Vascular actions and metabolism of purines in humans

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Vascular actions and metabolism of purines in humans

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Voor mijn ouders

Contents

Chapter 1 is divided into two parts; chapter 1A covers an extensive introduction, in the form of a review. The specific questions that are dealt with in this thesis follow in chapter 1B.

CHAPTER 1A

Cardiovascular pharmacology and possible clinical applications of adenosine, ATP and diadenosine polyphosphates in humans.

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Abstract

This review concentrates on the extracellular actions of adenosine, ATP, and diadenosine polyphosphates in the human cardiovascular system.
Adenosine has important cardiovascular actions: it induces vasodilation, inhibits noradrenaline release from sympathetic nerve endings, inhibits thrombocyte aggregation and has anti-arrhythmic properties. It plays an important role in ischemic preconditioning. Adenosine is generated from enzymatic degradation of ATP. The formation of adenosine is enhanced during ischemia. In humans, adenosine evokes a sympatho-excitatory reflex mediated by chemically sensitive receptors and afferent nerves in the kidney, heart and forearm. This reflex may be active during exercise and ischemia. New therapies are being developed to harness the tissue-protective properties of adenosine against ischemic injury.
ATP is released from aggregating thrombocytes, endothelium, and from sympathetic nerve endings. ATP acts on P_{2X} purinoceptors on vascular smooth muscle cells to induce vasoconstriction. Stimulation of P_{2Y} purinoceptors on endothelial cells induces vasodilation. The mechanism of ATP-induced vasodilation in humans is not elucidated yet. ATP might induce vasospasm at sites of impaired endothelial function and thrombus formation.
Diadenosine polyphosphates are endogenous compounds derived from ATP. They are stored and released from thrombocytes, adrenal medulla, and sympathetic neurons.The functions of the intact molecules as well as their receptor(s) and second messengers are not fully characterized yet. They induce vasodilation as well as vasoconstriction depending of the phosphate chain length. Diadenosine pentaphosphate has received much attention lately, being a possible mediator in the pathogenesis of hypertension, but experimental data are conflicting.
Currently, purine-agonists and antagonists are being developed as therapies against ischemia and other cardiovascular disorders.

1. Introduction

Although cardiovascular actions of extracellular purines have been known for many years now [1], insight into their interstitial formation, metabolism, and mode of action has only recently been acquired [2]. Most of the pharmacologically characterized purine receptors have been characterized at a molecular level. Purine-agonists and antagonists are being developed as therapies for important cardiovascular diseases such as cardiac arrhythmias, heart failure and ischemic syndromes or as a diagnostic tool to detect reversible cardiac ischemia or flow reserve. Purines participate in the regulation of vascular tone, central nervous system, immune function, and the coagulation cascade. They can influence the function of virtually all innervated organs by interacting with the autonomic nervous system [3].
This article will focus on the cardiovascular actions of extracellular adenosine, ATP, and the recently identified class of diadenosine polyphosphates [4].
The interested reader is referred to other authors for recent reviews on the potential role of purine receptors in the brain with implications for the cardiovascular system [5;6].

2. FORMATION AND METABOLIC FATE OF PURINES

2.1 Adenosine

Extracellular adenosine can originate from both intracellular and extracellular sources. The first route of intracellular adenosine-production comprises sequential dephosphorylation of intracellular ATP to adenosine-5'-diphosphate (ADP), adenosine-5'-monophosphate (AMP) and adenosine. This pathway is accelerated when energy demand exceeds supply, and probably represents the main route of adenosine formation during ischemia. The hydrolysation of AMP is catalysed by 5`-nucleotidase, which is present within (cytosolic form) and outside cells (membrane-bound form) [7;8] and is the rate limiting step in this pathway of adenosine formation [9].
Norepinephrine-induced activation of ecto 5`-nucleotidase may provide a functional link between the sympathetic nervous system and adenosine formation [10;11]. A second pathway yielding intracellular adenosine comprises hydrolysation of ATP via S-adenosylmethionine to S-adenosylhomocysteine (SAH), which is further hydrolysed to adenosine and L-homocysteine. This oxygen-insensitive reaction is catalysed by S-adenosylhomocysteine-hydrolase and seems to be of minor importance during ischemia. During non-ischemic conditions however, the SAH pathway may define the transmembrane gradient of adenosine which is the driving force of cellular uptake of extracellular adenosine [12;13]. Recent observations in hyperhomocysteinemic rats in which the formation of adenosine from SAH is inhibited, support this view [14].
Adenosine formation also takes place extracellularly, where ATP is rapidly degraded to adenosine by widespread ecto-nucleotidases present at the outer surface of endothelium and vascular smooth muscle cells [15;16].
During normoxia, intracellular adenosine and AMP-concentrations are low, and extracellular adenosine will be rapidly transported into the cell, where it is phosphorylated by adenosine kinase (main route at physiological adenosine-

concentrations) [17], or it is deaminated to the inactive compound *inosine*, which is further metabolised into *hypoxanthine* and *uric acid* [18]. Although an extracellular form of adenosine deaminase exists, its role is species-dependant and probably plays a minor role in humans [19].
Adenosine diffuses through cellular membranes from extracellular to intracellular and vice versa, depending on the concentration gradient. This bidirectional transport is facilitated by nucleoside transporters located in the plasma membrane of many cells, such as endothelial cells, erythrocytes, vascular smooth muscle cells and cardiomyocytes [20]. Two types of adenosine transporter can be distuinguished: so-called secondary active transporters driven by the transmembranous sodium gradient, and facilitated-diffusion (equilibrative) carriers. Facilitated-diffusion carriers are subdivided based on their sensitivity to the transport inhibitor nitrobenzylthioinosine (NBMPR)] into sensitive (*es)* and insensitive (*ei)* carriers. Dipyridamole and draflazine are more potent inhibitors of the *es* carrier than of the *ei* carrier. Sodium-dependent adenosine transporters are expressed in the gastro-intestinal tract and in the kidney [21], but do not play a major role within the cardiovascular system. Uptake into erythrocytes and endothelial cells is the major mechanism that terminates the cardiovascular effects of luminally applied adenosine [13;22]. Therefore, adenosine transport inhibition is a suitable tool to harness the tissue-protective properties of adenosine against ischemic injury in a site-and event specific way.
Physiological plasma-concentrations of adenosine in humans are 0.1 to 1 µM. The plasma half life of adenosine in this concentration range is very short: less then 10 seconds [23].
Evaluation of the pathophysiological role of adenosine in different organs is often estimated from plasma concentrations. This requires rapid blood collection with immediate addition of enzyme blockers and transport inhibitors, and the reproducibility of this method is low in humans in-vivo [13]. The microdialysis technique seems a promising new tool to study adenosine metabolism, because cells and enzymes are too large to pass the dialysis membrane [24;25]. Once adenosine has passed the dialysis membrane, it is protected from degradation. Besides, intravascular as well as intramuscular and subcutaneous adenosine concentrations can be measured, even simultaneously.

2.2 Adenosine-5'-triphosphate

ATP is an ubiquitous intracellular compound, which is released by exocytosis from storage granules in aggregating thrombocytes, endothelial cells, the adrenal medulla, and from many nerve types, including sympathetic nerve endings [3;26;27]. ATP is also released from smooth muscle cells [28], ischemic myocytes [29] and possibly from exercising muscle [30]. Triggers for ATP release from endothelial cells are shear stress [31;32], sympathetic nerve stimulation [33] and application of vasodilators such as acetylcholine and bradykinin [34].
ATP is co-released with norepinephrine from sympathetic nerves, with acetylcholine in some parasympathetic nerves, and with serotonin and dopamine in nerves in the brain [35]. Nerves utilizing ATP as their principal transmitter, as determined with biochemical or pharmacological techniques, have been named `purinergic` [36].
ATP is degraded sequentially to ADP, AMP and eventually adenosine by ectonucleotidases which are co-released with ATP from sympathetic nerve terminals

[37-39], and are expressed on the surface of many cells, among which endothelial cells. The resulting adenosine is taken up in cells which is facilitated by the nucleoside transporter (see paragraph 2.1).

2.3 Diadenosine Polyphosphates

Diadenosine polyphosphates form a recently identified group of naturally occuring molecules derived from ATP [40]. They consist of two adenosine molecules linked by two to seven phosphate groups, and are commonly abbreviated as $AP_n A$ [40]. AP_4A is the most extensively characterized member of this family [41]. Recently described polyphosphates in humans are AP_7A, identified in thrombocytes [42], and AP_2A in myocardial granules [43]. Diadenosine polyphosphates can be released from storage granules in thrombocytes [44-46], from chromaffin cells in the adrenal gland [47;48] and from autonomic nerves [49-51], and have been identified in human [43] and guinea pig hearts [52]. It is known that they are synthesized intracellularly from amino acids and ATP, a reaction catalysed by aminoacyl-tRNA synthetases [41] and other ligases [53] and their degradation involves asymmetrical specific and non-specific hydrolases primarily yielding AMP and the remaining AP_{n-1} as products [15;54]. These hydrolases are inhibited by mononucleotides, and depend on bivalent cations for their activity. An alternative degradation route is catalysed by phosphorylases introducing orthophosphate, yielding two AP_n-moieties [54]. AP_n-moieties can be degraded by specific hydrolases as well as alkaline phosphatases, phosphodiesterases, and some diadenosine polyphosphate hydrolases. These enzymes are not only abundantly present in the vascular wall, but they can be cleaved from the plasma membrane and circulate as active enzymes. Ectohydrolases degrading diadenosine polyphosphates have been described on endothelial cells [55-57] vascular smooth muscle cells [57] and on the surface of many other cells [58].
The current view is that AP_nA are relative stable compounds in the extracellular space compared to mononucleotides, with half lives of several minutes [41;59]. They seem to derive this stability from the absence of specific degratory enzymes on blood cells. In contrast, ectoenzymes that degrade mononucleotides are present on erythrocytes, leucocytes, and thrombocytes [59]. The half-life of diadenosine polyphosphates is further extended by co-released ATP and ADP, which competitively inhibit AP_nA hydrolases [59].
Diadenosine polyphosphates do not penetrate intact membranes of healthy cells [56] but uptake of AP_4A into tumor cells has been reported [60].
The physiological concentration of AP_nA can be expected to be between the nM and low µM range [61]. Local concentrations at sites of platelet aggregation could potentially rise to 100 µM initially [41]. As described below, it is still a matter of debate whether responses to diadenosine polyphosphates are mediated by the intact molecules or by their metabolites. A simplified overview of extracellular purine metabolism is provided in figure 1.

3. PURINERGIC RECEPTORS

Extracellular purines mediate their effects via membrane-bound receptors (see table 1 for an overview). These receptors are divided into two families [62]. P1 or

adenosine receptors have a potency order of adenosine > AMP > ADP > ATP. P2 receptors have a potency order of ATP > ADP > AMP > adenosine. P1- but not P2 receptors are antagonised by xanthine derivates such as caffeine and theophylline [63].

3.1 P1 receptors

Adenosine receptors are subdivided into four subtypes based on pharmacological and molecular profiles, i.e. A_1, A_{2A}, A_{2B} and A_3 -receptors, all of which are G protein-coupled [2]. As outlined in tabel 1, many celltypes express various types of P1 receptors, which often mediate opposite actions. The final action of adenosine is determined by the relative expression of these receptors, differences between P1 subtype receptors in affinity for adenosine and differences between various cell types in coupling of P1 receptors to second messenger systems. This diversity even exists at the level of a single cell [64] which complicates the extrapolation of in vitro observations to the effects of adenosine in an intact organism.
A_1-receptors are coupled to adenylate cyclase via G_i -proteins, resulting in decreased intracellular levels of the second messenger cAMP. Stimulation of A_1-receptors also activates intracellular phospholipase C [65;66] and opens K_{ATP}-channels that are located in both the plasma membrane and the inner mitochondrial membrane [67]. A_1 -receptor-induced opening of mitochondrial K_{ATP} channels mediate the protective effects of adenosine in the phenomenon of ischemic preconditioning [68;69]. Opening of K_{ATP} channels in the plasma membrane is involved in adenosine-mediated vasodilation [67;70].
A_2 receptors, linked to G_s-proteins, stimulate adenylate cyclase activity, which results in a subsequent increase in intracellular cAMP levels. A_{2A} and A_{2B} receptors are expressed on vascular smooth muscle and endothelial cells [71-73], where both mediate vasodilation. In comparison to A_{2B} receptors, the affinity of A_{2A} receptors for adenosine is relative high and they have a wider tissue distribution. The physiological importance of this pharmacological subdivision in A_2 receptors is not yet known.
The signal transduction mechanism of the A_3 receptors comprises inhibition of adenylate cyclase activity [74] as well as stimulation of phospholipase C [75], resembling the A_1 receptor. A_3 receptors are typically xanthine-resistant. The physiological significance of the A_3 receptor is not well understood, but there is increasing evidence for a role in ischemic preconditioning [76].

3.2 P2 receptors

On the basis of their specific transduction pathways, P2 receptors are subdivided into two families: the P2x ionotropic ligand-gated ion-channel receptors and the P2y metabotropic G-protein-coupled-receptors [77;78]. P2x receptors were originally cloned in excitable cells and mediate fast permeability changes to cations [79;80]. Signal transduction from P2y receptors occurs by activation of phospholipase C and/or stimulation or inhibition of adenylate cyclase. To date, at least seven mammalian P2x- and six P2y receptor subtypes have been cloned [2]. The most recently cloned P2y-12 receptor plays an important role in platelet aggregation [81]. This platelet P2y receptor is currently the only P2 receptor for which a specific antagonist (clopidogrel) is available for human use [82;83].

The P2x receptors are present in vascular smooth muscle cells (VSMC) and mediate vasoconstriction [2]. Recently, the expression of P2x receptors in human endothelial cells was reported [84]. Their functional significance remains to be elucidated, they appear to be associated with cell adhesion and permeability [35] and may play a role in shear stress-mediated vasodilation [84;85]. P2y receptors are present on vascular endothelial cells and on VSMC and mediate relaxation [2].
The lack of specific antagonists hinders investigation of the (patho)physiological sigificance of the various P2 receptor subtypes. The drug suramin acts as a P2 receptor antagonist with limited specificity and efficacy [86]. It does not antagonise all P2 receptors, and it does not discriminate between P2x and P2y receptors. Furthermore, suramin inhibits ecto-nucleotidase [87] and neural diadenosine polyphosphate hydrolases [88], which further complicates the interpretation of ligand-binding studies. Its carcinogenic properties hinder its use in human *in-vivo* studies. Pyridoxal-5-phosphate (P5P) and pyridoxalphosphate-6-azophenyl 2`,4`-disulfonic acid (PADS) are often used to block P2x receptors, but these antagonists are not selective [89].

