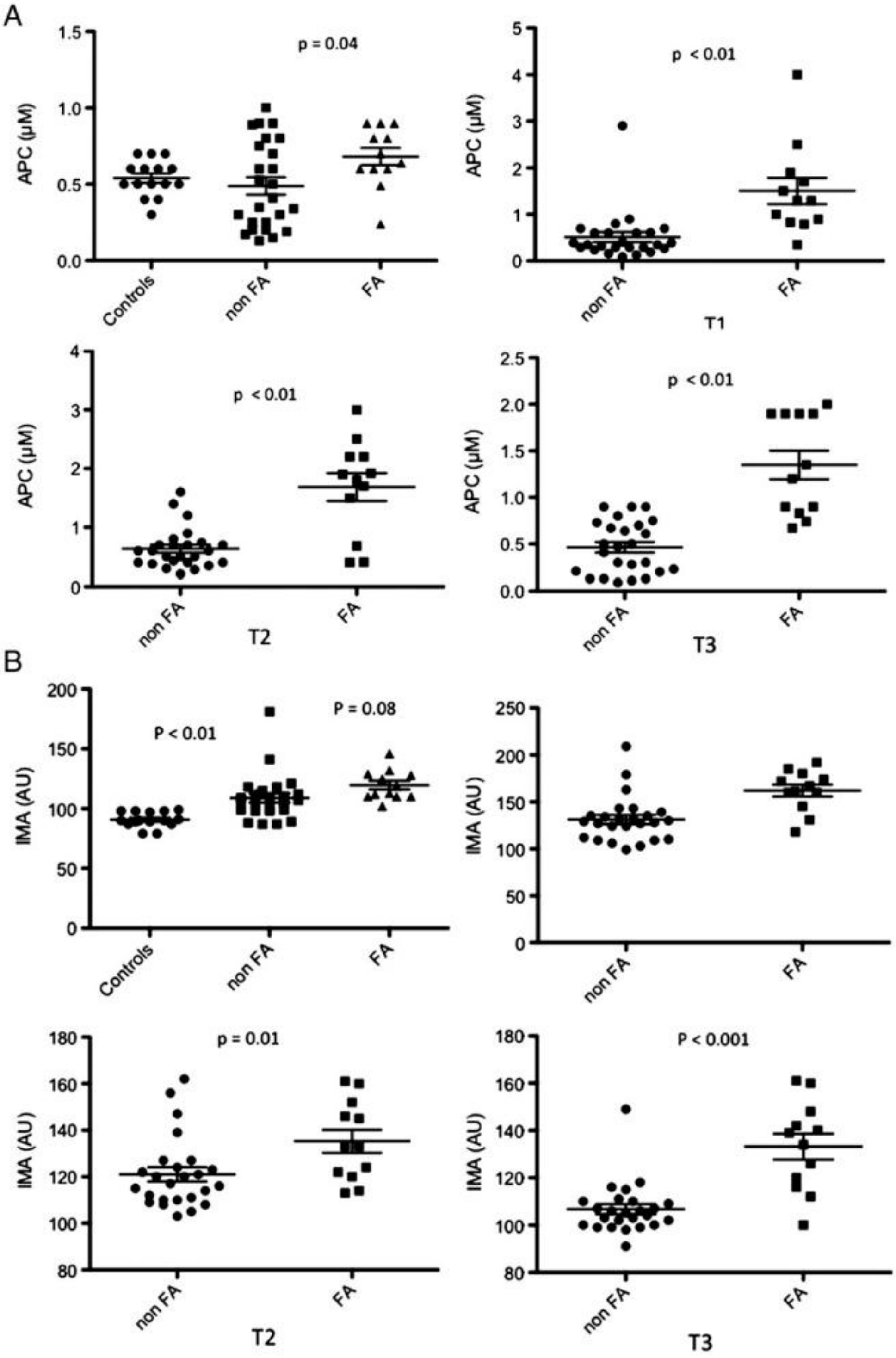

Fig. 1. Adenosine plasma concentration (APC, A) and ischaemia modified albumin (IMA, B) evaluated at baseline (TO, beginning of cardiopulmonary bypass), at T1 (end of CBP), T2 (the morning following surgery), and T3, 24 h later. AF: atrial fibrillation (n = 12); non FA patients without AF (n = 25). Data are expressed as means and SD. Mann–Whitney *U* test was used for APC or IMA levels comparisons.

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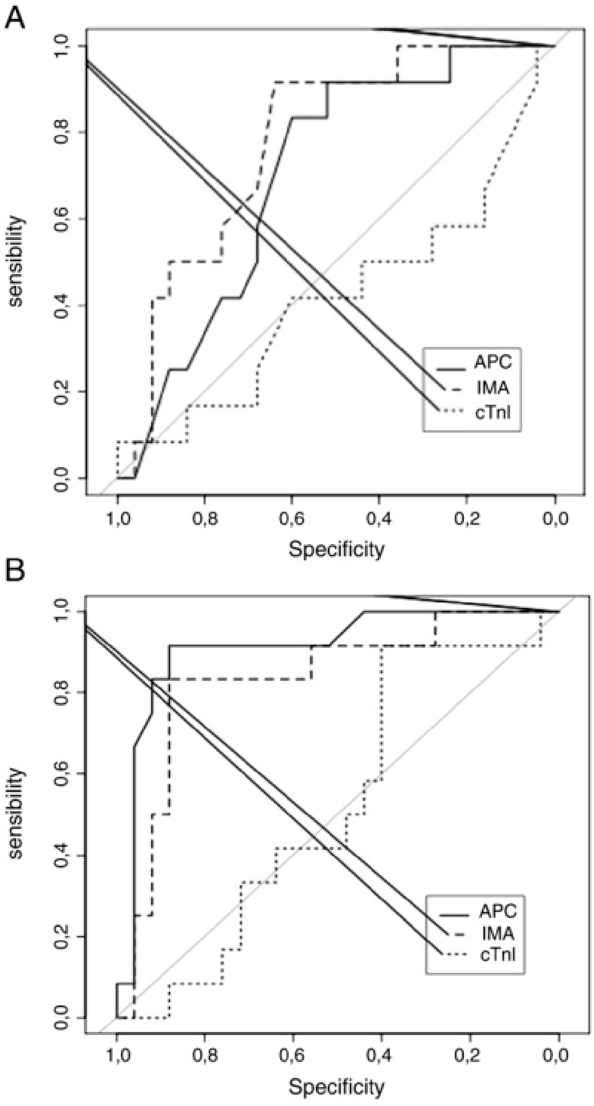

Fig. 2. Receiver operating characteristic (ROC) curves at T0 (panel A), and T1 (panel B) concerning adenosine plasma level (APC), ischaemia modified albumin (IMA), or cardiac troponin (cTnI). For each of them, the areas under the curve (AUC) were computed, with the 95% confidence interval (CI). Logistic regression, systematically adjusted for age, left ventricular ejection fraction, and PaO_2 was used to quantify the predictive value of APC, IMA, and cTnI. When the areas under the curves were compared, we used a Bonferroni correction to ensure an overall type I error rate of 5% and, therefore, an adjusted $P<0.05/3=0.016$ was considered significant in these cases. Analyses were performed with SPSS for Windows release 13.5 (SPSS Inc., Chicago, IL) and with the R free software, release 2.6.1, using ucR package.

vs. non AF patients: 794 vs. 87 ± 5 mm Hg; $p=0.04$). At the other times, PaO_2 did not differ significantly between groups. At baseline (T0), APC and IMA were significantly higher in patients with AF than in the non AF group or in controls (Fig. 1). At T1, APC and IMA increased significantly in patients with AF and remained high at T2 and T3 when compared with T0 or with the non AF group (Fig. 1). No difference was found in cTnI concentrations between groups. ROC curve analysis at T0 and T1 showed that APC and IMA were both predictive of AF occurrence (Fig. 2). After adjustment for age, left ventricular ejection fraction, and PaO_2, relative variation of APC between T1 and T0 was the best predictive parameter of AF occurrence (adjusted odd ratio: 3.72, 95% CI: 1.38–14.76; $p=0.02$).

The main finding of this clinical study is that high APC and high IMA levels are predictive factors of postoperative AF after cardiac surgery with CBP. The source of adenosine release is mostly due to hypoxia or oxidative stress, which occurs during cardiac surgery with CBP [5]. We observed that PaO_2 was significantly lower at baseline in patients with AF than in those without 2AF. IMA is also released during ischemia or hypoxia in response to free radical release or in response to decreased blood flow which can induce a relative ischemic process in most tissues [7]. Whereas IMA is only a marker of oxidative stress which occurs during hypoxia or ischemia, adenosine acts on the cardiovascular system, and its release during surgery with CBP may participate in AF occurrence. AF can be induced by intravenous administration of exogenous adenosine [8]. Adenosine accelerates AF drivers and increases excitation frequency in humans with paroxysmal or persistent AF. By increasing potassium channel conductance through activation of inward rectifier potassium channels adenosine shortens action potential duration and the refractory period [4]. Theophylline administration reduces AF secondary to myocardial infarction. These effects are secondary to A_1 adenosine receptor blockade [9]. However, human AF was found associated with a high expression of A_{2A} adenosine receptors and calcium release [10]. Thus, both A_1 and A_{2A} receptors could be implicated in adenosine release-induced AF. As APC was higher at baseline in patients with postoperative AF, these patients may initially have less than adequate O_2 supply. Our results suggest that adenosine release is involved in postoperative AF and supports the hypothesis that adenosine receptor blockers could be useful to decrease the incidence and morbidity of AF during cardiac surgery.