3.3 Binding sites for diadenosine polyphosphates

The receptor(s) for diadenosine polyphosphates and their second messengers have yet to be fully characterized. Consequently, at present it is preferable to avoid the use of terms such as P4, $P2y_{AP4A}$ or dinucleotide receptors in favour of more general terms such as binding sites [90]. APnA can activate subclasses of P2x- and P2y receptors, as well as specific binding sites [2;91]. A binding site specific for AP_4A has been reported in mouse brain [92], mouse heart [93] and in rat brain [94]. It was shown recently that diadenosine polyphosphates activate calcium-dependent potassium conductance in smooth mucle cells derived from porcine aorta. This action is most likely mediated via P2y-receptors and possibly also partially by a specific receptor for AP_4A [95].

4. CARDIOVASCULAR EFFECTS OF PURINES

4.1 Vascular tone

Depending on the site of adenosine formation (luminal versus interstitial) [96], adenosine stimulates A_2-receptors [97] on either endothelial [71] or vascular smooth muscle cells [2], resulting in endothelium dependent [71;98] or independent vasorelaxation [99].
In humans, adenosine infusion into brachial or coronary arteries causes a dose-dependent vasodilation [100;101] which is mediated by P1-receptors, because it can be blocked by either caffeine or theophylline. Forearm vasodilation induced by intra-brachial adenosine-infusion is, at least partially, mediated by nitric oxide [102] supporting the importance of the endothelium in the vasodilator response to luminal adenosine.
Stimulation of A_1-receptors in renal glomerular afferent arterioles [103] results in vasoconstriction which may play a role in tubuloglomerular feedback [104]. In other

vascular beds, vascular A_1 receptors may mediate vasorelaxation by opening ATP-dependent potassium channels [105-107].

ATP is capable of inducing both vasoconstriction or vasodilation, depending on the experimental conditions and the integrity of the endothelial cells. In general, P2x and P2y receptors on vascular smooth muscle cells mediate vasoconstriction, whereas P2y receptors on endothelial cells mediate vasodilation [77]. P2x receptors on vascular smooth muscle cells are stimulated by ATP released from perivascular sympathetic nerve endings, while ATP acting on endothelial receptors is derived from endothelial cells and aggregating thrombocytes. This dual vasomotor-action of ATP may have important clinical consequences: during thrombocyte aggregation at sites of severe atherosclerosis, locally released ATP might induce vasoconstriction mediated by P2x receptors on vascular smooth muscle cells, unopposed by P2y receptor-mediated vasodilation because of endothelial damage. It was recently shown that activation of endothelial P2y receptors induces release of EDHF, the vasodilator action of which can be counteracted functionally by smooth muscle cell P2x receptor stimulation [108;109]. The vasomotor action of intra-arterially infused ATP in the forearm of healthy volunteers is dominated by vasodilation which can not be explained by stimulation of P1 receptors. The mechanism of this ATP-induced vasodilation in humans has not been elucidated yet but does not seem to involve endothelial nitric oxide release [110;111].

The effects of diadenosine polyphosphates on vascular tone are a function of the number of phosphate groups in the molecule, the species and the origin of the vascular bed that is studied, and the presence or absence of intact endothelial cells. For example, Ralevic *et al.* demonstrated that diadenosine-polyphosphate-induced, P2x -receptor-mediated vasoconstriction in the isolated perfused rat mesenteric artery increases with the length of the phosphate chain [112]. Generally, in intact animals or vessels AP_2A, AP_3A and AP_4A are vasodilators and AP_5A and AP_6A are vasoconstrictors.
AP_3A and AP_4A have been shown to induce vasodilation in rabbit mesenteric and rabbit coronary vascular beds [113;114]. Continuous intravenous administration of AP_4A in anesthetized dogs induced hypotension by primarily dilating the arterial resistance vessels, without eliciting reflex tachycardia [115]. Endothelial release of nitric oxide and/or prostacyclin are involved in this vasodilator response [113;116;117].
AP_5A and AP_6A induce vasoconstriction in human umbilical vessels *in vitro* [118]. In rats, these dinucleotides may induce a rise in blood pressure and may be involved in the pathogenesis of essential hypertension [45].
Until recently data concerning the vasoactive properties of APnA in the human vasculature *in-vivo* were scarce. We recently demonstrated that intra-arterial infusion of AP_5A into the brachial artery of healthy volunteers evoked a dose-dependent forearm vasodilator response, equal to adenosine but less than ATP at equimolar doses [22]. This vasodilator action was inhibited by theophylline and augmented by dipyridamole, which implies involvement of P1-receptors. Kikuta *et al.* used a stable analogue of AP_4A in humans to induce controlled hypotension during surgical procedures [119]. It has been suggested that some diadenosine polyphosphates are involved in blood-pressure regulation [45] or the pathogenesis of essential hypertension [120], but direct evidence for these concepts is currently lacking.

4.2 Heart

In the heart, adenosine receptors are expressed in the sinus node, atrioventricular (AV) node, atrial and ventricular myocardial cells, coronary circulation and autonomic nerve endings. For the coronary circulation and autonomic nerve endings, the reader is refered to paragraph 4.1 and 4.5 respectively.
Within the sinus node, stimulation of A_1 receptors and subsequent reduction in intracellular cAMP and opening of potassium channels results in hyperpolarization of the cell membrane which reduces the spontaneous depolarization and subsequent firing rate. Likewise, in the AV-node A_1 receptor stimulation results in hyperpolarization and a subsequent reduction in AV-nodal conduction velocity which is responsible for adenosine-induced termination of AV-nodal tachycardias [19]. In atrial myocardial cells, A_1 receptor stimulation and subsequent sarcolemmal hyperpolarisation prevents calcium influx which reduces contractility, irrespective of sympathetic tone [121;122]. This direct negative inotropic action of adenosine is not observed in ventricular myocardial cells, where stimulation of A_1 receptors reduces contractility only in the presence of β-adrenoceptor stimulation. This interaction of adenosine with the sympathetic nervous system involves inhibition of β-adrenoceptor-induced activation of adenylate cyclase [19;123]. Finally, A_1 and A_3 receptor stimulation prevents ischemia-induced arrhythmias and cell death [124] (see chapter 5).
Intravenous infusion of ATP in humans exerts its negative chronotropic and dromotropic effects on the heart after degradation to adenosine [124]. In isolated cell and tissue preparations, ATP has a direct positive inotropic effect on atrial and ventricular myocardial cells, which is mediated by a P2x receptor mediated increase in intracellular calcium [125].
Diadenosine polyphosphates have been shown to influence both coronary blood flow and cardiac electrical activity [126]. $AP_{3\text{-}6}A$ exert a negative inotropic effect in canine and guinea pig myocardium, probably mediated by P1-receptors, but a yet undefined receptor may also be involved [127-129]. Recent reports suggest a role for diadenosine polyphosphates in cardioprotection against ischemia [130;131]. The role of degradation products of APnA must be clarified first however, as well as whether specific APnA-receptors on endothelial cells and VSMC exist.

4.3 Kidney

The vascular effects of adenosine in the kidney (glomerular afferent vasoconstriction and efferent vasodilation) have been reviewed [132]. Other adenosine mediated renal effects include A_1-receptor-mediated inhibition of renin release [133], mediation of tubuloglomerular feedback [104], and increased salt reabsorption in the proximal tubule [134]. Acting in concert, these actions of adenosine reduce diuresis by reducing glomerular filtration rate and increasing tubular salt reabsorption. However, medullary A_2 receptor-mediated vasodilation and subsequent increased diuresis may override A_1 receptor-mediated salt and water retention [135].
ATP induces vasoconstriction in rat afferent glomerular arterioles, and has no effect on the efferent arterioles [136]. For rabbit and human renal arteries, it has been shown that exogenous ATP mainly acts after degradation to adenosine [137].
The renal vascular effects of APnA are not completely clear [138-140]. Apart from differences in models and species, differences in metabolism may be basis of

discrepancies (see paragraph 4.1). Diadenosine polyphosphates also have a mitogenic effect on mesangial cells, which leaves a possible role for them open in accelerating the process of glomerulosclerosis [141].

4.4 Haemostasis

Adenosine inhibits platelet aggregation [142], whereas [143] adenosine-5'-diphosphate (ADP) induces thrombocyte aggregation mediated by a thrombocyte specific P2y-12 receptor. ATP is an endogenous antagonist for the P2y-12 receptor [81]. The clinical importance of this action is underlined by results from recent clinical trials which show additional benefit of P2y-12 receptor antagonists on survival in patients with unstable angina (see chapter 6). AP_3A is a potent stimulator of platelet aggregation, mediated by its metabolite ADP [144]. In contrast, AP_4A and AP_5A inhibit ADP-induced platelet aggregation [145;146]. ATP strongly induces release of the anti-thrombotic factor tissue-type plasminogen activator (tPA) from the intact human vascular bed *in vivo* [145;147]. The clinical relevance of this finding is not known yet.

4.5 Adenosine and the sympathetic nervous system

4.5.1 Adenosine and sympathetic afferents

In conscious humans and some larger animals such as dog, adenosine stimulates carotid chemoreceptors [148] and sympathetic afferent nerves in heart [149], kidney [150] and forearm muscle [151;152]. In the carotid body of the rat and rabbit, both A_1 and A_2 receptors are expressed [153;154]. The A_2 receptor is most likely involved in adenosine-induced carotid body excitation [155] but human in-vivo data are lacking. Adenosine-sensitive afferent nerves in heart, kidney and skeletal muscle are possibly involved in the sympatho-excitation that accompanies ischemia in these organs. The receptor subtype that is involved in adenosine-mediated activation of these sympathetic afferents is largely unknown, but studies in the dog heart suggest the involvement of A_1 receptors [156].
Since adenosine is rapidly taken up by erythrocytes, the site and mode of adenosine administration determine which adenosine sensitive afferents are stimulated. During continuous intravenous infusion of adenosine as performed during adenosine-Thallium stress testing, the stimulation of carotid body chemoreceptors are probably solely responsible for the sympatho-excitation [157]. This results in stimulation of the sympathetic nervous system and the respiratory system [151;158], and a subsequent increase in systolic blood pressure, plasma renin activity and ventilation [159]. These effects are not elicited during anesthesia, because an intact autonomic reflex loop is essential [160]. The rise in systolic blood pressure is not always observed during intravenous adenosine infusion in healthy volunteers, due to differences in the degree of caffeine abstinence [161].
Several mechanisms for involvement of adenosine-induced sympatho-excitation in the (patho)physiology of the cardiovascular system have been put forward. First, adenosine may be an important trigger of the exercise-induced pressor reflex [162]. This hypothesis is supported by the observation that theophylline blunts the

sympatho-excitation that is evoked by isometric exercise [163] and increased levels of forearm interstitial adenosine during isometric handgrip as determined using microdialysis. However, this last observation may be confounded by mechanical cell rupture due to the presence of a microdialysis probe in a contracting muscle and subsequent increases in interstitial adenosine.
Second, adenosine may be an afferent signal for sympatho-excitation in heart failure which is an important marker of reduced survival in this condition [164]. Patients with heart failure have increased levels of adenosine in proportion to disease severity [165], as well as an augmented sympathetic response to isometric exercise which can be partially reversed by the adenosine receptor antagonist caffeine [166]. In healthy subjects, the angiotensin II receptor antagonist losartan blunts the generalized sympatho-excitation that is induced by forearm infusion of adenosine [152]. These results suggest that the beneficial effect of inhibition of the renin-angiotensin system in patients with heart failure is partially mediated by inhibition of the adenosine-induced sympatho-excitation.
Third, ischemia-induced formation of interstitial adenosine and subsequent sympatho-excitation could potentially be involved in remote ischemic preconditioning: a phenomenon in which ischemia of a remote organ such as kidney, gut, or skeletal muscle protects the heart against subsequent ischemic injury [167;168] (see chapter 5).
Fourth, sympatho-excitation in response to intravenous adenosine infusion may play a role in the pro-arrhythmic action of adenosine that sometimes occurs a few minutes after a bolus injection of adenosine when used to terminate AV-nodal tachycardias [169].
Finally, adenosine may be involved in hypoxia-induced stimulation of peripheral chemoreceptors and the subsequent increase in ventilation [170].
Recently, adenosine-induced activation of hepatic sympathetic afferents have been suggested, which may be implicated in sympathetic nervous system-mediated renal salt retention. Whether this reflex is clinically important and involved in salt retention in patients with liver cirrhosis remains to be elucidated [171;172].

4.5.2. Adenosine and central regulation of sympathetic outflow

At the area postrema, circulating adenosine can easily access the nucleus tractus solitarius [173]. Micro-injections of adenosine in the caudal part of the nucleus tractus solitarius of the rat induce contrasting effects on blood pressure, depending on the adenosine receptor type that is stimulated: A_1 receptor activation induces an increase in blood pressure whereas A_{2a} receptor activation reduces blood pressure and sympathetic outflow [174;175]. The role of these central actions of adenosine in the hemodynamic effects of intravenous infusion of adenosine is not known.

4.5.3. Adenosine and sympathetic efferents

Adenosine inhibits norepinephrine release by stimulating A_1 receptors on sympathetic nerve endings [176]. This local action of adenosine on sympathetic efferents functionally counteracts the central or reflex-mediated increase in sympathetic nerve traffic and subsequent release of norepinephrine. This action has been demonstrated in various in vitro and in vivo models including in humans in vivo [152;177]. Human in

vivo data on the involved adenosine receptor subtype are lacking. This action of adenosine is possibly involved in the local sympatholysis in exercising muscle [178] which contributes to the local vasodilation in exercising muscle. In the heart, this sympatho-inhibitory action of adenosine at sympathetic nerve terminals may contribute to the anti-arrhythmic action of adenosine in the ischemic myocardium [179]. In vitro, an A_{2a} receptor mediated increase in sympathetic norepinephrine release has been described, but the functional significance of this modulation is not known [180;181].

4.6 Integrated cardiovascular action of adenosine in humans

The cardiovascular effect of parenteral adenosine administration in conscious humans depends on the route of administration (intra-arterial versus intravenous) and the velocity of infusion (bolus versus continuous infusion) [148;158]. These differences are explained by the kinetics of extracellular adenosine (rapid uptake by endothelial cells and erythrocytes) and the relative contribution of sympatho-excitation as elicited by adenosine sensitive sympathetic afferents [13]. In addition, the period of caffeine abstinence is critical for the evaluation of cardiovascular responses to adenosine [161].
After a typical intravenous bolus injection of 6-12 mg, the inhibitory actions of adenosine on sinus node firing rate and AV conduction velocity predominate the response [148]. Therefore, this method of administration is prefered when adenosine is used to terminate supraventricular tachycardias [182]. When adenosine is infused continuously at a frequently used infusion rate of 140 $\mu g \cdot kg^{-1} \cdot min^{-1}$, bradycardias or AV block are only rarely observed. In stead, an increase in heart rate is the typical response which is frequently accompanied by some chest discomfort, an increase in tidal volume, sympatho-excitation and, if caffeine is abstained for at least 24 hours, an increase in systolic blood pressure [157;161;183]. Of note, continuous intravenous administration of adenosine does not result in forearm vasodilation [184]. Apparently, these conditions facilitate stimulation of carotid chemoreceptors as supported by its inhibition during 100% oxygen breathing and observations in patients in whom the carotid chemoreceptors have been removed surgically [157;183].
Continuous infusion of adenosine into the brachial artery at rates < 0.5 $mg \cdot min^{-1}$ induces forearm vasodilation and inhibits norepinephrine release in the forearm. Although heart rate and blood pressure are not affected under these conditions, careful monitoring reveals generalized sympathoexcitation probably resulting from stimulation of metabolic afferents in the forearm skeletal muscle [152;185]. Bolus injections of adenosine into the brachial artery result in more profound sympatho-excitation as reflected by an increase in heart rate and peroneal muscle sympathetic nerve activity possibly resulting from the higher local peak concentrations as compared with continuous infusions [186]. Injection of adenosine into the femoral artery also results in sympatho-excitation but this does probably not represent local stimulation of muscle sympathetic afferents but rather reflects local vasodilation with a subsequent decrease in blood pressure and baroreflex activation or originates from systemic spillover of locally infused adenosine [187].

5. INVOLVEMENT OF ADENOSINE IN ISCHEMIC PRECONDITIONING

Extracellular formation of adenosine is increased during hypoxia and ischemia [188]. Adenosine is a protective agent in the setting of ischemia-reperfusion: in the heart, adenosine reduces oxygen-demand by its negative inotropic and chronotropic effects and improves oxygen supply by coronary vasodilation. Its electrophysiological effects reduce the risk of ischemia-induced arrhythmias. Adenosine also inhibits noradrenaline release and sympathetic neurotransmission during ischemia, which could limit catecholamine-induced arrhythmogenesis and vasoconstriction [179]. Additionally, during ischemia and reperfusion adenosine inhibits thrombocyte aggregation [143] and adhesion of neutrophil cells, inhibits cytokine release and free radical formation [189].
Ischemic preconditioning, i.e. the concept that brief periods of ischemia and reperfusion reduce the rate of cell death during a subsequent prolonged period of ischemia, was first described in the dog myocardium by Murry *et al.* [190], but has been observed in skeletel muscle [191], liver and brain [192;193]. In humans in vivo, this phenomenon is likely to occur as well [194;195]. However, definite prove is difficult to obtain due to the lack of a good model to study ischemic injury in humans in vivo (see table 2).
Endogenous adenosine, acting through its A_1-receptors, triggers and mediates the process of ischemic preconditioning in various animal models and human in-vitro experiments with myocardial cell cultures or isolated atrial tissue preparations [76;196;197]. The second messenger system includes protein kinase C activation and subsequent opening of mitochondrial K_{ATP} channels [198;199]. The final mechanism of retarded cell death is not known but may involve inhibition of ischemia/reperfusion-induced apoptosis [200-203].

6. POTENTIAL CLINICAL APPLICATIONS OF PURINES

Adenosine is the drug of first choice for treatment of atrio-ventricular node re-entry tachycardia, for which it has FDA approval. Nowadays, selective and stable adenosine A_1 receptor agonists are being developed, in order to slow atrioventricular conduction at concentrations that do not cause the significant coronary vasodilation or systemic hypotension, that is associated with bolus injections of adenosine [204]. In addition, these compounds are not expected to cause chemoreceptor mediated sympatho-excitation although this property of A_1 agonists has not been studied in humans yet.
The vasodilator properties of adenosine are exploited in the adenosine-Thallium stress test. The advantage of adenosine over dipyridamole is its short lasting action which improves its tolerability [205].
A_1 receptor antagonists may prove valuable in the prevention of radiocontrast-mediated renal injury [206;207]. Furthermore, these agents have diuretic properties which may be promising in patients with congestive heart failure. In these patients A_1-antagonists may prevent prerenal failure because their diuretic properties are associated with renal vasodilation that is limited to the afferent arterioles [208]. A_1 - and A_3 receptor agonists may also exert tissue-protection in the setting of cardiac bypass graft surgery, limb ischemia, and reconstructive surgery.

Systemic side effects (sympatho-excitation, AV block, vasodilation) are the major problems when systemic administration of adenosine is used to exploit its protective actions against ischemic injury. In theory, various strategies are possible to circumvent these drawbacks among which are the use of selective adenosine receptor agonists, the administration of a nucleoside transport inhibitor or the local infusion of adenosine. Before selective A_1- or A_3 receptor agonists can be used for this purpose, more research is needed to determine the involvement of the various adenosine receptor subtypes in the systemic actions of adenosine in humans. Because extracellular adenosine is also formed in non-ischemic conditions, high grade inhibition of nucleoside transport also results in systemic side effects that are similar to the effects of intravenous infusion of adenosine [13]. Therefore, systemic administration of a nucleoside transport inhibitor needs carefull dose titration to exploit the benficial effects of endogenous adenosine in the setting of ischemia without inducing undesired systemic actions. An alternative approach to prevent systemic side effects is the local, intra-arterial, administration of adenosine or a nucleoside transport inhibitor. Several trials in humans have shown cardioprotective actions of intracoronary infusion of adenosine in the setting of (acute) coronary angioplasty [209-211]. Likewise, intracoronary infusion of the nucleoside transport inhibitor dipyridamole reduced the ischemia-induced decrease in left ventricular function and ischemia-induced changes in ECG [212;213].
The antithrombotic drugs *clopidogrel* and *ticlopidine,* acting as antagonist on the platelet P2y-12 receptor have proven their capacity to reduce the risk of recurrent heart attacks and strokes [82;83].
Stable and potent analogues of APnA are being developed for different purposes such as inhibition of platelet aggragation [214-216].

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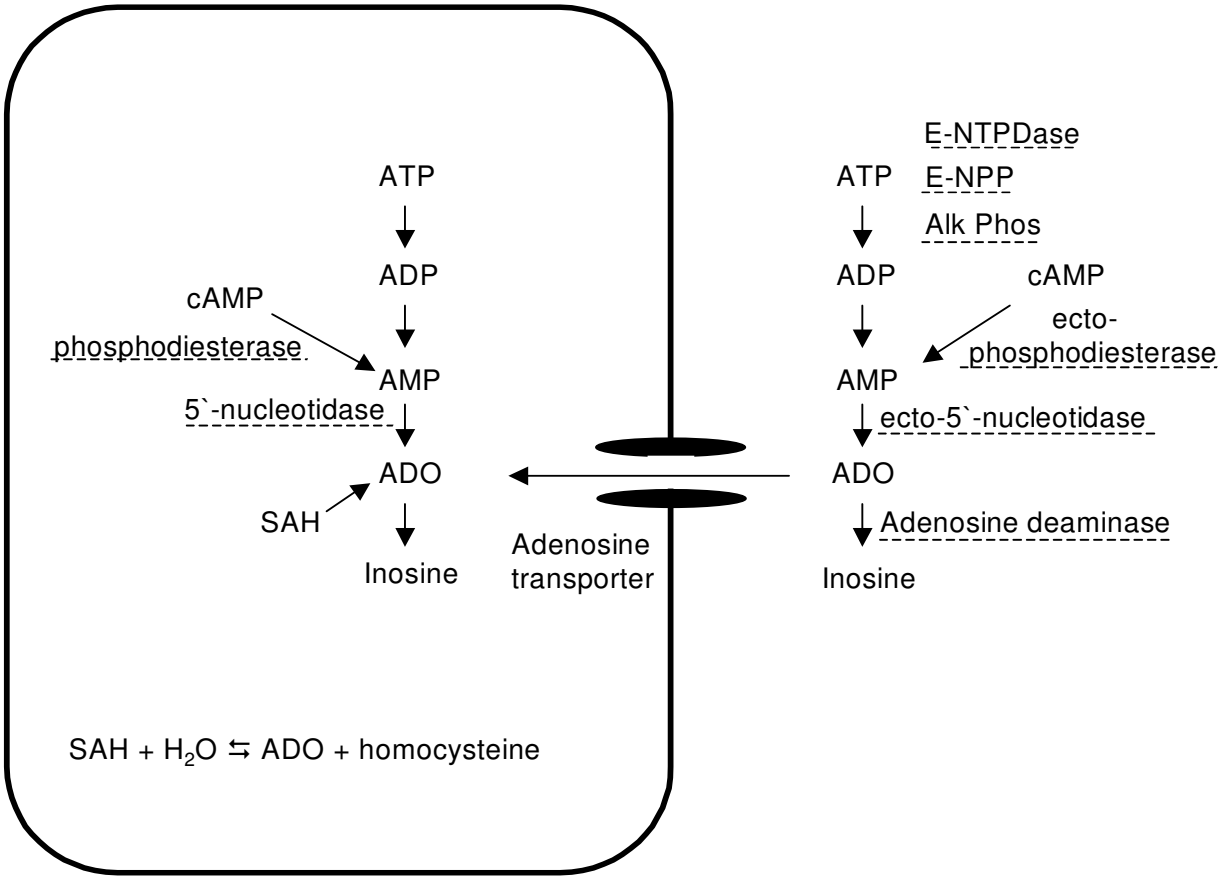

ADO = adenosine

SAH = S-Adenosylhomocysteine

E-NTPDase = Ecto-nucleoside triphosphate diphosphohydrolase

E-NPP = Ecto-nucleotide phosphodiesterase

Alk Phos = Alkaline phosphatase

Figure 1: simplified overview of purine metabolism

Table 1: Cardiovascular distribution and effects of purine receptors

Class	Sub-type	Second messenger system(s)	Tissue distribution	Cardiovascular effects
P1	A_1	$G_{i/o}$ coupled, ↓cAMP ↑ PLC, ↑ IP_3, ↑PKC, ↑ K_{ATP}-conductance, ↑ MAP kinase ↑ IP3K/Akt pathway	Brain stem Carotid chemoreceptors Sympathetic efferents Sinus and AV node Atrial cardiomyocytes Ventricular cardiomyocytes Endothelial cells Vascular smooth muscle cells Juxtaglomerular cells Renal tubular cells	Increased sympathetic outflow [217] Functional significance unknown [154] Inhibition of norepinephrine release [176] Sinus bradycardia; AV conduction delay (AV blockade) [19] Decrease in contractile force both in the absence and presence of catecholamines [121;122]; ischemic preconditioning [218] Decrease in contractile force only in the presence of catecholamines [19;123]; ischemic preconditioning [194] NO release, vasodilation; ischemic preconditioning [219;220] vasoconstriction in glomerular afferents [221;222] Reduced renin release [133] Sodium retention (A_2 mediated medullary vasodilation overrides this tubular effect of adenosine) [135;223]
	A_{2a}	G_s coupled, ↑ cAMP ↑ K_{ATP} conductance	Brain stem Carotid body chemoreceptors Endothelium Vascular smooth muscle cell Platelets	Hypotension [217] Increase in sympathetic afferents, reflex mediated sympatho-excitation [155] Hyperpolarization and NO release, vasodilation [102;107;224] Vasodilation [225] Inhibition of thrombocyte aggregation [226]
	A_{2b}	As A_{2a}	As A_{2a}	As A_{2a} (functional significance not well known, probably similar to A_{2a})
	A_3	G_i G_q coupled, as A_1	Widely distributed	Physiological significance largely unknown. Involved in ischemic preconditioning [227]. May serve as a 'backup' for the A_1 receptor
P2	$P2X_1$	Ion channel, ↑ intracellular Ca^{2+}	Vascular smooth muscle cell Brain stem Cardiac myocytes	Vasoconstriction [228;229] Increase in sympathetic outflow (rostral VLM) or decrease in sympathetic outflow (caudal VLM) [230] Increased contractility [231]
	$P2X_{4,5,7}$	Ion channel, ↑ intracellular Ca^{2+}	Endothelial cells	Functional significance not well known, role in shear stress induced vasodilation? [232-234]
	$P2Y_{1,2}$	$G_{q/11}$ coupled, PLC, PKC activation, IP_3 formation, ↑intracellular calcium	Endothelial cells Smooth muscle cells Cardiac myocytes	Vasodilation [235] $P2Y_1$: vasodilation, proliferation; $P2Y_2$: vasoconstriction, proliferation [236] Increased contractity [237]
	$P2Y_{12}$	↓ cAMP	Platelets	Thrombocyte aggregation [81]

Table 2: Evidence for ischemic preconditioning in humans

Author	Method	Observation	Limitations
Ikonomidis et al [194]	Cultured non- beating ventricular cells	IPC increases survival	Highly artificial conditions
Speechly-Dick et al [218]	Isolated, superfused, isometrically contracting atrial trabeculae	Hypoxia + glucose deprivation reduces contractile dysfunction; role for adenosine, subsequent PKC activation and opening of K_{ATP}-channels	No direct measurement of cell death
Cleveland et al [238]	Idem	Idem, sulphonylurea derivatives prevent 'IP'	Idem
Yellon et al [76]	Coronary artery bypass surgery/aortic cross clamping	Repeated global myocardial ischemia reduces intracellular ATP loss.	Idem
Deutsch et al [239]	Coronary angioplasty with ECG monitoring and venous lactate measurements	The increase in coronary venous lactate and ECG changes are reduced after repeated ischemia	Idem
Tomai et al [240;241]	Coronary angioplasty with ECG monitoring	Glibenclamide and bamiphylline prevent 'IP'.	Idem
Napoli et al [242]	Epidemiological observation	Predromal angina reduces infarct size	Possible confounding by reduced time to reperfusion

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CHAPTER 1B

Aim of the study

Introduction

The first report about the cardiovascular effects of nucleosides and nucleotides dates back from amply seventy years ago (Drury and Szent-Gyorgi, 1929). Nowadays, endogenous purines are known to exert a multitude of cardiovascular effects. These were briefly outlined in chapter 1A, with a focus on adenosine. The endogenous purine adenosine acts on cell surface receptors, resulting in numerous cardio- and vasoprotective effects, like modulation of vascular tone, presynaptic inhibition of noradrenaline release, ischaemic preconditioning, and inhibition of platelet aggregation. Many details about the cardiovascular pharmacology of purines in humans remain to be answered, however, and the following chapters deal with some of them.