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Conclusion

Les concentrations en IMA ainsi que les concentrations en adénosine plasmatique de base étaient plus élevées chez les patients qui ont présenté une FA en post-opératoire. Si l'IMA n'est qu'un marqueur des phénomènes hypoxiques et du stress oxydatif, l'adénosine, au contraire, peut participer au déclenchement d'épisodes de FA. Il serait intéressant d'évaluer l'efficacité d'un antagoniste de l'adénosine sur l'incidence de la FA en post-opératoire de chirurgie cardiaque.

B. *L'adénosine dans les phénomènes d'hypoxie tissulaire*

1. Origine de la sécrétion d'adénosine chez l'insuffisant cardiaque

Introduction

L'adénosine est libérée au cours de phénomènes de stress oxydatif et lors d'une hypoxie tissulaire. Elle peut avoir un effet bénéfique sur le myocarde défaillant. Il a été démontré que les concentrations en adénosine plasmatique étaient élevées chez l'insuffisant cardiaque (Funaya H et al 1997). Cependant, la source de libération d'adénosine n'était pas établie : est ce le myocarde en souffrance lui même qui est la source de libération d'adénosine ou bien la libération d'adénosine est elle secondaire à une hypoxémie tissulaire périphérique par chute du débit cardiaque ? Pour répondre à cette question, nous avons réalisé des prélèvements sanguins au niveau d'une veine brachiale ainsi qu'au niveau du sinus coronaire de manière simultanée au cours de procédures de resynchronisation cardiaque. Il s'agit de procédures de stimulation cardiaque qui s'adressent aux insuffisants cardiaques sévères (FEVG inférieure à 35%) et symptomatiques. Techniquement, il est nécessaire de mettre en place une sonde de stimulation cardiaque dans une branche du sinus coronaire, à l'aide d'une gaine. Il est alors possible de réaliser un prélèvement de sang veineux provenant du sinus coronaire par l'intermédiaire de cette gaine. Nous avons également mesuré les concentrations périphériques en IMA, pour déterminer si la baisse du débit cardiaque pouvait influencer la production de celle ci, l'IMA étant comme nous l'avons vu précédemment un marqueur sensible du défaut d'apport en oxygène aux tissus.

Ce travail est détaillé dans l'article : **"Peripheral plasma adenosine release in patients with chronic heart failure"** ci dessous.

Publication 7

“Peripheral plasma adenosine release in patients with chronic heart failure”

Franceschi F, Deharo JC, Giorgi R, By Y, Montserrat C, Condo J, Ibrahim Z, Saadjian A and Guieu R

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Peripheral plasma adenosine release in patients with chronic heart failure

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ABSTRACT

Objective: Chronic heart failure (CHF) is accompanied by increased adenosine plasma levels (APLs). It is unknown whether adenosine release occurs at the peripheral level or whether the myocardium itself is the source of adenosine release. To answer this question, we evaluated APLs in the coronary sinus of CHF patients during a resynchronisation procedure and compared the values with those at the peripheral level. We also investigated a possible correlation between APLs and ischaemia-modified albumin (IMA) levels, a useful marker of tissue ischaemia.

Methods: 19 men and seven women were prospectively included. Blood samples for APLs were collected simultaneously from a brachial vein (peripheral) and from the coronary sinus. Blood samples for brain natriutretic peptide (BNP) and IMA were collected from a brachial vein.

Results: APLs from the brachial vein were higher than those from the coronary sinus (1.69 vs 0.75 μM $p<0.01$). IMA levels were correlated with APLs from the brachial vein ($r = 0.59$, $p<0.01$). BNP concentrations were correlated with APLs from the brachial vein ($r = 0.73$, $p<0.001$) but not with APLs from the coronary sinus ($r = 0.38$, $p>0.05$). BNP concentrations and IMA levels were correlated ($r = 0.71$, $p<0.001$).

Conclusions: In CHF patients, adenosine release occurs at a peripheral level and not at the myocardium level.

Chronic heart failure (CHF) results in the release of many neurohumoral factors, including catecholamines, renin-angiotensin and cytokines. Most of them worsen cardiac performances,[1–3] but some, like adenosine, could have primary beneficial effects on myocardial function.[4] Adenosine is a ubiquitous nucleoside that comes partly from ATP dephosphorylation and partly from cysteine metabolism.[5] Adenosine is released in the case of even minimal oxidative stress[6 7] or during ischaemia.[6 8–10]

Increased adenosine plasma levels (APLs) have been reported in CHF patients, and it has been suggested that this increase is secondary to an ischaemic process.[11 12] It is not known, however, whether the release of adenosine occurs at the peripheral level or comes from the impaired myocardium itself. To answer this question, we compared APLs measured simultaneously in the coronary sinus and at the peripheral levels of CHF patients. Furthermore, ischaemia-modified albumin (IMA) and adenosine are early and sensitive markers of ischaemia,[8 13 14] so we also looked for a relation between peripheral and coronary sinus APLs and between IMA level and APLs at the peripheral level. Indeed, establishing such a relation would support the assumption that increased APLs are related to an ischaemic process.

METHODS

Subjects and study design

Twenty-six patients with severe congestive heart failure and undergoing cardiac resynchronisation therapy (CRT) (19 men and seven women, mean age 69 (9) years; range 49–85) were included prospectively during a 6-month period and were classified according to the New York Heart Association (NYHA) functional classification. Ischaemic cardiomyopathy was defined as left ventricular systolic dysfunction associated with at least 70% narrowing of at least one of the three major coronary arteries or a documented history of myocardial infarction. Dilated cardiomyopathy was defined as left ventricular systolic dysfunction without coronary stenosis. Indications for CRT and study inclusion criteria according to the European Society of Cardiology guidelines, were (1) patients with severe heart failure (NYHA class III-IV) remaining symptomatic despite a complete pharmacological treatment, (2) low left ventricular ejection fraction ⩽35% (echocardiography or ventriculography), and (3) left bundle branch block >120 ms[15]. Every patient remained in NYHA class III or IV, and no intravenous inotropic drugs were required. Heart failure was also assessed by measuring brain natriuretic peptide (BNP). Every patient had a coronary angiography in the 6 months preceding the CRT. We excluded patients in NYHA class I or II, patients with a recent acute coronary syndrome (<3 months) or a valvular stenosis, patients who had sepsis or infectious disease in the last 3 months and patients with an evolutive neoplasia. None of the patients was receiving a xanthine oxidase derivative.

Control subjects were 14 age-matched healthy staff members of the biochemistry laboratory and the cardiology department (six men and eight women, mean age 54 (6) years, range 47–65). None of the control subjects were taking medication, whether statins, angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers. Patients and controls were instructed to avoid drinking coffee or tea 72 hours before the study. The investigation conformed with the principles outlined in the Declaration of Helsinki. All patients and control subjects gave written informed consent to participate in the study. The protocol was approved by the ethics committee for human research of the Centre Hospitalo-Universitaire de Marseille, Université de la Méditerranée.

Sample collection for APL measurement

Samples were collected during cardiac resynchronisation procedures. After local anaesthesia

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(xylocaine 1%), a subclavian puncture was performed and an 8 F sheath (CPS Direct SL, St Jude Medical) was advanced. Then the coronary sinus was cannulated. Adenosine sample collection and treatment have been described.[9 16] For the coronary sinus samples, the lumen of the sheaths was washed out and filled with 1 ml of papaverine and 1 ml of dipyridamole, injected through the lateral entry of a three-way stopcock just before blood sampling. Blood (3 ml each) was taken through the axial entry of the stopcock using an ice-cold syringe containing 3 ml of the cold stop solution (dipyridamole 0.2 mM; Na2 EDTA 4.2 mM; (9-erythro-3 nonyl) adenine (EHNA) 5 mM; α-β-methylene adenosine 5′ diphosphates (AOPCP) 79 mM; heparin sulfate 1 UI/ml; Na Cl 0.9%) to prevent both adenosine uptake by red blood cells, deamination into inosine and ATP dephosphorylation by 5′nucleotidases.[16 17] For patients and healthy subjects, peripheral blood samples (3 ml) were collected in 3 ml of cold stop solution as previously described.[18 19] For patients, blood samples were collected simultaneously in the coronary sinus and in a brachial vein.

Sample collection for c-TnI, IMA and BNP measurement

Before sample collection for APL determination, 2 ml of whole blood was collected from a brachial vein in patients and healthy subjects.

Adenosine assay

Adenosine assay has been described.[18 20] In brief, samples were immediately centrifuged (4°C, 1500 *g*), haematocrits and proteins were evaluated, and then samples were deproteinised (perchloric acid, 70%) and lyophilised before being analysed by chromatography. A modular system with a diode array detector (Hewlett Packard, HP 1100, Palo Alto, CA, USA) was used. Lyophilised samples were dissolved in 1 ml of phosphate buffer and eluted with a methanol gradient (0–35% in 60 minutes) on a Merck LiChrospher C18 column (Nottingham, UK). Adenosine was identified by its elution time and by spectrum and quantified by comparison of peak areas with those given by known quantities of adenosine. The sensitivity threshold was 2 pMol/ml of plasma matrix. The intra-assay and inter-assay coefficients of variation ranged from 1% to 3%. Absence of xanthine derivatives was checked on each chromatogram.

Table 1 Clinical and biological characteristics of the study patients and controls

	Patients (n = 26)	Controls (n = 14)
Age (years, mean (SD))	69.0 (9.0)	54.0 (6.0)
Sex (men/women)	19/7	6/8
NYHA class (n)		
III	25	NA
IV	1	NA
Left ventricular ejection fraction (n)		
<0.20	5	0
0.20–0.24	14	0
0.25–0.30	7	0
Diabetes (n)	7	0
Chronic obstructive pulmonary disease (n)	5	0
Systemic hypertension (n)	6	0
Current smoker (n)	4	6
Atrial fibrillation (n)	4	0
Current treatment (n)		
Digitalis	2	0
Amiodarone	11	0
Aldosterone receptor antagonists	4	0
β-blockers	15	0
Diuretics	26	0
ACE inhibitors	19	0
Angiotensin II receptor blockers	6	0
Creatinine plasma levels (μM, mean (SD))	120.7 (48.0)	71.0 (12.0)
Urea plasma levels, (mg/l, mean (SD))	9.3 (4.5)	4.5 (2.7)
Coronary sinus APLs (μM, mean (SD))	0.75 (0.31)	NA
Brachial vein APLs (μM, mean (SD))	1.69 (0.68)	0.65 (0.19)
Brachial vein IMA (arbitrary units)	108.7 (15.0)	87.9 (10.6)
BNP (ng/ml, mean (SD))	506.8 (277.0)	

*$p<0.05$ compared with controls; **$p<0.05$ compared with values obtained in the coronary sinus.

ACE, angiotensin-converting enzyme; APLs, adenosine plasma levels; BNP, brain natriuretic peptide; IMA, ischaemia-modified albumin; NA, not applicable; NYHA, New York Heart Association.

Ischaemia-modified albumin measurement

IMA was evaluated as previously described.[21] In brief, the albumin cobalt binding (ACB) test is a quantitative in vitro diagnostic test that detects IMA by measuring the cobalt binding capacity of albumin in human serum (Ischaemia Technologies, Denver, CO, USA). IMA level (arbitrary units) was determined with a Synchron LX 20 analyser (Beckman Coulter, Villepinte, France). The coefficient of variation was <5%.

BNP measurement

Samples were collected on sodium EDTA. BNP assay was performed on the ADVIA Centaur analyser (Siemens Medical Solutions Diagnostics SAS, 92815, Puteaux cedex France). The ADVIA Centaur BNP assay is a fully automated two-site sandwich immunoassay using direct chemiluminescent technology, with two monoclonal antibodies. This assay measures the physiologically active BNP molecule (77–108), and antibodies are specific to the C-terminal portion. The assay range is from 2–50 ng/ml. The coefficient of variation (CV) at 2 pg/ml ranges from 10–20% according to the manufacturer's data.

Cardiac troponin I assay

Samples were collected on lithium heparin. Cardiac troponin I (c-TnI) was measured by immunoassay using the ADVIA Centaur analyser, according to the manufacturer's recommendations. This assay is three-site sandwich immunoassay using direct chemiluminometric technology. The manufacturer recommends an assay range of 0.06–50 μg/l. At 0.03 μg/l, the coefficient of variation was <10%.

Reagents

Adenosine (crystallised, 99%) and dipyridamole were supplied by Boehringer Mannheim; AOPCP, deoxycofrormycin and EDTA were supplied by Sigma Aldrich; EHNA was supplied by Burroughs Welcome; heparin sulfate was supplied by Sanofi Aventis (Paris, France); methanol and other reagents were supplied by Merck, France.

Statistical analysis

Patients' data were expressed as mean (SD). Correlations between biological parameters were quantified and tested using Spearman rank correlation coefficient. Comparisons of biological parameters between patients and controls were performed using non-parametric Mann-Whitney U test (for independent variables). the non-parametric Wilcoxon paired test was used to compare biological values obtained in the coronary sinus with those obtained at the peripheral level. All statistical tests were two-sided and p values less than 0.05 were considered statistically significant. Analysis was performed with SPSS software.

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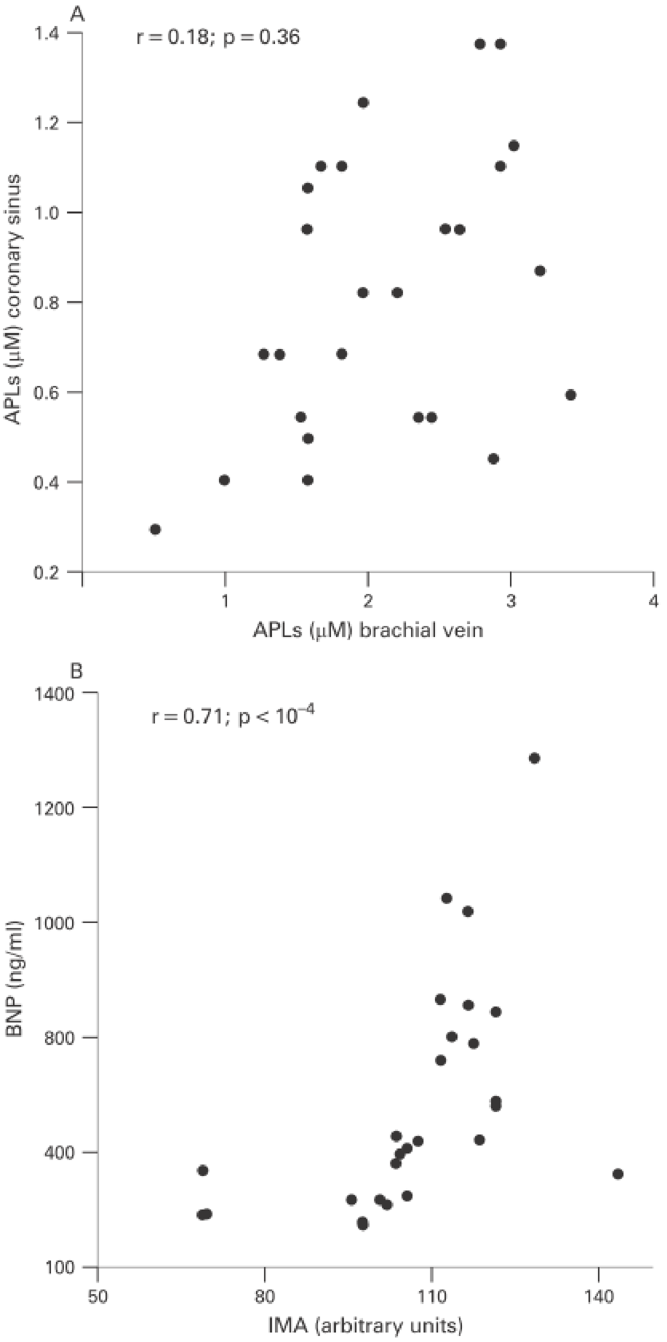

Figure 1 Correlation curves between adenosine plasma levels (APLs) (A); samples were collected simultaneously in the brachial vein and in the coronary sinus, in 26 patients with chronic heart failure. In (B), correlation curve between ischaemia-modified albumin (IMA) concentrations and brain natriuretic peptide (BNP) concentrations evaluated in samples collected from a brachial vein. r, Spearman's correlation coefficient.

RESULTS

Among patients, 25 were NYHA class III and one was NYHA class IV (see table 1). Systolic blood pressure (mean (SD)) was 131 (4) mm Hg and diastolic blood pressure was 78 (4) mm Hg. Heart rate was 72 (8) mm Hg. Ejection fraction was 25.38% (3.4%).

BNP concentrations

BNP concentration was higher than 200 ng/ml in every patient (see table 1). No significant difference in BNP concentration was found between patients with ischaemic heart disease and those with non-ischaemic heart disease (442 (228) vs 562 (311) ng/ml, $p = 0.41$).

c-TnI concentrations

Mean c-TnI concentrations remained low (see table 1). No significant difference was found between patients with ischaemic cardiomyopathy and those with dilated cardiomyopathy (0.09 (0.09) vs 0.08 (0.06), $p = 0.32$).

IMA concentrations

IMA level was significantly higher in patients than in controls (mean + 24%, $p<0.01$). No significant difference was found in IMA concentration between patients with ischaemic cardiomyopathy and those with dilated cardiomyopathy (100 (15) vs 116 (12.6); $p = 0.14$).

APLs

APLs were higher (mean 2.25-fold) in the brachial vein than in the coronary sinus (table 1). No significant difference was found in APLs between the patients with ischaemic cardiomyopathy and those with dilated cardiomyopathy (coronary sinus: 0.73 (0.35) vs 0.77 (0.30; $p = 0.59$; brachial vein: 1.41 (0.47) vs 1.92 (0.77); $p = 0.94$). On the other hand, APLs evaluated in the coronary sinus were not different from those measured in the brachial vein of healthy subjects (table 2).

Correlation data

No correlation was found between APLs in the coronary sinus and in the brachial vein (Spearman's $r = 0.18$, $p = 0.36$). In contrast, APLs in the brachial vein were correlated with both IMA and BNP concentrations (fig 1). APLs in the coronary sinus were not correlated with IMA or BNP concentrations (see fig 2). A correlation was observed between IMA and BNP concentrations, but no correlation was found between c-TnI and IMA or between c-TnI and APLs.