This thesis deals with the following study questions:

1. What is the effect of intra-arterially infused dipyridamole and adenosine on interstitial and intravascular adenosine concentrations (chapter 2).
   As outlined in chapter 1, adenosine plays an important role in the pathophysiology of ischemia and reperfusion. In particular, its protective actions against the sequels of ischemia are of therapeutic interest in humans. However, it is still not known how these effects could optimally be exploited in a clinical context. Furthermore, adenosine is frequently used as a pharmacological tool to detect reversible perfusion defects in the heart.
   The biological availability of intravenously or intra-arterially infused adenosine has not been well characterized in humans in vivo. Since the pharmacological actions of adenosine will vary depending on its site of action, this knowledge could improve the clinical application of adenosine and dipyridamole as tools to diagnose ischemia and prevent ischemia-reperfusion injury.
2. What is the role of adenosine in the pressor response to exercise in healthy volunteers (chapter 2).
   Several lines of evidence suggest a role for adenosine in the exercise-pressor reflex. This reflex is augmented in patients with heart failure and may play a role in the deleterious activation of the sympathetic nervous system in these patients. Therefore, we studied the possible role of adenosine in this reflex in healthy volunteers.
3. Is there a difference in the mechanism of vasodilation between interstitial and intravascular adenosine (chapter 2 and 3).
   Partially based on observations in chapter 2 and previous observations with another adenosine uptake inhibitor draflazine, we argued that dipyridamole and adenosine may both induce vasodilation by stimulating adenosine receptors. However, these agents could differ in the involved cell populations that are responsible for vasodilation: adenosine could stimulate endothelial cells whereas dipyridamole could preferentially stimulate vascular smooth muscle cells. This difference could result in differences in post-receptor pathways that are involved in vasodilation with potential importance for the clinical use of these drugs to detect reversible perfusion defects in the heart.
   In particular the interaction with glibenclamide, a blocker of ATP-sensitive potassium channels could differ between dipyridamole and adenosine with important consequences for patients with type 2 diabetes mellitus who are

treated with glibenclamide and scheduled for a pharmacological stress-thallium scan.

4. What is the vasomotor action of AP_5A in humans, and what is the role of adenosine in AP_5A-induced changes in vascular tone (chapter 4).
   Animal studies suggest a vasoconstrictor action of the complex endogenous diadenosine AP_5A. Furthermore, platelet derived AP_5A has been suggested to cause hypertension. However, these studies do not definitely exclude confounding by vascular actions of degradation products of AP_5A such as adenosine. Furthermore, human in-vivo data are lacking. Therefore, we decided to study the effect of AP_5A on forearm vascular tone in healthy volunteers.
5. Do benzodiazepine-derivates inhibit cellular adenosine uptake in the human forearm (chapter 5).
   Clinically, benzodiazepines are frequently used in the setting of an acute myocardial infarction. In-vitro studies suggest that these substances inhibit nucleoside transport which could theoretically improve tolerance to ischemia-reperfusion injury by augmenting the protective action of endogenous adenosine that is released in ischemic cardiac tissue. This study was performed to explore a possible effect of benzodiazepines on adenosine uptake in humans in vivo.
6. What is the mechanism of ATP-induced vasodilation in the human forearm (chapter 6).
   Although the potent endothelium-dependent vasodilator action of adenosine-5'-triphosphate (ATP) is known for some time, its mechanism of action in humans in vivo is still not completely resolved. Since ATP, released from aggregating thrombocytes, may induce paradoxical vasoconstriction at sites of dysfunctional endothelium, it is of potentially clinical importance to elucidate the mechanism of ATP-induced vasodilation as it may reveal new targets for pharmacological treatment of patients with atherosclerosis. In this chapter, the role of cyclo-oxygenase products and various key molecules that mediate vasodilation by endothelium-derived hyperpolarizing factors (ATP-sensitive potassium channels, Ca^{2+}-sensitive potassium channels, Na^+/K^+-ATPase) is investigated.
7. Is the vasodilator response to adenosine reduced in patients with uncomplicated insulin dependent diabetes mellitus (chapter 7).
   Diabetes mellitus is associated with increased cardiovascular morbidity. The vascular effects of adenosine are impaired in animal models of diabetes. Is the presence of type 1 diabetes perse associated with a reduced action of adenosine? To answer this question, we evaluated the vasodilator response to adenosine in patients with uncomplicated insulin dependent diabetes mellitus and compared these observations with a matched control group.

Chapter 2 up to chapter 5 inclusive are centered around nucleoside transport. Nucleoside transport is characterized both biochemically -using microdialysis- and pharmacologically, using dipyridamole as a nucleoside transport inhibitor and the vasodilator response to intra-arterially infused adenosine as a biomarker of adenosine receptor stimulation. Chapter 5 focuses on a possible adenosine-transport inhibiting effect of two different compounds; diazepam and midazolam. Chapter 6 involves the vasodilatory mechanism of the nucleotide ATP. Chapter 7 deals with a

more clinical issue: the vasodilator response to adenosine in patients with uncomplicated insulin dependent diabetes mellitus. The last chapter provides a summary of the thesis.

Methods applied

The perfused forearm technique

In the experimental studies described in this thesis, the perfused forearm technique was applied to quantify actions of endogenous or exogenous substances on vascular tone. The brachial artery is cannulated for infusion of purines and other vasoactive compounds and for blood pressure recording. Intra-arterial infusion of vaso-active substances allows the use of very low cumulative doses that reach local concentrations in the infused forearm that are sufficient to induce a local effect without relevant systemic spill-over to induce confounding systemic actions. Calibrated strain-gauges are attached around the forearms at the level of maximal diameter to measure forearm blood flow (FBF; venous occlusion plethysmography). The rate of swelling of the forearm during occlusion of venous return is used to assess the rate of arterial inflow. To confine the measurement to the muscular vascular bed as much as possible, the hand circulation is impeded by pediatric cuffs around the wrists. Concomitant measurement of FBF in both arms enables detection of relevant systemic effects on forearm vascular tone. The ratio of FBF in both forearms is sometimes used to correct for generalized random fluctuations in vascular tone that are not directly related to the intra-arterial infusions. Alternatively, to correct for small changes in blood pressure that sometimes occur during the experiment, Forearm Vascular Resistance (FVR) can be calculated from the quotient of mean arterial blood pressure (MAP) and FBF and is expressed in Arbitrary Units (AU).

Microdialysis

After local anesthesia, a small double-lumen catheter is inserted into the musculus flexor digitorum superficialis or in a cubital vene. The tip of the probe is constituted of a semi-permeable membrane. The probe is perfused with NaCl 0.9% by a small pump. Small molecules (<20.000 KD) in the interstitial space or in blood, such as adenosine, can diffuse through the membrane but cells and enzymes can not. Adenosine within the probe is therefore protected against degradation. Samples were collected every 15 minutes and frozen until analysis by HPLC. After finishing the protocol, the dialysis catheters were brought to the laboratory for *in vitro* calibration of adenosine recovery (see chapter 2 for a more detailed description).

Finally: the perfused forearm technique is the basis for all studies described in this thesis. The microdialysis technique was applied -as described in chapter 2- to gain more insight into the compartmentalization of adenosine.

CHAPTER 2

The effect of dipyridamole on interstitial and circulating adenosine: Implications for the role of adenosine in the exercise-pressor reflex

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Submitted

Abstract

In the present study, microdialysis was used to study the effects of the nucleoside uptake inhibitor dipyridamole on interstitial and intravascular adenosine kinetics. We hypothesized that dipyridamole [1] augments the exercise-pressor reflex by interstitial adenosine uptake inhibition and [2] improves extravasation of intra-arterially infused adenosine. Firstly, interstitial adenosine was measured with a microdialysis probe in the forearm flexor muscle of healthy volunteers, during rhythmic handgripping. Infusion of dipyridamole into the brachial artery (12 $\mu g \cdot min^{-1} \cdot dl^{-1}$ forearm) potentiated the exercise-induced rise in dialysate adenosine (from 0.30 ± 0.08 to 0.48 ± 0.10 $nmol \cdot ml^{-1}$, n=9, P<0.05) but not the exercise-induced rise in blood pressure (SBP/DBP 9.6 ± 2.4 / 4.5 ± 2.0 versus 10.4 ± 2.2 / 7.0 ± 1.3 mmHg, n=9, P>0.1). Secondly, a microdialysis probe was inserted into forearm muscle and antecubital vein for simultaneous measurement during infusion of adenosine into the brachial artery (5.0, 15 and 50 $\mu g \cdot min^{-1} \cdot dl^{-1}$). Adenosine infusion did not significantly increase dialysate adenosine from either probe. However, co-infusion of adenosine (5.0 $\mu g \cdot min^{-1} \cdot dl^{-1}$) and dipyridamole (100 $\mu g \cdot min^{-1} \cdot dl^{-1}$) increased dialysate adenosine from the intravascular probe (0.07 ± 0.02 to 0.39 ± 0.14 $nmol \cdot ml^{-1}$, n=9, P<0.05), without affecting muscle interstitial adenosine.
Conclusions: (1) Dipyridamole significantly inhibits intravascular as well as interstitial adenosine uptake, without potentiating the exercise-pressor response, indicating that interstitial adenosine is not involved in the pressor response in these healthy volunteers. (2) There is a strong blood-muscle barrier for adenosine, which is not significantly reduced by inhibition of dipyridamole-sensitive nucleoside transport. This observation supports the concept that the pharmacological actions of intra-arterially infused adenosine are mediated by endothelial or circulating cells.

Introduction

The endogenous nucleoside adenosine exerts several physiological effects via stimulation of specific membrane-bound adenosine receptors [1]. Adenosine receptor stimulation induces vasodilation in most vascular beds, inhibits noradrenaline release from sympathetic nerve endings, inhibits thrombocyte-aggregation and induces anti-arrhythmic effects [2-5]. It plays an important role in ischemic preconditioning, a naturally occuring phenomenon in which ischemia-induced cell death is reduced by a previous non-lethal bout of ischemia [6;7]. Acting in concert, these effects of adenosine provide this molecule with unique protective properties against the sequelae of ischemia. In addition, adenosine may prevent the development of atherosclerosis by preventing proliferation of vascular smooth muscle cells and by inhibition of vascular inflammation [8;9]. Finally, adenosine is considered a potential trigger for the exercise-pressor reflex, which is defined as a reflex rise in blood pressure resulting from isometric exercise, by stimulation of muscle afferent fibers after release during exercise [10-12]. Infusion of adenosine into the brachial artery of healthy volunteers results in forearm vasodilation, inhibition of noradrenaline release from local sympathetic nerve endings and generalized activation of the sympathetic nervous system by activating local metabolic afferents [4;12;13]. Although there is not much doubt that these actions are mediated by adenosine receptors, there is an ongoing debate about the cell types that mediate the effects of intravascular adenosine. For example, vasodilation may occur by stimulation of adenosine receptors on endothelial cells or vascular smooth muscle cells and the relative contribution of these cell types on adenosine-induced vasodilation is not known [14;15]. Indeed, a recent report from our group suggests a difference in vasodilator mechanism between circulating and interstitial adenosine (Glibenclamide inhibits dipyridamole-induced forearm vasodilation but not adenosine-induced forearm vasodilation, Bijlstra and van Ginneken *et al*, Clin Pharm Ther 2004). Similarly, it is unknown whether intra-arterial infusion of adenosine directly stimulates metabolic afferents or, alternatively, stimulates the endothelium to release a substance that subsequently triggers generalized sympatho-excitation. The same uncertainty exists for the inhibiting action of intravascular adenosine on local release of noradrenaline: does adenosine act directly on presynaptic nerve endings or is this action mediated by an endothelium-derived factor. Likewise, the protective effect of intra-arterially-infused adenosine against ischemic cell death, as shown in various models [7;16;17], may result from a direct effect on interstitial cells or from the increased release of an endothelium-derived factor.

To provide more insight in the contribution of the various cell types in the action of endogenous or infused adenosine, it is important to be accurately informed about the kinetics of extracellular adenosine in interstitial as well as intravascular compartments. Although much has been learned from animal experiments and *in vitro* studies, human *in vivo* data are scarse. Recently, the microdialysis technique has been introduced to determine tissue adenosine concentrations in humans. Using this method, previous studies showed an increase of muscle interstitial adenosine concentration during exercise [12;18-21]. The present studies were conducted to characterize the effect of dipyridamole, a nucleoside transport inhibitor, on the kinetics of interstitial and circulating adenosine. In addition, intra-arterial infusion of dipyridamole in combination with intramuscular and intravascular monitoring of adenosine was performed to test two hypotheses: (1) adenosine receptor stimulation mediates the pressor response to exercise and therefore, this pressor response is

augmented by inhibition of cellular uptake of adenosine; (2) dipyridamole sensitive cellular uptake of adenosine prevents the passage of intravascular adenosine into the interstitial compartment.

Methods

Subjects

The study-protocols were approved by the local ethics comittee, and each subject gave written informed consent before participation. The demographic data of the 26 participants of the substudies are shown in table 1. The subjects were normotensive nonsmokers and were not taking any medications except for oral contraceptives. The subjects underwent a physical examination including an ECG before entering the study. Subjects with a history of pulmonary or cardiovascular disease were excluded. Participants were asked to abstain from food-intake 2 hours prior to the study. For subjects participating in protocol 1, caffeine-containing beverages were not allowed 24 hours before the start of each study. Protocol 2 was performed without caffeine abstinence to prevent systemic side effects from the adenosine infusion in the presence of a high dose of intra-arterially infused dipyridamole.