DISCUSSION

The present study shows that in patients with severe CHF, APLs are significantly higher at the peripheral level (brachial vein) than in the coronary sinus. This finding suggests that adenosine release in these conditions occurs mainly at the peripheral level.

Cause of high levels of adenosine and IMA

Adenosine release is likely to occur during an ischaemic process,[8 22] which can also result in an increased IMA level.[13 14] The increases observed at the peripheral level in APLs and in IMA, as well as the correlation between these two variables, support the hypothesis that the adenosine release was secondary to a peripheral ischaemic process. In a few cases, APLs were increased and IMA levels were normal. These data suggest that APLs could be a more sensitive marker of ischaemia than IMA.

At the myocardial level, most of the adenosine comes from ATP dephosphorylation and it has been established that myocardial ATP remains normal even at advanced degrees of heart failure.[23–25] Furthermore, low myocardial adenosine level was found in an animal model of cardiac failure.[26] These observations and experimental findings are consistent with our finding that the myocardium is not the source of the high APLs in CHF patients.

Compared to controls, patients with severe CHF had high IMA levels. IMA results from the modification of the N-terminus cobalt-binding sites of albumin caused by the release

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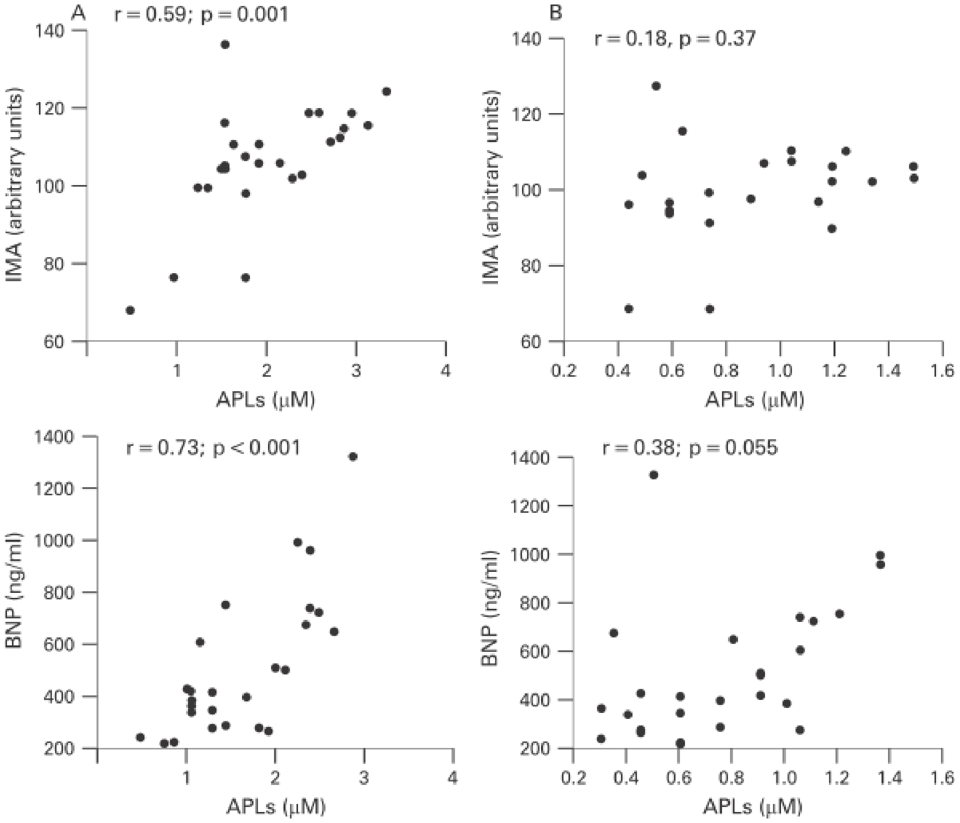

Figure 2 Correlation data between adenosine plasma levels (APLs) and ischaemia-modified albumin (IMA) or brain natriuretic peptide (BNP). Blood samples were collected from a brachial vein (A) and the coronary sinus (B). r, Spearman's correlation coefficient.

of free radicals from ischaemic tissue. The IMA concentration increases only a few minutes after the beginning of even a minimal ischaemic process, and its level is correlated with the size of the ischaemic tissue.[13] Thus, IMA can be a potential marker of transient myocardial[27 28] or non-myocardial ischaemia.[29] The present study strongly indicates that an ischaemic process occurs outside the myocardium, at a peripheral level. In CHF patients the decrease in blood flow can induce a relative ischaemic process in most tissues, which is responsible for the release of adenosine and the increase in IMA concentration. Indeed, adenosine and IMA are very sensitive markers of tissue ischaemia. The increase in BNP level is associated with the severity of CHF[30] and its concentration is inversely correlated with the left ventricular ejection fraction.[31] That IMA was correlated with BNP concentrations supports the hypothesis that a peripheral ischaemic process secondary to a decreased blood flow occurs in severe CHF.

Adenosine release can also happen at the renal level through the 5′ nucleotidase activation and dephosphorylation of AMP,[32 33] especially during an ischaemic process.[34] APLs can also be increased in patients with chronic renal failure, but this increase has been observed only in patients with end-stage renal failure under haemodialysis treatment.[35 36]

Nine patients among 26 had a weak increase in c-TnI level (between 0.1 ng/ml and 0.39 ng/ml). This could be considered in such patients without acute coronary syndrome as an independent prognostic marker of in-hospital mortality.[37] Also, CHF patients with a weak elevation in cardiac troponin T (c-TnT) level (>0.012 ng/ml) have more severe CHF and worse outcome.[38] In the present study, however, the number of patients is too small to allow any conclusion on the prognostic significance of c-TnI levels.

Possible effects of adenosine release in CHF patients

It was suggested that dipyridamole, which increases APLs,[39] improves ventricular systolic function in patients with CHF.[40 41] Extracellular adenosine has cardiac actions, mediated through the activation of adenosine receptor subtypes, called A_1, A_{2A}, A_{2B} and A_3 depending on the pharmacological properties of receptor subtypes.[42 43] It is well known that adenosine protects the heart during ischaemic reperfusion via the activation of A_1 and A_3 receptors.[44 45] However, the effects of activation of A_1 adenosine receptors on left ventricular function are debated. In patients with left ventricular dysfunction, the activation of A_1 adenosine receptors had no significant effects on haemodynamic changes.[46] A more recent experimental study indicates that activation of these receptors may attenuate cardiac hypertrophy in murine left ventricular pressure-overload model.[47] However, A_1 adenosine receptor blockade could have beneficial effects on CHF patients by improving renal function and diuresis.[48]

Adenosine increases coronary blood flow via the activation of A_{2A} and A_{2B} receptors.[49 50] Activation of A_{2A} receptors improves contractile performances of the myocardium[51–53] and inhibits cytokine production and inflammatory response.[54] Cytokine production and inflammatory response, however, are involved in cardiac depression.[55] Finally, endogenous adenosine also reduces myocardial oxygen consumption in the failing heart[48] and decreases oxygen radical release from activated neutrophils, thus preventing endothelial cell damage.[10]

Study limitations

Although the present study included enough patients to reach statistical significance, additional studies including a larger number of patients are needed. Indeed, only patients with

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severe CHF have been included. It would be of interest to include patients with less severe CHF in further studies.

CONCLUSION

High APLs in CHF patients do not result from a myocardial release but are secondary to a peripheral release. This release is probably caused by an ischaemic process, probably because of a decreased blood flow secondary to left ventricular failure.

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Competing interests: None.

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Conclusion

Chez l’insuffisant cardiaque sévère, les concentrations en adénosine plasmatique sont plus élevées en périphérie qu’au niveau cardiaque. L’origine de la libération d’adénosine est donc périphérique, faisant suite à une hypoxie tissulaire et à des phénomènes de stress oxydatif. Par ailleurs, les concentrations en adénosine plasmatiques sont corrélées aux taux d’IMA. De plus, BNP et IMA sont corrélés. Notre conclusion est donc que la chute du débit sanguin, objectivée par la baisse de la fraction d’éjection, engendre une hypoxémie tissulaire périphérique qui est la source de libération d’adénosine. Le myocarde lui même, ne libère pas d’adénosine comme en témoigne les concentrations en adénosine mesurées dans le sinus coronaire. Par ailleurs le fait qu’il existe une corrélation entre IMA et adénosine semble indiquer que c’est bien l’hypoxémie périphérique qui est en cause.

On ignore en revanche si l’adénosine est élevée chez l’insuffisant cardiaque moins sévère, ou dont la fonction systolique VG est préservée. Par ailleurs, l’adénosine n’agit-elle que comme un marqueur de la sévérité de l’hypoxie tissulaire, ou agit-elle comme un protecteur cardiaque ? Il serait intéressant de tenter de corréler les taux d’adénosine endogène au pronostic des patients.

2. Régulation adénosinergique d'une lignée de lymphocytes T humains dans des conditions d'hypoxie in-vitro

Introduction

L'hypoxie tissulaire existe in-vivo au cours de phénomène inflammatoires. Il est bien établi que l'adénosine extracellulaire libérée par l'endothélium possède des effets anti-inflammatoires, en particulier en stoppant la prolifération de lymphocytes T et la sécrétion de cytokines pro-inflammatoires par l'intermédiaire de récepteurs A_{2A}. Cette voie de régulation pourrait avoir des conséquences thérapeutiques importantes. En revanche, aucune donnée n'était disponible concernant le comportement de lymphocytes T isolés dans des conditions hypoxique par rapport à leur propre régulation adénosinergique. Nous avons soumis une lignée de lymphocytes T isolée à des conditions hypoxiques in vitro. Nous avons dans ces conditions, suivi l'évolution de la production d'HIF 1 alpha, des concentrations extracellulaires en adénosine, de l'activité de l'adénosine déaminase et de l'expression des récepteurs A_{2A}.

Ce travail est détaillé dans l'article : **"Fall in oxygen tension of culture medium stimulates the adenosinergic signalling of a human T cell line"** ci dessous.

Publication 8

“Fall in oxygen tension of culture medium stimulates the adenosinergic signalling of a human T cell line”

By Y, Jacquin L, **Franceschi F**, Durand-Gorde JM, Condo J, Michelet P, Guieu R, Ruf J

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BRIEF COMMUNICATION

Fall in oxygen tension of culture medium stimulates the adenosinergic signalling of a human T cell line

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Abstract We examined the short-course expression of various parameters involved in the adenosinergic signalling of a human T cell line during in vitro decrease of the medium culture oxygen tension mimicking in vivo hypoxia. Fall of 92 mmHg in oxygen tension of culture medium induced in CEM, a CD4+ human T cell line, a continuous production of hypoxia-inducing factor-1α with a plateau value at 9 h, a rapid increase in adenosine production peaking at 3 h and a decrease in adenosine deaminase peaking at 6 h. The adenosine A_{2A} receptor ($A_{2A}R$) protein level of CEM cells was enhanced with a peak at 6 h. Intracellular 3′,5′-cyclic adenosine monophosphate accumulated in CEM cells with a maximal level at 9 h. These results show that a human-cultured T cells line can upregulate its own adenosine production and $A_{2A}R$ expression during exposure to acute hypoxia. Hypoxia-increased stimulation of the adenosinergic signalling of T cells may have immunosuppressive properties and, consequently, $A_{2A}R$ agonists may have therapeutic relevance.

Keywords Hypoxia · HIF-1α · Adenosine · $A_{2A}R$ · T cells

Introduction

Oxygen deprivation, ranging from hypoxia to anoxia, is a physiologic stimulus which occurs transiently in vivo within inflamed sites and growing tumours [1]. According to a previously proposed model [2], inflammatory damage to blood vessels induces low oxygen supply and hypoxia-driven increase of extracellular adenosine which accumulates because of altered expression of several key enzymes involved in the adenosine triphosphate (ATP) metabolism [3]. Adenosine, an endogenous purine nucleoside that is produced intra- or extracellularly by injured tissue cells, binds to four types of receptor, namely A_1, A_{2A}, A_{2B} and A_3, which are members of the G protein-coupled family of receptors [4]. It is well established that extracellular adenosine released from endothelium cells stops T cell proliferation, expansion and secretion of pro-inflammatory cytokines by stimulation of their A_{2A} surface receptors ($A_{2A}R$) that trigger a rise in intracellular 3′,5′-cyclic adenosine monophosphate (cAMP) through activation of adenyl cyclase [5]. This pathway has potential physiopathological and therapeutic consequences to downregulate the immune response due to spontaneous accumulation of adenosine at the T cell surface [6]. At inflamed site, recruited or resident T lymphocytes, as endothelium cells, are exposed to reduced oxygen pressure but little is known about the hypoxia effect on their own adenosinergic regulation. Because adenosine can be released from a single cell in an autocrine way [7] or be self surface produced by ectoenzymes [8], we have tested here whether in vitro exposure to reduced oxygen tension (mimicking hypoxia) can upregulate per se both the adenosine production and $A_{2A}R$ expression of a cultured T cell line.

Materials and methods

Cell culture conditions

CEM, a $CD4^+$ human T lymphoma cell line endogenously expressing $A_{2A}R$ [9], were purchased from the American

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Tissue Culture Collection and grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 mM Hepes, 10% foetal calf serum and penicillin/streptomycin (100 U/ml, 100 µg/ml) in incubator at 37°C under 5% CO_2 and 95% air. When cells reached 70% confluence, they were either exposed to normal or hypoxia culture medium conditions. In normal conditions, 90 ml of culture medium containing 1×10^6 cells/ml were set into a 250-ml sterile infusion bottle under sterile laminar air flow at atmospheric pressure before closure of the cap. Hypoxia in vitro conditions was achieved by slowly flushing the 90-ml culture medium with 0.22 µm-filtered N_2 for 60 min into a 250-ml sterile infusion bottle with a needle inserted through a butyl rubber stopper transiently equipped with an air hole to expulse O_2. Flushed culture medium was then allowed to equilibrate at positive N_2 pressure in the capped bottle overnight at 37°C before cell exposure. Samples of cells cultured in normal and hypoxia conditions were harvested at the onset and after 3, 6, 9 and 12-h incubation at 37°C by the syringe puncture through the cap. The samples were immediately used for cell counting, pO_2, pH and lactate measurements. For cell parameters, the samples were distributed into tubes and centrifuged at 4°C ($1{,}500 \times g$, 15 min) to discard supernatant. Cell pellets were kept frozen at −80°C until use.

Viable cell counting

At 3-h time intervals, the CEM cells in culture bottle were suspended and withdrawn by a syringe for cell counting. Viable cells count was performed using the trypan blue dye exclusion method and a Malassez haemocytometer.

pO_2, pH and lactate measurements

Two millilitres of culture medium from bottles in normal and hypoxia conditions was taken into a dedicated syringe to monitor pH, pO_2 and lactate levels using a RAPIDSystem blood gas analyser (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA).

Adenosine assay

The technique used was previously described [10] with minor modifications. Intracellular adenosine concentration was measured using high-performance liquid chromatography equipped with a diode array detector (Chromsystems, Munich, Germany). Frozen cells (5×10^6) were mixed with 1 ml of cold stop solution (0.2 mM dipyridamole, 4.2 mM Na_2EDTA, 5 mM (9-erythro-3 nonyl) adenine, 79 mM α-β methylene adenosine 5′-diphosphates and 1 IU/ml heparin sulphate in NaCl 0.9%) to prevent adenosine degradation. Aliquot of 500 µl was injected into a 1-ml loop and eluted with a methanol gradient (0–30% *v*/*v*) in ultrapure water containing 0.1% trifluoroacetic acid for 37 min on a LiChrospher C18 column (Merck, Darmstadt, Germany). Adenosine was identified by its elution time and spectrum. Measurement was made by comparison of peak areas with those given by standards. The intra-assay and inter-assay coefficients of variation ranged from 1% to 3%.

ADA activity

Adenosine deaminase (ADA) activity measurement was carried out as previously described [11]. Briefly, 1×10^6 frozen cells were lysed in 500 µl of ultrapure water. One hundred twenty-five microlitres of lysate was mixed with 750 µl of 28 mM adenosine and 125 µl of 7% BSA in 0.9% NaCl and incubated for 36 min at 37°C. The reaction was stopped by cold immersion at 4°C, and the quantity of ammonium formed was determined with a Synchron LX 20 analyser (Beckman Coulter Inc, Villepinte, France). The intra-assay and inter-assay coefficients of variation ranged from 3% to 5%.

cAMP assay

Intracellular cAMP level was measured on 1×10^6 frozen cells with the Amersham Biotrak kit (GE Healthcare Life Sciences, Buckinghamshire, UK) by a competitive enzyme immunoassay according to the manufacturer's instructions.

HIF-1α and $A_{2A}R$ measurements

Hypoxia-inducing factor-1α (HIF-1α) production and $A_{2A}R$ expression were assessed by quantitative Western blotting procedure as previously described [9]. Briefly, frozen cell pellets were solubilised with 4% SDS aqueous solution by 30-min sonication at 47 kHz. After protein quantification by microBCA (Pierce Biotechnology, Rockford, IL, USA), 20 µg of cell solubilisate were diluted in 65.2 mM Tris–HCl buffer, pH 8.3, containing 10% glycerol, 0.01% bromophenol blue and 5% mercaptoethanol and subjected to standard electrophoresis procedure in Mini Protean II system (Bio-Rad, Hercules, CA, USA). Separated proteins in 12% acrylamide minigel were transferred onto a PVDF membrane. Blotted membrane was placed into the blot holder of the SNAP i.d. protein detection system (Millipore, Billerica, MA, USA), saturated with non-fat dried milk and incubated 20 min with the appropriately diluted mouse monoclonal antibody, anti-$A_{2A}R$ (Adonis) [12] and anti-HIF-1α (clone 241809, R&D Systems, Minneapolis, MN, USA). Blots were visualised by horseradish peroxidase labelled anti-mouse IgG Fab-specific antibodies and enhanced chemiluminescent substrate (SuperSignal West Femto, Pierce Biotechnology, Rockford, IL, USA) using a Kodak Image Station 440CF

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(Eastman Kodak Company, Rochester, NY, USA). The staining intensities of the bands were densitometrically measured with the public domain NIH Image software developed at the US National Institutes of Health.