Table 1: demographic characteristics (mean ± SD)

	Study 1 Dipyridamole	Study 1 Time control	Study 2
Number (M/F)	9 (1/8)	8 (5/3)	9 (5/4)
Age (years)	20.5 ± 1.7	21.0 ± 1.2	23.7 ± 2.8
BMI (kg m^2)	22.9 ± 2.3	21.9 ± 2.6	22.9 ± 2.1
SBP (mmHg)*	120.0 ± 5.2	123.5 ± 11.4	122.7 ± 5.3
DBP (mmHg)*	73.0 ± 7.8	72.8 ± 6.2	73.9 ± 8.0
HR (bpm)§	61.8 ± 10.8	63.5 ± 9.7	66.9 ± 9.7
Cholesterol ($mmol \cdot l^{-1}$)	4.6 ± 0.5	4.0 ± 0.7	4.0 ± 0.7
Triglycerides($mmol \cdot l^{-}1$)	1.0 ± 0.2	0.9 ± 0.5	1.0 ± 0.4
Glucose ($mmol \cdot l^{-1}$)	4.5 ± 0.4	4.6 ± 0.4	4.7 ± 0.3

* Auscultatory measurement after 5 minutes rest in supine position
§ Measured by pulse frequency counting after 5 minutes of supine rest

General outline of the procedure

The experiments started in the morning in a quiet room with stable temperature (23°C), with the subjects in supine position. Heart rate was monitored continuously with surface ECG. After local anaesthesia (xylocaine 2%), the brachial artery of the non-dominant arm was cannulated (Angiocath, 20 gauge, Deseret Medical, Becton Dickinson Sandy, UT, USA) for drug infusion (syringe infusion pump, type STC-521, Terumo Corp., Tokyo, Japan) and blood pressure recording (Hewlett Packard GmbH, Böblingen, Germany). In the first protocol, blood pressure was measured at two-minute intervals at the dominant arm, using an automated device (Dinamap). Drug- and volume infusion rates (50 $\mu l \cdot min^{-1} \cdot dl^{-1}$ forearm) were adjusted to forearm volume, which was measured for each person by water displacement.
The rate of infused volume and the amount of connected syringes was kept constant throughout each experiment.
After local anesthesia, a microdialysis probe (CMA 70 brain microdialysis catheter, Stockholm, Sweden) was inserted into the flexor digitorum superficialis muscle of the non-dominant arm, guided by a venflon cannula (14 G, Ohmeda, Sweden). The probe had a dialysis tubing of 10 x 0.6 mm with a membrane cut-off of 20.000 Dalton. In the second experiment ('protocol 2') a second, identical probe was inserted retrogradely into a deep antecubital vein of the same arm, guided by a venflon cannula (16 G, Ohmeda, Sweden). The probes were continuously perfused with NaCl 0.9% (perfusate) with a microdialysis pump (CMA 107, Stockholm, Sweden) at a rate of 2 $\mu l \cdot min^{-1}$. The effluent (dialysate) was collected at 15 minute intervals to obtain 30 µl samples. Dialysate collections were shifted with 2.5 minutes in relation to the exercise period because of lag time due to the volume of the collecting tube. Samples were kept on ice and protected from light before they were stored at –20 ^{o}C untill analysis.
After finishing the second protocol, the dialysis catheters were brought to the laboratory for *in vitro* callibration of adenosine recovery, as described before (see also 'analytical procedures') [18].

Study 1: Interstitial adenosine measurements during repeated handgrip with and without dipyridamole

Maximal force of handgrip was determined after insertion of the microdialysis probe (Baseline Hydrolic Hand Dynamometer, Fabrication Enterprise Inc., Irvington, NY, USA). Sampling of microdialysate started immediately after insertion of the microdialysis probe. Immediately after insertion of the probe, interstitial levels of adenosine are known to be particularly high, likely due to insertion-related muscle fiber injury. In a pilot study (data not shown), we found that dialysate concentrations of adenosine returned to a stable baseline level within 1.5 hours. Therefore, baseline resting values were determined in 2 consecutive 30 µl dialysate samples that were obtained 1.5 hours after insertion of the microdialysate probe. Thereafter, subjects performed intermittent handgrip during 15 minutes, with 5-second contractions at 50% of maximal force every 10-seconds while dialysate sampling continued. One hour later, this procedure was repeated in the absence (n=8) or presence (n=9) of dipyridamole infusion into the brachial artery (12 $\mu g \cdot min^{-1} \cdot dl^{-1}$) which started 15 minutes prior to the second period of contractions and was continued throughout the exercise.

Study 2: Simultaneous intramuscular and intravascular adenosine measurements during intra-brachial infusion of adenosine with and without dipyridamole

In a separate group of 9 volunteers, 1.5 hours after instrumentation, baseline samples were obtained during intra-brachial infusion of NaCl 0.9%. Thereafter, increasing doses of adenosine (5, 15 and 50 $\mu g \cdot min^{-1} \cdot dl^{-1}$) were co-infused with saline. Each infusion lasted 20 minutes and the succeeding infusions were interrupted by a 5 min drug free interval. After one hour, recontrol values were obtained during infusion of NaCl 0.9% followed by dipyridamole 100 $\mu g \cdot min^{-1} \cdot dl^{-1}$. Subsequently, the lowest dose of adenosine was repeated together with dipyridamole. Infusion of the two higher doses of adenosine appeared to be impossible in the presence of dipyridamole, due to the occurance of systemic side effects that resemble the action of intravenous infusion of adenosine such as chest pain, hyperventilation and an increase in heart rate.

Analytical procedures

Dialysate samples were analyzed for concentrations of adenosine using high performance liquid chromatography, equipped with a UV detector set at 254 nm. The nucleotides were separated on a Lichrosorb RP18-column. A binary low-pressure gradient elution was used with eluent A consisting of di-potassium-hydrogen-phosphate (0.1 M) and tetra-butyl-ammonium-hydrogen-sulfate (10 mM) as the ion-pair forming agent. The pH was adjusted to 6.5 with HCl. Solvent B contained, in addition, 40% (v/v) methanol. In addition, in the time-control study of protocol 1 creatine and phosphocreatine were measured spectrophotometrically with Merck kit nr. 12320, without use of creatininase. In protocol 2, *in vitro* recovery of the purines was measured in 9 intramuscular catheters and in 6 intravenous catheters (3 catheters were damaged due to removal). After the experiment was finished, the catheters were removed from the arm and placed in a homogeneously mixed solution that contained 0.8 µM adenosine. The catheters were perfused with NaCl 0.9% at a rate of 2 $\mu l \cdot min^{-1}$. The dialysate was collected in two 10-minute fractions. The percentage recovery was calculated by dividing the measured dialysate adenosine concentration by the concentration from a sample taken directly from the solution.

Drugs and solutions

All solutions were freshly prepared. Adenosine (*Adenocor*, Sanofi-Synthelabo) and dipyridamole (*Persantin*, Boehringer Ingelheim) were diluted in NaCl 0.9% to reach the necessary concentrations.

Statistical analysis

Blood pressure was measured at two-minute intervals at the dominant arm, using an automated device (Dinamap). All blood pressure values during the 15 minute-contraction periods were averaged to one value. This value was compared with the

mean value of a baseline period of 15 minutes immediately before contraction. All results are expressed as mean ± SE, unless indicated otherwise.
T-tests on absolute values of adenosine concentrations and blood pressure were used to explore the effects of exercise with and without dipyridamole. To avoid multiple comparison, the effect of dipyridamole on interstitial versus intravascular adenosine concentrations was assessed with repeated measures ANOVA. The presence of dipyridamole and adenosine doses were used as within subject factors. $P<0.05$ (two sided) was considered statistically significant.

Results

Study 1: Interstitial adenosine measurements during repeated handgrip with and without dipyridamole

Two cycles of handgrip without intra-arterial infusion of dipyridamole increased dialysate-adenosine by 0.25 ± 0.10 and 0.15 ± 0.07 nmol·ml^{-1} respectively ($p<0.05$ for the effect of exercise; $p> 0.1$ for the comparison between the first and the second period of contractions; n=8, see figure 1). Creatine (C) en creatine-phosphate (CP) (summed as (phospho)creatine) concentrations were measured in seven from the eight volunteers as indicator of muscle cell damage. Due to insertion-damage, dialysate (phospho)creatine was high immediately after insertion (330.1 ± 82.3 nmol·ml^{-1}, n=7). One and a half hour later, (phospho) creatine had stabilized (64.6 ± 11.4 nmol·ml^{-1}). During subsequent exercise, dialysate (phospho)creatine increased by 150.5 ± 58.1 nmol·l^{-1}. Prior to the second bout of exercise, (phospho)creatine had returned to baseline (78.4 ± 14.3 nmol·ml^{-1}) and increased by 44.5 ± 18.8 nmol·ml^{-1} during the second period of exercise. The ratio of dialysate (phospho)creatine to adenosine just after insertion did not significantly differ from the ratio during the exercise periods (1291 ± 477 vs 1033 ± 434 and 805 ± 181, $P>0.1$).
The handgrip-induced rise in blood pressure (SBP/DBP) was 15.1 ± 2.8 / 9.8 ± 2 for the first contraction period and 17.3 ± 3.7 / 14.3 ± 1.6 mm Hg for the second contraction period (p=NS for SBP, $p<0.05$ for DBP for comparison between first and second period of contractions; see figure 2).
In a separate group of 9 volunteers, infusion of dipyridamole 12 µg·min^{-1}·dl^{-1} into the brachial artery significantly potentiated the exercise-induced increase in dialysate-adenosine from 0.30 ± 0.08 to 0.48 ± 0.10 nmol·ml^{-1} ($p<0.05$ for effect of dipyridamole). There was a significant difference in exercise-induced increase in dialysate-adenosine between the group with and without dipyridamole (p=0.01; unpaired t-test). As opposed to the dialysate adenosine concentration, dipyridamole did not potentiate the exercise-induced increase in blood pressure; 9.6 ± 2.4 / 4.5 ± 2.0 and 10.4 ± 2.2 / 7.0 ± 1.3 mm Hg in the presence and absence of dipyridamole, respectively (see figure 2).

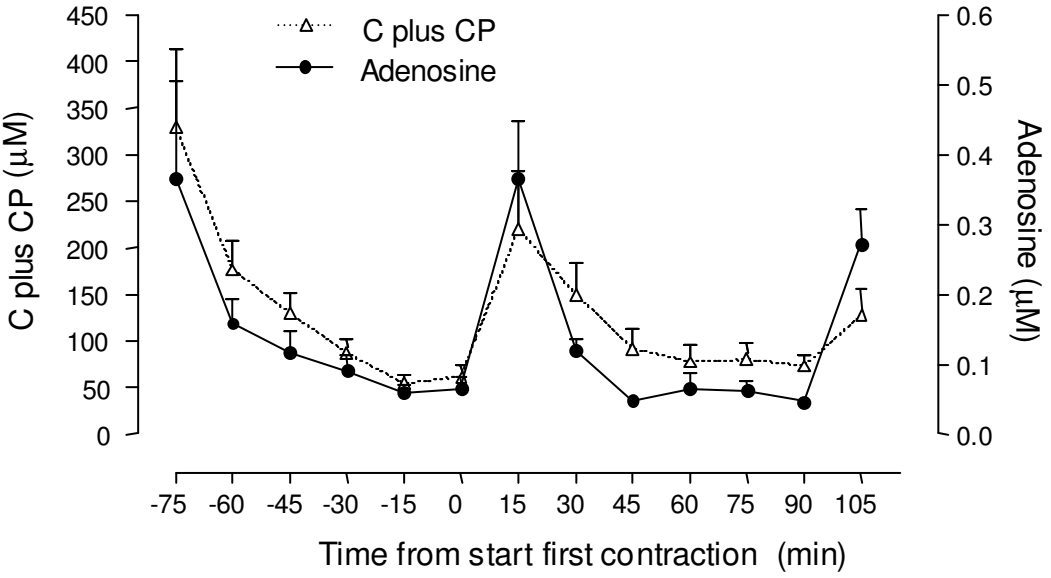

Figure 1. Course in dialysate adenosine (µM) and creatine + creatine phosphate (µM), during study 1 (repeated hangrip without dipyridamol infusion, n=8).

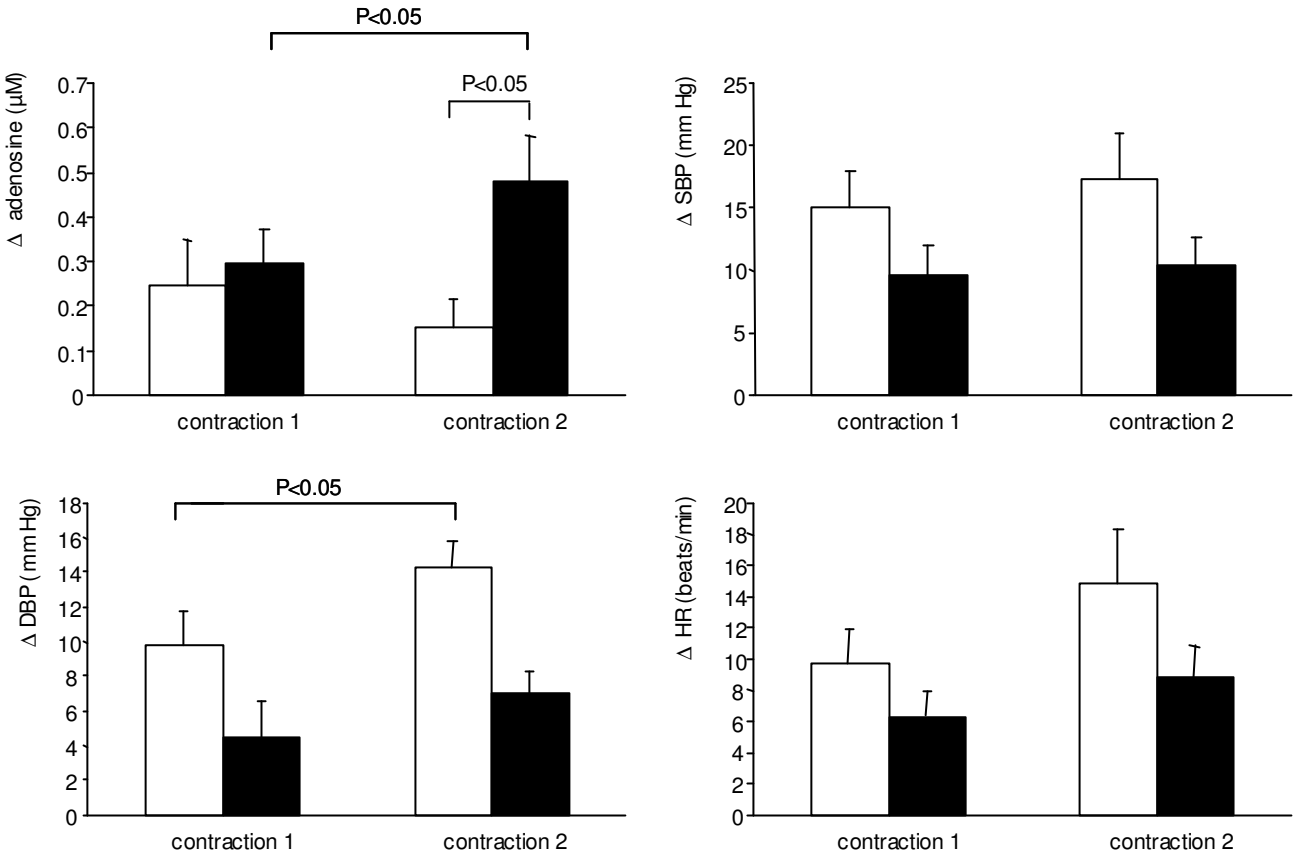

Figure 2. Delta dialysate adenosine (µM), delta Systolic Blood Pressure, delta Diastolic Blood Pressure and delta Heart Rate during repeated handgrip with (black, n=9) and without (white, n=8) intra-arterial infusion of dipyridamole 12 $\mu g \cdot min^{-1} \cdot dl^{-1}$.