Results and discussion

In vitro hypoxia conditions

The non-physiological relevance of classic in vitro culture conditions was particularly emphasised for hypoxia studies that obviously can only be done in vivo like most of physiopathological experiments [13, 14]. However, in vitro cell culture is required for testing comportment of unique types of cells in special conditions deprived of influence of their natural environment. To mimic at best the in vivo process of hypoxia by using in vitro cultured T cells under 5% CO_2 and 95% air (i.e. hyperoxia conditions), the culture medium was flushed with N_2 to obtain a 92-mmHg fall in oxygen tension that shift from hyperoxia (~145 mmHg) to moderate hypoxia (~53 mmHg) in vitro conditions, and we postulated that this change was representative of a similar shift which can occur in vivo from normal oxygen tension in blood vessels with circulating lymphocytes (40–100 mmHg) to anoxia or severe hypoxia conditions for tissue lymphocytes at inflamed site (4–20 mmHg). So, in this study, we used the terms of normal and hypoxia conditions for comparing our in vitro conditions. Figure 1a shows that pO_2 remained stable throughout the 12-h experiment both in normal (145.5±5.6 mmHg) and hypoxia (53.4±9.2 mm Hg) in vitro conditions. Figure 1b shows that pH of culture medium gradually diminished over the time in both conditions, but hypoxia conditions systematically induced a 0.2±0.04 pH increase of culture medium. This rise of pH was due to the flush of N_2 that lowered the CO_2 as the O_2 in the culture medium (not shown). However, pH values always remained compatible with cell culture conditions along the 12-h incubation. Effectively, as shown in Fig. 1c, CEM cells continuously grow to reach a plateau value at 9 h, and the number of viable cells remained unchanged throughout the experiment both in normal and hypoxia in vitro conditions. Furthermore, blue dead cells were never observed during the 12-h experiment in both conditions, and longer timings were excluded to avoid cell death in hypoxia

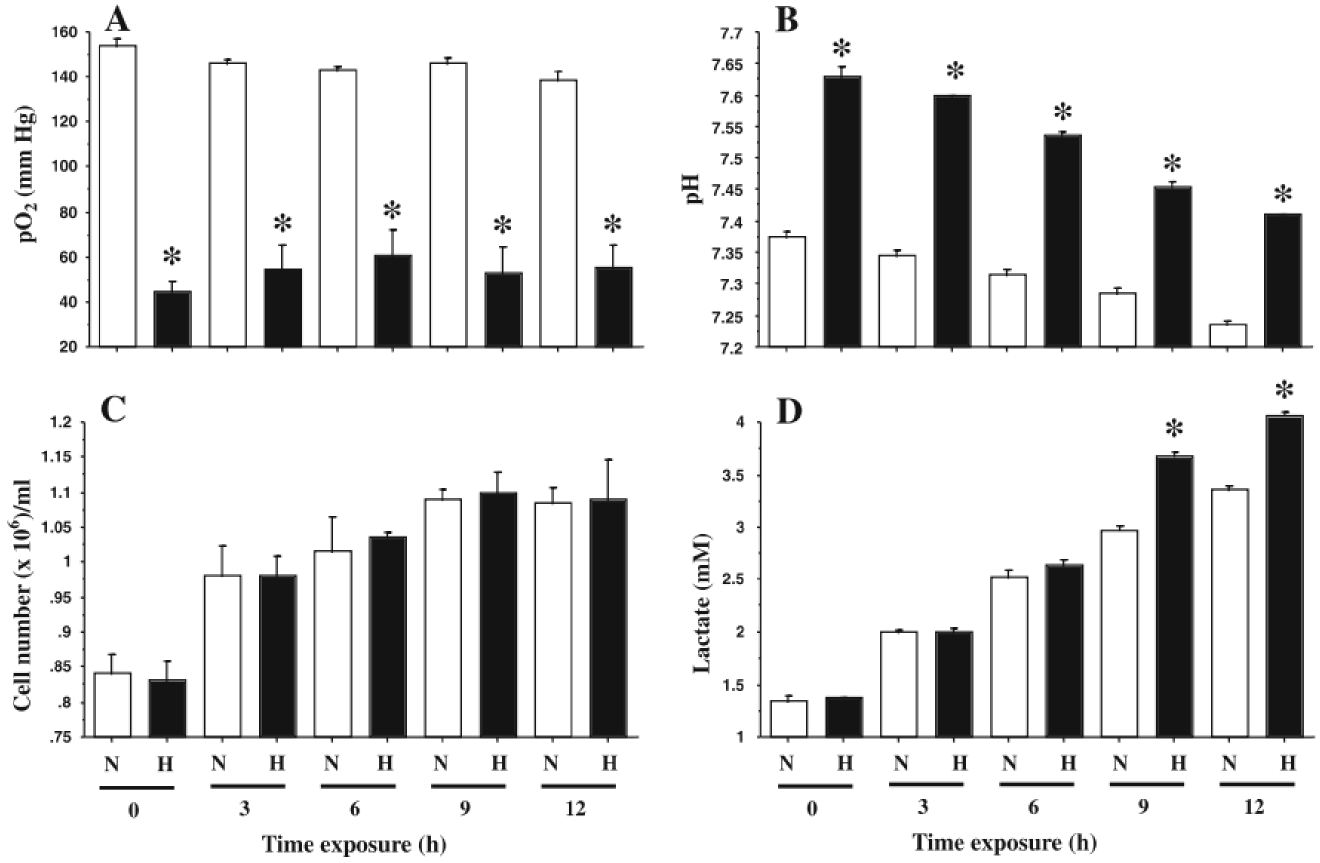

Fig. 1 Effect of acute hypoxia on CEM cell culture. CEM cells were seeded in sterile bottles under normal (*N*) and hypoxia (*H*) conditions for 12 h. Medium with cultured cells were sampled at the onset (*0*) and *3*, *6*, *9* and *12*-h incubation. Data shown are the temporal profile in the culture medium of oxygen pressure (**a**), pH (**b**), cell number (**c**) and lactate production (**d**). Results are mean ± SD of triplicates. *$p<0.05$ for hypoxia value compared to normal value by *t* test

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conditions. Under aerobic conditions, the source of cellular energy is glycolysis coupled to oxidative phosphorylation. During glycolysis, glucose is converted to pyruvate with the production of ATP. Then, pyruvate enters the tricarboxylic acid cycle to produce electrons kept by oxygen through the mitochondrial respiratory chain. This chain of electron transport reactions produces ATP through the action of ATP synthase and this is the oxidative phosphorylation [15]. Under hypoxia conditions, glucose is converted to pyruvate which does not reach mitochondria and accumulates in cytoplasm. As a result, pyruvate is reduced in lactate and released from the cell. Figure 1d shows that CEM cells used glycolysis as their main source of energy irrespective of the pO_2 in culture medium because they continuously produced lactate in normal and hypoxia conditions. However, we noticed that hypoxia increased the anaerobic glycolysis in CEM cells by significantly increasing the lactate production in the culture medium at 9 and 12 h. This is in agreement with previous observations that, after stimulation, the glucose catabolism in immune cells increased [16] and that inhibitors of glycolysis impaired the function of immune cells whereas inhibitors of oxidative phosphorylation did not [17–19]. Our results also fit nicely with a recent report that concludes to a role of hypoxia-induced adenosine via $A_{2A}R$ in high glucose and lactate plasma levels [20].

Hypoxia-adenosinergic mechanism

During hypoxia, ATP is dephosphorylated to adenosine via endo-apyrase and endo-5′-nucleotidase, and adenosine kinase salvage pathway is suppressed leading to increased intracellular adenosine levels. Extracellular ATP is directly catabolized into adenosine by ecto-apyrase (CD39) and ectonucleotidase (CD73) cell membrane enzymes. Adenosine can be degraded into inosine by ADA localised inside the cell or linked to CD26 at the cell surface. Adenosine level in and out of the cell is also dependant of equilibrative nucleoside transporters. Under hypoxia conditions, extracellular adenosine accumulates due to decreased cell uptake by transcriptional repression of transporters, and thus, adenosine stimulates the $A_{2A}R$ to produce cAMP. HIF-1α is the oxygen-regulated subunit of the transcription factor heterodimer HIF-1. Under hypoxia, HIF-1α is stabilised and can dimerize with the constitutively expressed HIF-1β to coordinate the transcription of genes involved in adenosine metabolism and transport (for a review, see [21]). Figure 2 shows the temporal profile of the production or expression of five hypoxia-related parameters: HIF-1α, adenosine, ADA, $A_{2A}R$ and cAMP levels in CEM cells. Levels of all these products changed with time in hypoxia conditions showing various temporal profiles. Figure 2a shows that HIF-1α is immediately produced upon hypoxia conditions and raised a plateau value at 9 h. Based on recent literature [22], we suggest that hypoxia allowed HIF-1α to repress the transcriptional activation of adenosine kinase. Consequently, adenosine rapidly increased, peaking as soon as 3 h (Fig. 2b), and conversely, ADA continuously decreased until 6 h and remained almost stable up to 12 h (Fig. 2c). In turn, $A_{2A}R$ expression was upregulated peaking at 6 h and rapidly returned to normal values (Fig. 2d). Finally, cAMP accumulated with a peak at 9 h (Fig. 2e). Adenosine has immunomodulatory properties on $A_{2A}R$ [23], and as expected in hypoxia conditions, we observed a rise in adenosine intracellular T cell level which was probably representative of the adenosine level released by the cells in the culture medium (not tested). The observed decrease of total (cytoplasmic and cell surface CD26 associated) ADA activity is in agreement with the enhancement of adenosine metabolism and fits very well with previous studies which revealed inhibition of ADA and adenosine kinase as a metabolic adaptation to elevated adenosine levels in rat pheochromocytoma (PC12) cells chronically exposed to hypoxia [7]. High adenosine levels with decreased ADA activity were also observed in mononuclear cells of hemodialysed patients with immune defect [11]. In contrast, hypoxia was reported to increase total and cell surface ADA protein level and enzymatic activity in cultured endothelial cells [24]. However, the ADA rise was evidenced in a 12–72-h range of hypoxia exposure but not for a lesser timing (3–12 h) as reported here. We have not extended hypoxia exposure of T cells in our method because cells started to die after 12 h in hypoxia conditions. We can only speculate that ADA first decreased and then increased in a second time (after 12 h) by feedback regulation to counteract excessive adenosine production as suggested by the virtually V-shaped curve of ADA (Fig. 2c) and the weak level of adenosine at 12-h hypoxia exposure (Fig. 2b). Alternatively, because ADA was only tested for its enzymatic activity, it was possible that ADA protein expression immediately increased (as soon as 3 h) but its enzymatic activity was transiently inhibited under hypoxia conditions by unknown regulatory mechanism. However, the observed rise both in adenosine production and $A_{2A}R$ expression may explain the increased level of intracellular cAMP was able to inhibit the effector functions of T cells [25]. These results support the view that the increase in extracellular adenosine in inflamed tissue microenvironment is due to local hypoxia [26]. They further indicate that hypoxia upregulates adenosine production and $A_{2A}R$ expression in T cells which are key partners of the immune system.