Study 2: Simultaneous intramuscular and intravascular adenosine measurements during intra-brachial infusion of adenosine with and without dipyridamole

In the absence of dipyridamole, intra-arterial infusion of adenosine did not significantly affect dialysate adenosine concentrations from either intravascular and intramuscular probe (see figure 3). Likewise, infusion of dipyridamole alone (100 $\mu g \cdot dl^{-1} \cdot min^{-1}$) did not significantly affect dialysate adenosine concentration from either probe. However, infusion of adenosine together with dipyridamole significantly increased adenosine in dialysate from the intravascular probe from 0.07 ± 0.02 to 0.39 ± 0.14 $nmol \cdot ml^{-1}$ ($p<0.05$; n=9), without affecting adenosine in the dialysate from the intramuscular probe (0.08 ± 0.02 $nmol \cdot ml^{-1}$ during dipyridamole versus 0.08 ± 0.02 $nmol \cdot ml^{-1}$ during dipyridamole plus adenosine; $p>0.1$, n=9).
The *in vitro* adenosine-recovery from the intramuscular and intravenous catheter was measured in 6 from 9 persons and mounted 42 ± 4 versus 49 ± 6%, respectively ($p>0.1$).

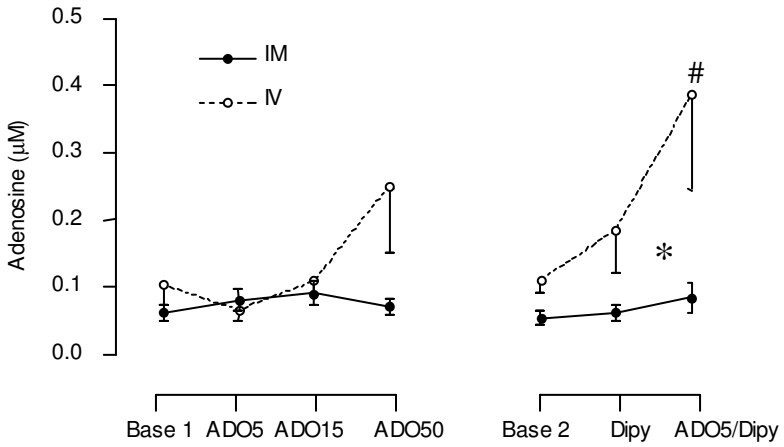

Figure 3. Course in dialysate adenosine during study 2 (n=9); $p<0.05$ for IV vs IM, $p<0.05$ for IV-adenosine with versus without dipyridamole. *:$p<0.05$ IV vs. IM. #: $p<0.05$ vs. ADO 5.

Discussion

In this study we report three main findings in man: intra-arterial infusion of dipyridamole significantly inhibits the cellular uptake of both interstitial and circulating adenosine; the pressor response to rhythmic handgrip is not affected by inhibition of cellular uptake of interstitial adenosine, indicating a minor role for adenosine in this hemodynamic response to exercise in healthy volunteers; finally, circulating adenosine does not significantly enter the interstitial compartment and this barrier does not depend on an intact dipyridamole-sensitive equilibrative nucleoside transporter.

The pressor response to rhythmic handgrip is not mediated by adenosine.

The exercise-pressor reflex is defined as a sympathoneural activation and a subsequent rise in blood pressure in response to exercise. This reflex contributes to a redistribution of blood flow in favor of exercising muscle. Afferent signals from the exercising muscle that trigger this reflex originate from stimulated mechano- and metaboreceptors. Several substances that locally accumulate during exercise have been implicated in the stimulation of metaboreceptors, including adenosine, potassium and lactate [12;21]. Experimental evidence for adenosine as a trigger of the exercise-pressor reflex are threefold: first, infusion of adenosine into the brachial artery stimulates local afferents which results in a generalized sympatho-excitation [10;12;13]. Second, infusion of theophylline, an adenosine receptor antagonist, into the brachial artery inhibits the forearm exercise-induced sympatho-excitation and increase in blood pressure [11]. Finally, graded hand-grip exercise results in a graded increase in interstitial adenosine as measured with microdialysis which correlates with the sympathetic arousal [12]. An increase in hand-grip intensity from 15% to 50% of maximal force resulted approximately in a doubling of the exercise-induced increase in interstitial adenosine as well as of the increase in sympathetic arousal. This observation triggered us to perform the first study in this report. Our observation indicates that, in contrast to the reported effect of graded exercise, pharmacological augmentation of the exercise-related increase in adenosine concentration did not potentiate the pressor response to exercise. Based on this finding, we further hypothesized that the previously reported correlation between interstitial adenosine and exercise-induced sympatho-excitation is confounded by mechanical injury which is associated with exercise-intensity and which is limited to muscle fibers that are located near the microdialysis probe. To further assess the role of muscle fiber injury as a source of interstitial adenosine during exercise, creatine and creatine phosphate were measured in microdialysate that was obtained during the time-control study. Creatine and creatine phosphate are found in high intracellular concentrations in muscle cells and cannot diffuse freely out of these cells [22] but easily pass the semipermeable microdialysis membrane. We assumed an increase of these substances in the microdialysate to indicate muscle fiber rupture. Since exercise results in dephosphorylation of creatine phosphate, the sum of creatine and creatine phosphate (refered to as (phospho)creatine) was used as a marker of muscle fiber injury. Immediately after insertion of the microdialysis probe, dialysate levels of adenosine and (phospho)creatine are elevated, reflecting pure mechanical injury of muscle fibers. During exercise, a similar increase in (phospho)creatine and adenosine occured and the ratio of (phospho)creatine to adenosine did not significantly differ between samples obtained during exercise and those obtained immediately after insertion of the probe. This observation indicates that mechanical injury of muscle fibers significantly contributes to the increase of adenosine in the microdialysate during exercise and reflects an artefact that is due to the presence of a microdialysis probe in the exercising muscle. Dipyridamole significantly potentiated this exercise-induced increase in dialysate adenosine concentration, indicating significant inhibition of cellular uptake of interstitial adenosine. Nevertheless, dipyridamole did not potentiate the exercise-induced increase in blood pressure. Thus, in healthy volunteers, muscle interstitial adenosine is not involved as a trigger of the pressor response to exercise. This conclusion is supported by a recent study in healthy volunteers and patients with heart failure. In this study, systemic infusion of the adenosine receptor antagonist caffeine inhibited the sympatho-excitation during

post handgrip ischemia in patients with heart failure but had no effect in healthy volunteers [23] suggesting that adenosine may play a more important role in the exercise-pressor reflex in patients with reduced skeletal muscle perfusion as compared with healthy volunteers. In this context, it is important to note that the isometric exercise in the present study, as in previous studies with microdialysis, was performed rhythmically to allow volunteers to sustain the exercise for a sufficient period of time to complete microdialysis sampling. Therefore, skeletal muscle perfusion was restored during each 5 second interval of relaxation. This experimental set-up differs from studies in which caffeine or theophylline were used to block adenosine receptors during exercise. In these studies, sustained isometric exercise was used and sometimes combined with occlusion of forearm circulation. Isometric exercise in combination with circulatory arrest produced a greater increase in muscle sympathetic nerve activity than isometric exercise alone [11]. The resulting ischemia probably augmented adenosine release and accumulation which increased the involvement of adenosine as a trigger of the exercise-pressor reflex. The intermittent restoration of muscle perfusion in the present study prevented accumulation of metabolites such as adenosine and explains why our conclusion differs from a previous report by others [11]. In the present setting, stimulation of mechanoreceptors probably fully accounts for the observed pressor response to exercise.

The blood-muscle barrier for adenosine is not reduced by dipyridamole.

Since the biological importance of circulating and interstitial adenosine differs and the concentration of adenosine may vary between these two compartments, it is important to be informed about the adenosine concentration in both compartments. Microdialysis could serve this goal. However, insertion of a microdialysis probe disrupts the microcirculation. Subsequent access of circulating blood to the intramuscular microdialysis probe could complicate the separate determination of interstitial and intravascular adenosine. The results from study 2 demonstrate that a significant increase in intravascular adenosine was not accompanied by an increase in adenosine in dialysate from the intramuscular probe, indicating that traumatic admixture of circulating blood with interstitial fluid does not occur. This finding supports extrapolation of previous observations in animal in vitro models to the human in vivo (micro)circulation that circulating adenosine has only minor access to the interstitial space [24-26]. Our observation is also in accordance with a study by Costa et al. in which they showed that 15 minutes of forearm ischemia significantly increased the intravenous adenosine concentration but did not affect the muscle interstitial concentration [27]. Interestingly, intravascular administation of adenosine has repeatedly been shown to prevent ischemia/reperfusion injury of myocardial and skeletal muscle cells [7;16;17]. Our present observations indicate that intravascular adenosine does not sufficiently reach the muscle cells to stimulate adenosine receptors on these cells and therefore suggest an important role for the endothelium or circulating blood cells in mediating this beneficial action of adenosine.
Despite significant inhibition of cellular uptake of circulating adenosine, dipyridamole did not improve access of infused adenosine to the interstitial compartment.This finding contrasts with a recent report from Gamboa et al. [28]. An important difference between the two studies is that we infused dipyridamole into the brachial artery, whereas Gamboa administered this substance intravenously. The intravenous

administration of a relatively high dose of the nucleoside transport inhibitor probably resulted in a generalized sympatho-excitation [29-31]. Consequently, the experimental design that was applied by Gamboa et al. may have resulted in a more pronounced perivascular release of noradrenaline and sympathetic co-transmitters such as adenosine-5'-triphosphate (ATP) [32] as compared to our present experimental set-up. Due to the abundent presence of ecto-phosphatases [33], this ATP is rapidly metabolized to adenosine and provides an alternative source for the rise in interstitial concentration of adenosine that was observed by Gamboa when intra-arterial infusion of adenosine is combined with intravenous administration of dipyridamole.

In conclusion, intra-arterial infusion of dipyridamole inhibits uptake of both interstitial and intravascular adenosine. Dipyridamole did not affect the blood pressure response to exercise nor the blood-muscle barrier for intravascular adenosine. These observations indicate that interstitial adenosine is not the trigger of the pressor response to rhythmic handgrip in healthy volunteers and that the dipyridamole-sensitive nucleoside transporter is not critically involved in the blood-muscle barrier for adenosine.

Acknowledgment

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CHAPTER 3

Glibenclamide inhibits dipyridamole-induced forearm vasodilation but not adenosine-induced forearm vasodilation

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Abstract

The mechanism of the vasodilator response to adenosine has not been elucidated in humans. Stimulation of adenosine receptors on endothelial and vascular smooth muscle cells with subsequent endothelial release of nitric oxide and opening of ATP-sensitive potassium (K_{ATP}) channels has been suggested. Aim of the study: to investigate the involvement of K_{ATP} channels in the vasodilator response to adenosine and the nucleoside transport inhibitor dipyridamole.
In healthy male volunteers, adenosine (0.6, 1.9, 5.6, 19, 57 and 190 nmol·min^{-1}·dl^{-1}) as infused into the brachial artery and forearm blood flow (FBF) was measured using strain gauge plethysmography. Adenosine increased the ratio in FBF (FBF-ratio = FBF experimental arm / FBF control arm) from 1.3 ± 0.2 to 1.2 ± 0.2, 1.5 ± 0.2, 2.8 ± 0.4, 7.3 ± 2.3, 11.1 ± 4.1 and 12.9 ± 3.7 for the 6 increasing adenosine doses respectively. Simultaneous infusion of glibenclamide, a blocker of K_{ATP}-channels, did not affect this response (from 1.7 ± 0.4 to 1.5 ± 0.2, 2.2 ± 0.3, 4.0 ± 1.0, 9.3 ± 4.0, 13.5 ± 6.4 and 15.9 ± 5.3 for the six increasing doses of adenosine respectively; p=0.439, N=6). The increase in FBF-ratio during infusion of the nucleoside transport inhibitor dipyridamole (20, 60 and 200 nmol·min^{-1}·dl^{-1}) was significantly reduced by glibenclamide: from 1.2 ± 0.1 to 1.7 ± 0.2, 2.4 ± 0.5 and 2.9 ± 0.4 during saline versus from 1.6 ± 0.2 to 1.8 ± 0.2, 2.1 ± 0.3 and 2.2 ± 0.4 during glibenclamide (p=0.010 for effect of glibenclamide on response from baseline, ANOVA for repeated measures; N=8). The vasodilator response to dipyridamole was significantly inhibited by the adenosine receptor antagonist theophylline.
Opening of vascular K_{ATP} channels is involved in the forearm vasodilator response to dipyridamole but not to adenosine. Differences in stimulated cell type (endothelium for adenosine versus smooth muscle cells for dipyridamole) may underly this divergent pharmacological profile.

Introduction

Extracellular adenosine stimulates specific adenosine receptors, which are classified as A_1, A_{2a}, A_{2b} and A_3 receptors [1]. Stimulation of A1 adenosine receptors triggers and mediates ischemic preconditioning of cardiac and skeletal muscle, a naturally occuring adaptive process that delays ischemia-induced cell death [2-4]. Furthermore, adenosine inhibits thrombocyte aggregation and leucocyte activation [5;6], it modulates sympathetic noradrenaline release [7;8], and it is a potent vasodilator [9]. With respect to the vasodilator action of adenosine, animal studies suggest an important role for the A_{2a} receptor on vascular smooth muscle cells and endothelial cells, with subsequent opening of ATP-sensitive potassium (K_{ATP}) channels and stimulation of endothelial release of nitric oxide [10;11].
The mechanism of the vasodilator action of adenosine in humans is less well characterized. In the human forearm vascular bed of healthy male volunteers, adenosine-induced vasodilation is inhibited by the non-selective A_1 and A_2 receptor antagonists caffeine and theophylline [12;13] and potentiated by the nucleoside transport inhibitor draflazine [14] indicating the involvement of specific adenosine receptors on the cell membrane. Furthermore, its vasodilator action is inhibited by the NO-synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) but not by the K_{ATP} channel blocker tolbutamide [15]. These initial observations suggested the involvement of endothelial nitric oxide but not K_{ATP} channels in adenosine-induced forearm vasodilation [15]. However, this study has some limitations. First, intra-arterially infused adenosine may not reach the vascular smooth muscle cells due to rapid endothelial uptake and metabolism of adenosine [16]. Adenosine receptors on endothelial and smooth muscle cells may differ in their capacity to open K_{ATP} channels [11]. Second, glibenclamide is a more potent blocker of vascular K_{ATP} channels than tolbutamide in the human forearm [17]. Therefore, the negative findings in our initial studies with tolbutamide do not exclude the involvement of vascular K_{ATP} channels in adenosine-induced forearm vasodilation.
We hypothesize that the nucleoside transport inhibitor dipyridamole increases endogenous levels of adenosine at the adventitial side of the endothelium (see figure 1). Consequently, intraluminally applied dipyridamole (as opposed to adenosine) will indirectly stimulate adenosine receptors on vascular smooth muscle cells, while intraluminally applied adenosine (as opposed to dipyridamole) stimulates endothelial adenosine receptors. This hypothesis is supported by previous observations with the nucleoside transport inhibitor draflazine: its hemodynamic actions resembled that of adenosine without increasing plasma adenosine concentrations [14] and it is able to modulate sympathetic release of norepinephrine presynaptically [8]. Further support comes from in vitro studies that emphasize the important barrier function of the endothelium for adenosine between the interstitial and intravascular compartment [16;18]. Based on this model of action of adenosine and dipyridamole, we asked ourselves the following questions:
1. Does the involvement of K_{ATP} channels differ between adenosine and dipyridamole?
2. Is the forearm vasodilator action of dipyridamole mediated by stimulation of adenosine receptors?

To answer the first question, glibenclamide was used as a blocker of K_{ATP} channels in the human forearm [19]. To assess the second question, we used the A_1 and A_2-adenosine receptor antagonist theophylline [12;13].

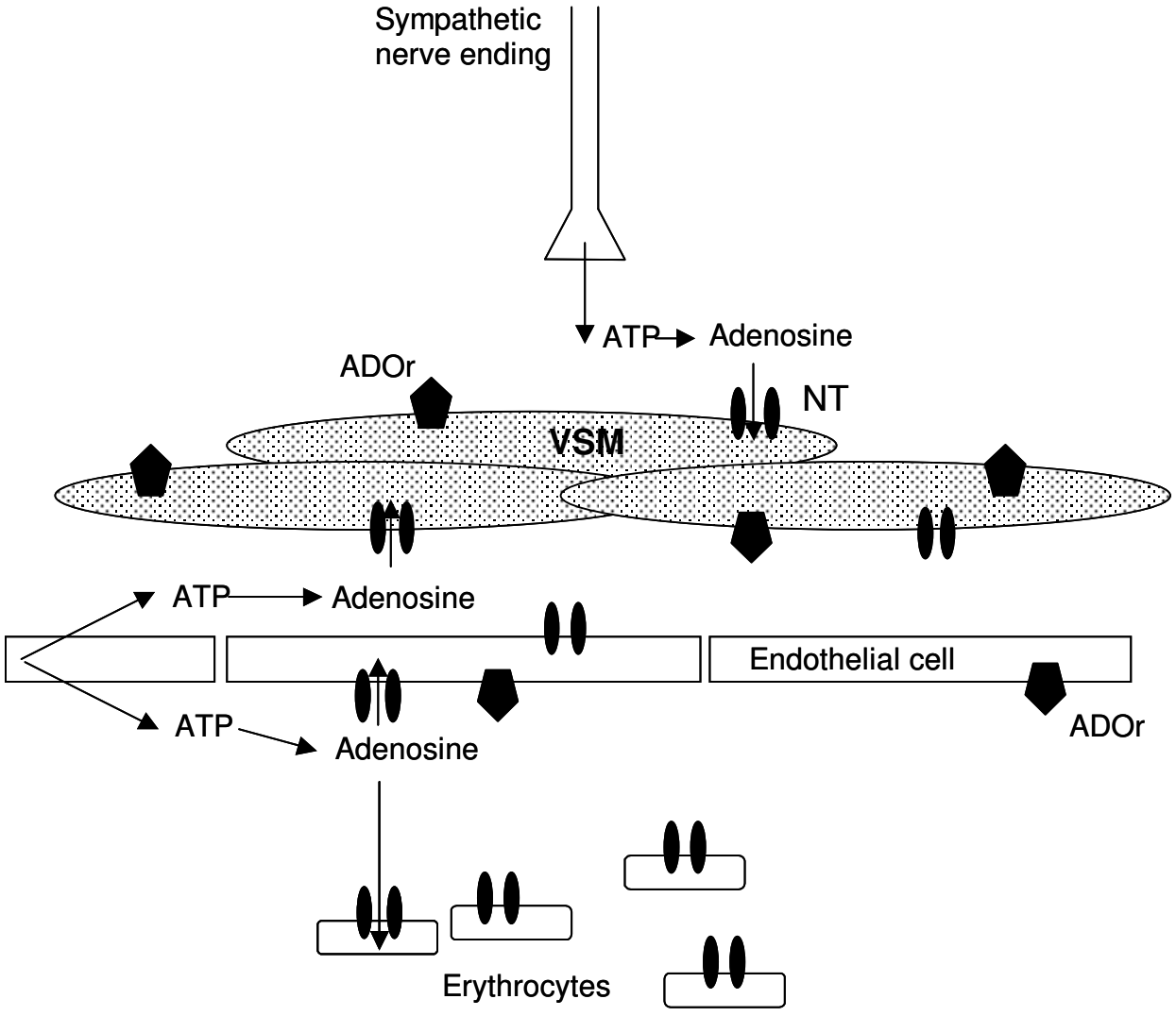

Figure 1: Schematic presentation of our working hypothesis for this study.
Intra-arterial infusion of adenosine will stimulate endothelial adenosine receptors (ADOr) resulting in (endothelium-dependent) vasodilation. Intraluminal adenosine is rapidly taken up by erythrocytes, endothelial cells and possibly also vascular smooth muscle cells (VSM). This process of facilitated diffusion is mediated by nucleoside transporters (NT) and prevents intraluminal adenosine from stimulating ADOr on VSM. Intraluminal infusion of dipyridamole, an inhibitor of NT, will result in accumulation of endogenous adenosine that may originate from endothelial cells and sympathetic nerve endings. This accumulated endogenous adenosine will stimulate ADOr on VSM.

Methods

Subjects

The study protocol was approved by the local ethics committee, and all participants gave written informed consent before entering the study. All experiments were performed in healthy male non-smoking volunteers with a normal history, physical examination and blood pressure. The characteristics of the subsets of volunteers are listed in Table 1. Each volunteer participated in only one experiment and was instructed to abstain from caffeine-containing beverages and alcohol for at least 24 hours before the experiment. Furthermore, they were asked to use a light meal at least 2 hours before the experiment was started and to abstain from further food intake until after the experiment.

Table 1: Characteristics of the healthy volunteers

Experiment involving:	Adenosine x glibenclamide	Dipyridamole Time-control	Dipyridamole x Theophylline	Dipyridamole x Glibenclamide	Adenosine (+ Dipyridamole)x Glibenclamide
N	6	7	8	8	6
Age (years)	22 ± 4	27 ± 10	27 ± 7	21 ± 4	22 ± 3
Weight (kg)	69 ± 6	74 ± 14	76 ± 5	75 ± 4	68 ± 10
Height (m)	1.82 ± 0.07	1.81 ± 0.05	1.84 ± 0.09	1.83 ± 0.07	1.74 ± 0.09
SBP (mm Hg)*	109 ± 3	125 ± 9	122 ± 8	118 ± 3	124 ± 5
DBP (mm Hg)*	83 ± 6	73 ± 6	67 ± 5	63 ± 10	79 ± 4
Heart rate (bpm)	60 ± 4	65 ± 14	63 ± 5	61 ± 7	60 ± 7

Data are presented as means (± SD).*Sphygmomanometrically obtained blood pressure after 5 minutes supine rest. SBP: Systolic blood pressure; DBP: diastolic blood pressure.

General outline of the procedures

The experiments were performed with the subjects supine in a quiet temperature controlled room (22^{o}C). A cannula was inserted into the brachial artery (Angiocath, 20 gauge, Deseret Medical Inc., Becton Dickinson and Comp., Sandy, Utah) for intra-arterial blood pressure measurement (Hewlett Packard monitor, type 78353B, Hewlett Packard GmbH, Böblingen, Germany) and infusion of drugs (automated syringe infusion pump, type STC-521, Terumo Corp., Tokyo, Japan). Deep antecubital veins of the left and the right arm were cannulated for blood sampling. During intra-arterial infusion of saline (NaCl 0.9%) or drugs, forearm blood flow (FBF) was measured three times per minute at both arms simultaneously, using ECG-triggered venous occlusion mercury-in-silastic strain-gauge plethysmography

(Hokanson EC4, D.E. Hokanson, Inc., Issaquah, Washington, USA). During all FBF recordings, the hand circulation was completely occluded using a wrist cuff which was inflated 100 mmHg above the systolic blood pressure to confine FBF measurements to the forearm skeletal muscle vascular bed [20].
At least 45 minutes after all cannulations were done, intra-arterial infusion of saline was started. Ten minutes thereafter, the vasodilator agonist (adenosine or dipyridamole) was infused at incremental doses. After a subsequent wash-out period to allow parameters to return to baseline levels, measurements during intra-arterial infusion of placebo were repeated. Then, the agonist was infused again, but now with concomitant infusion into the brachial artery of the antagonist (theophylline or glibenclamide) instead of saline. Drugs or saline were infused at a constant rate of 50 $\mu l \cdot min^{-1} \cdot dl^{-1}$.

Vasodilator response to adenosine

Adenosine-glibenclamide study

In 6 subjects, the effect of glibenclamide on the adenosine-mediated vasodilation was studied. After baseline measurements of blood pressure, heart rate and bilateral FBF were performed during intra-arterial infusion of saline, incremental doses of adenosine were infused (0.6, 1.9, 5.6, 19, 57 and 190 $nmol \cdot min^{-1} \cdot dl^{-1}$ forearm), 4 minutes per dose. After a subsequent equilibration period of 60 minutes, baseline measurements were repeated. Then, intra-arterial infusion of adenosine was recommenced, but now with concomitant infusion of glibenclamide instead of saline. Glibenclamide was administered at a rate of 0.7 $nmol \cdot min^{-1} \cdot dl^{-1}$ forearm. We have previously shown in the same experimental set up that this dose leads to local therapeutic concentrations of glibenclamide equivalent to concentrations reached in type 2 diabetes on oral glibenclamide therapy and significant blockade of vascular K_{ATP} channels without systemic effects [19].
Venous blood samples were collected from the non-experimental arm to determine insulin and C-peptide concentrations at start, after 30 minutes and at the end of glibenclamide infusion. Blood glucose and glibenclamide concentrations were monitored in venous blood from the experimental arm at regular intervals during the glibenclamide infusion.

Vasodilator response to the nucleoside transport inhibitor dipyridamole

Time-control study

In order to detect possible carry-over effects, dipyridamole was infused into the brachial artery at 20, 60 and 200 $nmol \cdot min^{-1} \cdot dl^{-1}$ forearm, 5 minutes per dose during the concomitant administration of saline. This procedure was repeated after an equillibration period of 120 minutes. Originally, this experiment was performed in 8

volunteers. For unknown reasons, one volunteer showed a reproducible reduction in FBF-ratio in response to dipyridamole. This vasoconstrictor response was not observed in any other volunteer in this study, including those who participated in the other substudies with dipyridamole. Since inclusion of this outlier would hinder a valid comparison of the vasodilator action of dipyridamole between the three substudies, we decided to exclude this volunteer from further analysis.

Dipyridamole-theophylline study

In 8 subjects, the involvement of adenosine receptors in the vasodilator action of dipyridamole was studied. The same protocol as for the time-control study was performed but saline in the second part was substituted by the adenosine receptor antagonist theophylline. We administered theophylline at a rate of 130 nmol·min^{-1}·dl^{-1} forearm, which is sufficient to inhibit adenosine-induced forearm vasodilation [12;21]

Dipyridamole-glibenclamide study

As for adenosine, the role of vascular K_{ATP} channels in dipyridamole-induced vasodilation was studied using glibenclamide. We repeated the above mentioned protocol with glibenclamide instead of theophylline. Blood samples were drawn as for the adenosine-glibenclamide study. Glibenclamide concentrations were not determined in this set of experiments.

The vasodilator response to adenosine in the presence of dipyridamole

First, the vasomotor action of 7.4, 14.8 and 22.2 nmol dipyridamole ·min^{-1}·dl^{-1} was studied in 8 volunteers (dose-finding study). Each dose was infused for 15 minutes. The lowest dose appeared to lack a significant vasomotor action (data not shown). This dose was used in 7 volunteers to test its ability to potentiate the vasodilator action of adenosine. Adenosine was infused at a dose of 0.6 and 6 nmol·min^{-1}·dl^{-1}. One hour later, these doses were repeated in the presence of dipyridamole (7.4 nmol min/dl). These two substudies have been published previously [21].
In a separate group of 6 volunteers, the effect of glibenclamide was studied on adenosine-induced forearm vasodilation in the presence of dipyridamole. We hypothesized that inhibition of endothelial nucleoside transport would increase the biological availability of intra-arterially-infused adenosine to stimulate adenosine receptors on vascular smooth muscle cells. First, adenosine (0.6, 1.9, 5.6, 19, 57 and 190 nmol·min^{-1}·dl^{-1} forearm, 4 minutes per dose) was infused in the presence of dipyridamole (7.4 nmol·min^{-1}·dl^{-1}). Sixty minutes later, these infusions (adenosine plus dipyridamole) were repeated in the presence of glibenclamide (0.7 nmol·min^{-1}·dl^{-1}).

Analysis of blood samples

For determinations of plasma glibenclamide concentrations venous blood samples were collected in glass tubes without additives. After 20 minutes the blood was centrifuged at 3000 r.p.m. for 10 minutes. Then serum was frozen at -20^{o}C. In these serum samples drug concentrations were determined at the laboratories of Hoechst AG, Frankfurt, Germany. Glibenclamide was determined, using a validated specific radioimmunoassay (RIA) [22]. The detection limit was 1-3 ng·ml^{-1} and concentrations higher than 200 ng·ml^{-1} were diluted before measured according to the standard procedure. Insulin and C-peptide concentrations were determined in venous blood samples collected in chilled glass tubes coated with lithium-heparin. The blood was centrifuged at 3000 r.p.m. for 10 minutes. Then plasma was frozen at -20^{o}C. In these samples insulin and C-peptide were determined in our laboratories using specific RIAs. C-Peptide was measured with a standard kit (D.P.C., Los Angeles, CA, USA) and insulin with a procedure using standard and tracer prepared from monocomponent human insulin (NOVO, Zoeterwoude, The Netherlands). Blood glucose was measured using an Accutrend glucose analyzer (type 1284851, Boehringer, Mannheim, Germany).