Clinical relevance and therapeutic implications

On T cells, $A_{2A}R$ are the predominant adenosine receptor subtype [25, 27]. The consensus emerging from numerous recent studies using adenosine receptor knockout mice is

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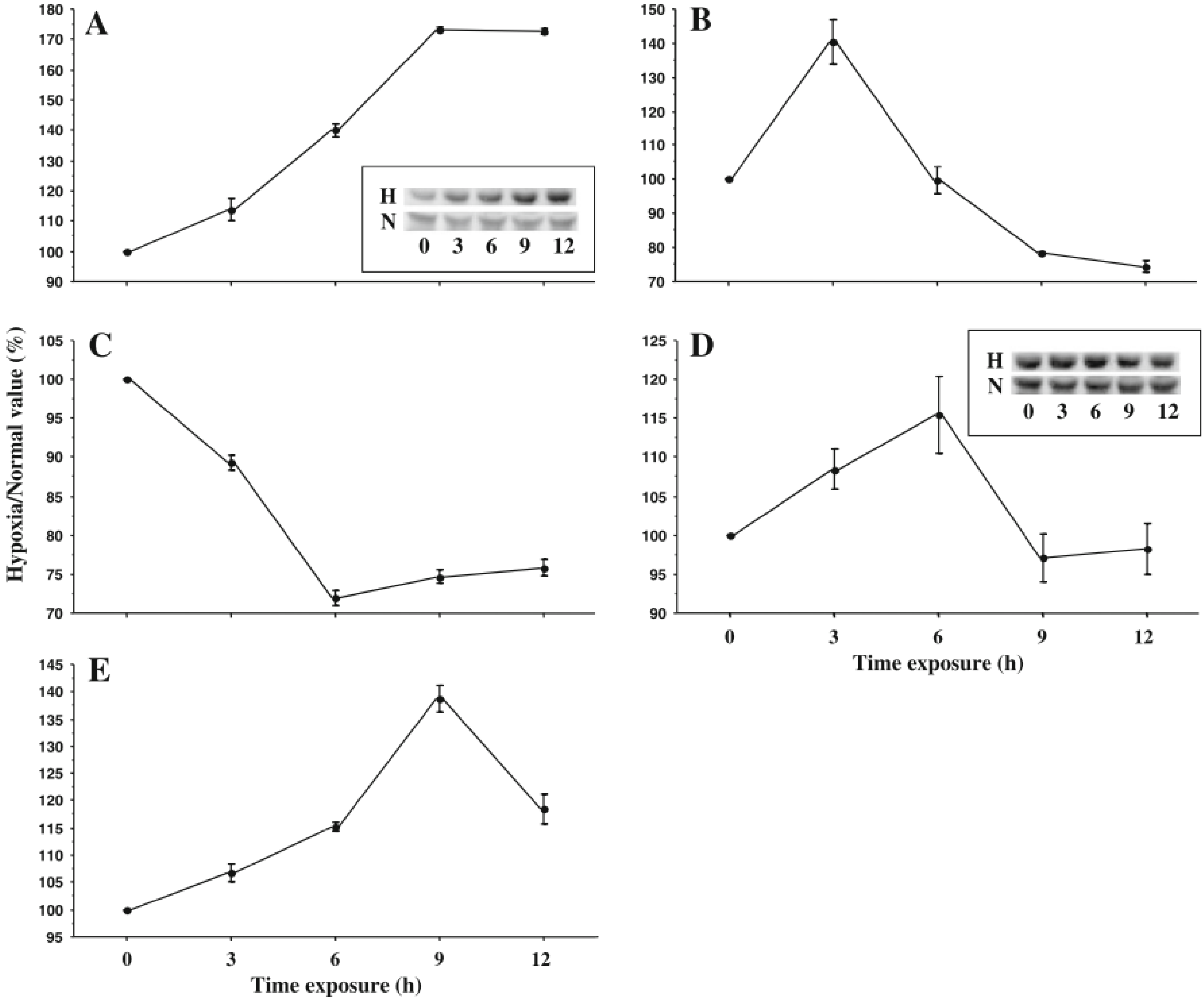

Fig. 2 Effects of acute hypoxia on five adenosinergic parameters in CEM cells. Experimental conditions were as in Fig. 1. Data shown are the temporal production/expression in CEM cells of HIF-1α (**a**), adenosine (**b**), ADA (**c**), $A_{2A}R$ (**d**) and cAMP (**e**). Results (mean ± SD of triplicates) are expressed as percentage of value in hypoxia conditions relative to value in normal conditions. Representative Western blots of triplicates for HIF-1α production and $A_{2A}R$ expression are inserted in **a** and **d**, respectively. The mean basal values±SD at the onset (time *0*) for adenosine production, ADA activity and cAMP level are 1.01±0.03 μM/5×10^6 cells, 34.7±1.2 IU/10^6 cells and 103.6±4.6 fmol/10^6 cells, respectively

that $A_{2A}R$ is the major receptor subtype in dictating lymphocyte responses [28]. $A_{2A}R$ agonists inhibit T cell activation by inducing intracellular cAMP production, and genetic deficiency or pharmacological antagonism of $A_{2A}R$ increase inflammatory tissue damage. In agreement with this reasoning, it was previously reported in experimental mouse model of induced T cell-dependent acute hepatitis that $A_{2A}R$-deficient mice are much more sensible to liver damage and exhibit high pro-inflammatory cytokines levels compared to wild-type mice [29]. In the same model, circulating adenosine increases in mice exposed to hypoxia which become resistant to liver damage [30]. Considering these data, the immunosuppressive effect of hypoxia would be of potential relevance in vivo to reduce tissue damages and loss of vital functions during inflammatory diseases. In contrast, hyperoxia appears deleterious especially for patients with pulmonary inflammation by weakening of $A_{2A}R$ signalling. Supporting this point is the feature that oxygenation of mice severely exacerbates inflammatory lung damage and even causes death in the majority of mice whereas hypoxia suppresses lung damage by the $A_{2A}R$-dependent mechanism [31]. When oxygen supplementation is required as for respiratory distressed patients, our results provide additional arguments for administration of $A_{2A}R$ agonists to restore protection by upregulating the adenosinergic immunosuppressive pathway as recently proposed

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[6]. In this regard, Adonis, the newly developed agonist-like monoclonal antibody [12] that inhibits the cell growth and upregulates the $A_{2A}R$ expression of CEM T cells [9] would be candidate as a therapeutic drug.

Conclusion

Accumulating evidences suggest that adenosine–$A_{2A}R$ pathway is involved in immune suppression via T regulatory cells [26]. T cells with regulatory phenotype (CD4+ CD25+) are controlled by hypoxia via HIF-1α [32] and produce themselves extracellular adenosine that contributes to $A_{2A}R$-mediated immunosuppression [8]. These assertions were hampered by the lack of reports on the complete sequence of hypoxia-related adenosine events on a same cell at a same time. The in vitro study reported here shows that a T cell line cultured alone without any other cells can detect a significant (92 mmHg) decrease in pO_2 of culture medium by producing HIF-1α to upregulate the expression of its own $A_{2A}R$ which are self-activated by auto-production of adenosine. We reasonably suggest that this finely tuned T cell mechanism occurs in vivo as part of immune regulation. Such a regulatory mechanism may auto-stop T cells hyperproducing adenosine under hypoxia environment in absence or deficit of $A_{2A}R$ on other surrounding cells. In contrast, in conditions of low adenosine levels relative to the $A_{2A}R$ cell surface expressed, administration of $A_{2A}R$ agonists may be required to help deficient T cells to weaken inflammatory damages.

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Conclusion

En réponse à l'hypoxie, on observe une augmentation rapide de la production d'HIF 1 alpha, d'adénosine, ainsi qu'une diminution de l'adénosine déaminase. L'expression des récepteurs A_{2A} augmente au niveau de la lignée cellulaire et il existe une accumulation d'AMPc intracellulaire. L'hypoxie aigüe expérimentale engendre une libération d'adénosine qui vient stimuler les récepteurs A_{2A} et augmenter la production d'AMPc intracellulaire. La baisse de l'activité ADA favorise le maintient de concentrations élevées d'adénosine extra cellulaire, favorisant la stimulation des récepteurs A_{2A}.

Il existe donc une activation du système adénosinergique en conditions d'hypoxie. Cette activation de la voie adénosinergique passe par l'activation de HIF-1α et peut avoir des effets positifs in vivo, en régulant le système immunitaire et en diminuant la réaction inflammatoire. Dans ce contexte, on émet l'hypothèse qu'il pourrait-être utile de traiter certains patients atteints d'inflammation pulmonaire contraints d'être soumis à l'oxygénothérapie pour détresse respiratoire avec un agoniste des récepteurs A_{2A}.

Chapitre trois

Discussion, perspectives et conclusion

A. *Discussion et perspectives*

Nos travaux et ceux de notre équipe ont permis des avancées dans la compréhension de l'implication de l'adénosine et de ses récepteurs dans le domaine de la rythmologie et de l'insuffisance cardiaque.

Dans le domaine des syncopes neurocardiogéniques.

Chez les patients présentant un test d'inclinaison positif, nos travaux ont permis de mettre en évidence plusieurs points :

- Les concentrations en endogène sont élevées et se majorent encore au cours de la syncope. Plus le taux basal est élevé, plus la syncope est rapide et la bradycardie importante.
- Il existe une surexpression des récepteurs A_{2A} au niveau des lymphocytes circulants, reflet très probable de son expression au niveau cardiovasculaire.
- La fonction de ces récepteurs est préservée. De plus, chez quelques patients, on constate la présence de récepteurs de réserve, présence dont l'incidence physiopathologique reste à déterminer.

En revanche, chez les patients présentant un autre type de syncope neurocardiogénique, avec test d'inclinaison négatif mais test à l'ATP positif, le profil est totalement différent : les concentrations en adénosine endogène et les niveaux d'expression des récepteurs A_{2A} sont normaux voire bas. Cette entité est moins bien connue, mais son profil adénosinergique permet de la distinguer des patients avec test-d'inclinaison positif. Il semble s'agir de deux populations différentes de patients, comme l'impression clinique le présageait.

L'ensemble de ces éléments est très en faveur d'une implication de l'adénosine et de ses récepteurs dans la physiopathologie des syncopes neurocardiogéniques. La raison de cette implication reste incertaine, mais pourraient être génétiquement déterminée, comme en témoigne l'étude des polymorphismes des récepteurs A_{2A}.

On peut faire l'hypothèse que dans les syncopes neurocardiogéniques avec tilt test positif la réponse vasoplégique est secondaire à l'activation des récepteurs A_{2A} de faible affinité. Les patients ayant un test à l'ATP positif ont une faible expression des récepteurs A_{2A} et des concentrations plasmatiques normales ou basses en adénosine. Lors du test à l'ATP on observe une bradycardie suggérant l'activation majoritaire des récepteurs A_1.

Les formes mixtes (vasoplégie et bradycardie) pourraient correspondre aux patients ayant des concentrations intermédiaires en adénosine plasmatique. Les récepteurs A1 sont malheureusement difficilement accessible puisque pas exprimés au niveau lymphocytaire. Notre équipe tente de mettre au point une méthode d'évaluation de l'expression des récepteurs A_1 au niveau des macrophages. Il serait alors possible de réaliser des travaux afin de connaître l'expression et la fonction de ces récepteurs chez les patients souffrant de syncope neurocardiogénique.

- Si on sait désormais que l'adénosine et le récepteur A_{2A} sont impliqués chez les patients présentant des syncopes neuro-cardiogéniques. On pourrait être tenté de tester un **antagoniste spécifique des récepteurs A_{2A}** pour traiter ces patients. Le problème essentiel est le caractère ubiquitaire de l'adénosine et de ses récepteurs. Il est probable que ce type de traitement expose à des effets secondaires, en particulier sur le système immunitaire. Par ailleurs, ces travaux sont difficiles à mener dans la mesure où la majorité des patients vagaux présentent peu de syncopes. Le nombre de sujet et la durée d'une étude pourraient être rédhibitoires compte tenu du faible nombre d'évènements attendus.

Dans le domaine de la fibrillation atriale

La prévalence de la fibrillation atriale augmente de manière rapide, en partie en raison du vieillissement de la population. Elle est une source importante de morbi-mortalité, avec en particulier un risque accru d'accidents vasculaires cérébraux. Le traitement anti-arythmique est peu efficace, le traitement radical par ablation est relativement efficace, en particulier sur les formes paroxystiques. On sait que l'adénosine possède des propriétés pro-arythmogènes. Notre équipe a pu démontrer que les taux d'adénosine en pré-opératoire de chirurgie cardiaque étaient liés au risque de développer une fibrillation atriale en post-opératoire.

En revanche, chez d'autres patients, **la fibrillation atriale survient sur un cœur sain**. Les mécanismes de cette dernière et les raisons qui conduisent à cette arythmie sont plus controversés. Nous réalisons au quotidien des procédures d'ablation de la fibrillation atriale. Elles s'adressent dans leur majorité à des patients sans cardiopathie, relativement jeunes et dont la fibrillation atriale est paroxystique. Récemment des travaux semblent impliquer les récepteurs A_{2A} et les mouvements calciques dans la genèse de ce type de FA (Llach A et al. 2011). A la lumière des effets agoniste d'Adonis, nous souhaiterions rechercher la présence d'anticorps anti récepteurs A_{2A} dans cette population de patients.

Dans le domaine de l'insuffisance cardiaque

L'insuffisance cardiaque par dysfonction systolique a un pronostic très péjoratif. On considère que lorsque la fraction d'éjection du ventricule gauche est inférieure à 35%, la mortalité annuelle est de l'ordre de 7%. Nous avons montré, dans cette population, que les taux d'adénosine endogène étaient élevés, et corrélés au taux d'albumine modifiée par l'ischémie. Nous avons, de plus, rapporté que l'adénosine libérée était périphérique et ne provenait pas du myocarde.

Plusieurs travaux de recherche restent à entreprendre. Nous avons étudié une population de patients présentant une dysfonction systolique VG sévère associée à une dyspnée importante, de stade III ou IV de la classification NYHA. Il existe une autre forme d'insuffisance cardiaque dite à fonction systolique conservée. Si son pronostic est péjoratif, le mode de présentation clinique est souvent moins bruyant. Il serait intéressant de connaître le profil adénosinergique des patients moins symptomatiques, ainsi que des patients insuffisants cardiaque à fonction systolique conservée. Par ailleurs, on ne sait pas si l'adénosine est un marqueur de pronostic péjoratif ou, au contraire, un facteur protecteur chez l'insuffisant cardiaque. Pour répondre à cette question, il serait nécessaire de corréler les concentrations en adénosine au pronostic, avec par exemple un critère combiné : mortalité et hospitalisation pour insuffisance cardiaque.

B. Conclusion

L'adénosine est une molécule dont le caractère ubiquitaire est majeur. C'est à la fois un dérivé de substrat énergétique universel (l'ATP) et en même temps un modulateur puissant des systèmes cardiovasculaire, neurologique et immunitaire.

Notre équipe a été un des premiers laboratoires en Europe à pouvoir réaliser son dosage dans des milieux biologiques, et à quantifier l'expression de certains de ses récepteurs. Nous avons modestement apporté notre pierre à l'édifice, notre activité de recherche ayant permis de révéler l'implication de l'adénosine chez les patients présentant des syncopes vagales, dans la fibrillation atriale et chez l'insuffisant cardiaque. Il s'agit aujourd'hui de comprendre les mécanismes physiopathologiques fondamentaux qui sont impliqués. La finalité est évidemment plus pratique, au lit du malade. Notre équipe a déjà été capable de développer un anticorps monoclonal anti-récepteur A_{2A} ayant des propriétés agonistes, dont les applications thérapeutiques potentielles sont nombreuses. Nous nourrissons l'espoir qu'une fois les mécanismes mieux compris, lorsque des agonistes et antagonistes très spécifiques auront été développés, le blocage ou l'activation sélective des différents récepteurs de l'adénosine auront des applications thérapeutiques très concrètes, dans le domaine cardiovasculaire en particulier. Les connaissances acquises au cours de ce travail, sur le système purinergique, semblent indiquer que l'adénosine par l'intermédiaire de ses récepteurs exerce un contrôle puissant sur l'appareil cardiovasculaire. Ce contrôle est tout aussi important quoiqu'avec des effets opposés, que celui exercé par le système catécholaminergique.

En revanche, les effets synaptiques de l'adénosine sur la conduction auriculo ventriculaire ne sont pas sans rappeler ceux exercés par l'acétylcholine (Deharo et al 2012, Brignole et al

JACC 2011). Restent de nombreuses questions sans réponse. Par exemple, quelle est la fonction des récepteurs de réserve identifiés chez certains patients ?

On a vu que les récepteurs purinergiques étaient activés par l'adénosine endogène, mais existe-t-il d'autres agonistes endogènes non identifiés à ce jour ?

D'autre part existe-t-il des antagonistes endogènes non encore identifiés ?

Enfin existe-t-il chez certains patients des auto-anticorps anti récepteurs de l'adénosine qui pourraient participer à la physiopathologie de certaines affections (FA, syncope…).

Tout ce champ reste à explorer.

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