Drugs and solutions

All solutions were freshly prepared on the study day. Dipyridamole (10 ml ampoules containing 5 mg dipyridamole ·ml^{-1}; Boehringer Ingelheim, Alkmaar, the Netherlands), adenosine (10 ml ampoules containing 20 mg of adenosine with NaCl 0.9% as solvent; Sigma Chemical Co., St Louis, MO) and theophylline (Euphyllin^{R}, 10 ml ampoules containing 24 mg·ml^{-1} aminophyllinum-hydricum; BYK Nederland, Zwanenburg,The Netherlands) were diluted in NaCl 0.9%. Lyophilized glibenclamide (2 mg per vial, Aventis Pharma, Frankfurt, Germany) was reconstituted with NaCl 0.9% on each study day.

Statistical analysis

Mean arterial pressure was measured continuously during each recording of forearm blood flow (FBF) and averaged per FBF registration. Experimental and control arms were analyzed separately. Since blood pressure gradually increased during the course of the experiment without relation to any intra-arterial drug infusion, forearm vascular resistance (FVR) was calculated as the quotient of simultaneously measured mean arterial pressure (MAP) and forearm blood flow (FBF) and expressed as arbitrary units (AU). Since intra-arterial drug infusions did not affect FVR in the control arm, random changes in forearm vascular tone were filtered out using the ratio of FBF in experimental and control arm which was taken as the main study parameter [23;24].
Based on previous experience in our laboratory with repeated infusion of intra-arterial adenosine [14], we calculated that a group of 6 volunteers would allow us to detect a minimal difference of 0.6 in absolute response or 61% in percentage response to 5.6 nmol adenosine min/dl, expressed as FBF ratio, with a power of 80% and alpha of 0.05. Similarly, a minimal difference of 6 in absolute response or 250% in percentage

response to 190 nmol adenosine min/dl can be detected with a power of 80% and alpha of 0.05.
Baseline parameters were compared by student t-tests. A two-way analysis of variance (ANOVA), with type of agonist (adenosine versus dipyridamole) as between group factor and presence of antagonist and agonist dose as within-subject factors, was used to test the hypothesis that the interaction with glibenclamide differed between adenosine and dipyridamole. For this test, only the first three adenosine doses were included since these doses induced an equal vasodilator response as compared with the three dipyridamole doses. Additionally, the effect of antagonists (or infusion order for the time-control study) on vasodilator responses were analysed by ANOVA for repeated measures with the used antagonist (or infusion order) and agonist dose as within-subject factors. A p-value of <0.05 was considered statistically significant. Results are presented as means ± SEM unless indicated otherwise.

Results

None of the infused drugs significantly affected forearm vascular resistance in the non-infused arm, blood pressure or heart rate, indicating the absence of a relevant systemic action of the intra-arterial drug infusions. Therefore, the data are presented as absolute (text) and percentage (figures) change in FBF ratio from control values.

Vasodilator response to adenosine

Adenosine-glibenclamide study

Adenosine increased the FBF ratio from 1.3 ± 0.2 at baseline to 1.2 ± 0.2, 1.5 ± 0.2, 2.8 ± 0.4, 7.3 ± 2.3, 11.1 ± 4.1 and 12.9 ± 3.7 during the six increasing adenosine doses. Sixty minutes later the FBF ratio had almost returned to baseline (1.9 ± 0.3; p=0.026 vs baseline). Repeated infusion of adenosine with glibenclamide increased FBF ratio from 1.7 ± 0.4 (glibenclamide alone; p=0.074 vs baseline and p=0.364 vs recontrol) to 1.5 ± 0.2, 2.2 ± 0.3, 4.0 ± 1.0, 9.3 ± 4.0, 13.5 ± 6.4 and 15.9 ± 5.3 during the six incremental doses of adenosine (p=0.439 for effect of glibenclamide; N=6). Similar results were obtained when percentage changes in FBF ratio were calculated (see figure 1). Intra-arterial glibenclamide did not affect blood glucose (from 4.6 ± 0.2 $mmol \cdot l^{-1}$ before to 4.4±0.4 $mmol \cdot l^{-1}$ at the end of glibenclamide infusion), plasma insulin (from 7.5 ± 1.2 to 8.8 ± 0.9 $mU \cdot ml^{-1}$) or plasma C-peptide concentrations (from 0.4 ± 0.1 to 0.4 ± 0.1 $ng \cdot ml^{-1}$). At the end of a 10 minute infusion of glibenclamide, the regional serum concentration averaged 162 ± 14 $ng \cdot ml^{-1}$. During simultaneous intra-arterial infusion of glibenclamide and adenosine, serum glibenclamide concentrations dropped to 43 ± 8 $ng \cdot ml^{-1}$ and 21 ± 4 $ng \cdot ml^{-1}$ at the end of 5.6 and 190 nmol adenosine $\cdot min^{-1} \cdot dl^{-1}$ respectively, reflecting adenosine-induced increases in forearm blood flow with subsequent dilution of glibenclamide.

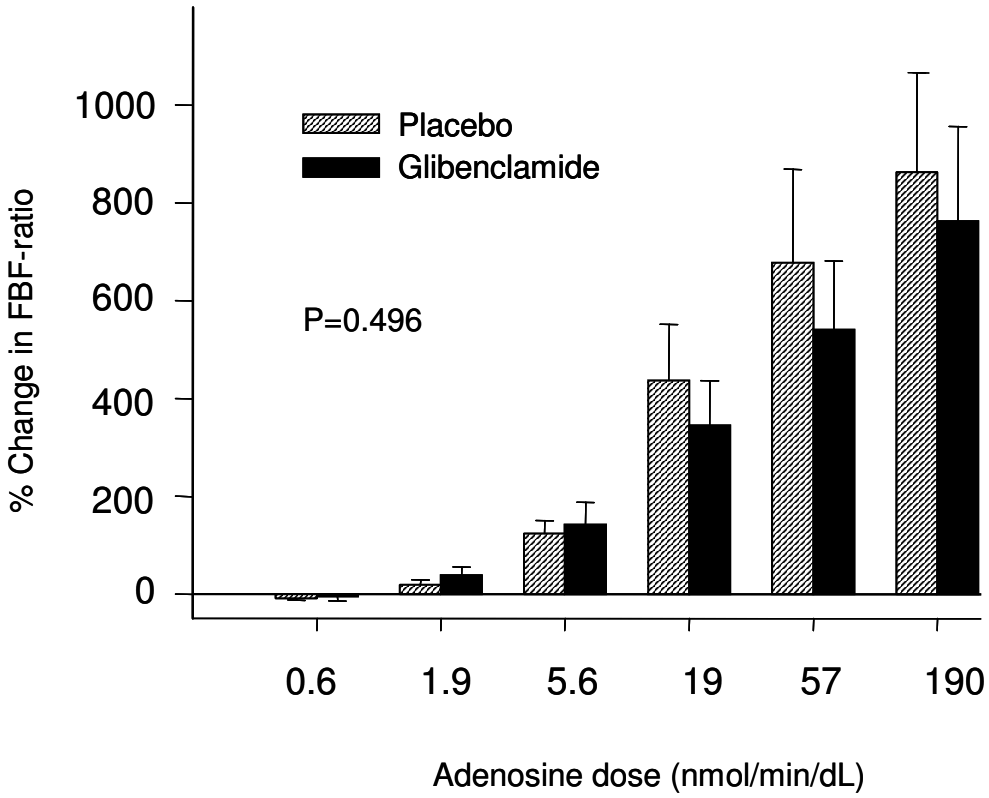

Figure 2. The effect of glibenclamide on the vasodilator response to adenosine expressed as percentage changes in forearm bloodflow (FBF) ratio from baseline. The p-value represents the level of significance for the effect of glibenclamide (ANOVA for repeated measures).

Vasodilator response to the nucleoside transport inhibitor dipyridamole

The vasodilator action of dipyridamole (pooled data of 23 volunteers).

Experimental conditions were similar during the first set of dipyridamole infusions for three subgroups (N=23). In this entire group, dipyridamole increased FBF-ratio from 1.2 ± 0.7 at baseline to 1.5 ± 0.1, 1.9 ± 0.2, and 2.3 ± 0.3 during the three increasing dipyridamole doses (p=0.000 for dipyridamole effect). When expressed as percentage changes form baseline similar results were obtained: 31.5 ± 4.2, 65.0 ± 15.4 and 101.4 ± 16.1% for the three subsequent dipyridamole doses respectively (p=0.000 for dipyridamole effect).

Time-control study

Dipyridamole increased FBF ratio from 0.9 ± 0.1 at baseline to 1.1 ± 0.1, 1.3 ± 0.3 and 1.5 ± 0.3 during the 3 increasing doses respectively. Two hours after the last dipyridamole infusion, FBF ratio returned to baseline (1.2 ± 0.1; p=0.160 vs baseline). During repeated infusion of dipyridamole, FBF ratio increased to 1.4 ± 0.2, 1.8 ± 0.3 and 2.1 ± 0.4 for the three incremental doses respectively (p=0.026 for dipyridamole effect; p=0.186 for time effect and p=0.266 for interaction between dipyridamole and time; N=7). Likewise, results expressed as percentage change in FBF ratio did not reveal a significant carry-over effect (p=0.021 for vasodilator effect of dipyridamole, p=0.819 for time effect and p=0.449 for interaction between dipyridamole and time; N=7).

Dipyridamole-theophylline study

Dipyridamole increased FBF ratio from 1.3 ± 0.1 at baseline to 1.7 ± 0.2, 1.8 ± 0.2 and 2.6 ± 0.4 during the three subsequent dipyridamole infusions. Two hours after the highest dipyridamole dose, FBF ratio returned to baseline (1.4 ± 0.2; p=0.510 vs baseline, N=8). During simultaneous infusion of theophylline, dipyridamole increased FBF ratio from 1.5 ± 0.2 (theophylline alone; p=0.325 vs recontrol) to 1.8 ± 0.4, 1.7 ± 0.3 and 2.1 ± 0.4 during the three subsequent dipyridamole doses (effect of theophylline: p=0.081; N=8). When results were expressed as percentage change in FBF ratio, theophylline significantly reduced the vasodilator response to dipyridamole (p=0.032 for effect of theophylline, see figure 3).

Dipyridamole-glibenclamide study

Dipyridamole increased FBF ratio from 1.2 ± 0.1 at baseline to 1.7 ± 0.2, 2.4 ± 0.5 and 2.9 ± 0.4 respectively. Two hours after the last dipyridamole infusion, FBF ratio returned to baseline (1.7 ± 0.3; p=0.196 vs baseline). During intra-arterial infusion of glibenclamide, FBF ratio increased from 1.6 ± 0.2 during glibenclamide alone (p=0.162 vs baseline and p=0.418 vs recontrol, N=8), to 1.8 ± 0.2, 2.1 ± 0.3 and 2.2 ± 0.4 during the three incremental dipyridamole doses respectively (effect of glibenclamide: p=0.010; N=8). Likewise, when results were expressed as percentage change in FBF ratio, glibenclamide significantly reduced the vasodilator response to dipyridamole (p=0.006 for effect of glibenclamide, see figure 2).
During these experiments blood glucose did not change (4.9 ± 0.2 $mmol \cdot l^{-1}$ at the start versus 4.8±0.2 $mmol \cdot l^{-1}$ at the end of the protocol). Likewise, insulin and C-peptide concentrations remained stable (10.7 ± 2.3 vs 9.2 ± 1.3 $pmol \cdot l^{-1}$ for insulin and 0.69 ± 0.11 vs 0.54 ± 0.05 $nmol \cdot l^{-1}$ for C-peptide; p>0.1 for both comparisons, N=8).
The two-way ANOVA for repeated measures showed that the percentage response in FBF ratio to the lowest three adenosine doses and the three dipyridamole doses significantly differed with respect to the effect of glibenclamide (p=0.024 for group x glibenclamide interaction). The vasodilator effect of dipyridamole and the lowest three adenosine doses in the absence of glibenclamide did not significantly differ

(p=0.212). However, in the presence of glibenclamide, the vasodilator response to dipyridamole was significantly less as compared with adenosine (p=0.035). Similar results were obtained with absolute responses in FBF ratio.

Vasodilator response to adenosine in the presence of dipyridamole

First, the functional significance of intra-arterial infusion of dipyridamole at a dose that does not result in forearm vasodilation was confirmed. In the absence of dipyridamole, adenosine increased the FBF ratio from 1.1 ± 0.1 at baseline to 1.2 ± 0.2 and 3.0 ± 0.7 during infusion of 0.6 and 6 nmol adenosine min/dl respectively. Subsequently, in the presence of dipyridamole (7.4 nmol·min^{-1}·dl^{-1}), adenosine increased the FBF ratio from 1.1 ± 0.1 (recontrol with dipyridamole; p=0.936 vs baseline, N=7) to 2.1 ± 0.2 and 4.9 ± 1 for 0.6 and 6 nmol adenosine min/dl respectively (p=0.002 for the effect of dipyridamole; N=7). Similar results were obtained when results were expressed as percentage change in FBF ratio.
Next, in a separate group of 6 volunteers, the effect of glibenclamide was studied on the adenosine-induced vasodilation in the presence of dipyridamole. In the absence of glibenclamide, adenosine increased the FBF ratio from 1.0 ± 0.1 (baseline with dipyridamole) to 1.3 ± 0.1, 2.0 ± 0.2, 3.7 ± 0.7, 4.9 ± 0.7, 9.7 ± 1.6 and 14.9 ± 2.6 for the six increasing adenosine doses respectively. In the presence of glibenclamide, adenosine increased the FBF ratio from 1.0 ± 0.2 (dipyridamole plus glibenclamide) to 1.6 ± 0.3, 2.7 ± 0.8, 5.6 ± 1.7, 7.1 ± 0.9, 12.1 ± 2.5 and 18.3 ± 3.6 for the six increasing adenosine doses respectively (p=0.240 for the effect of glibenclamide, p=0.550 for the interaction between adenosine dose and glibenclamide, N=6). Similar results were obtained when results were expressed as percentage change in FBF ratio.

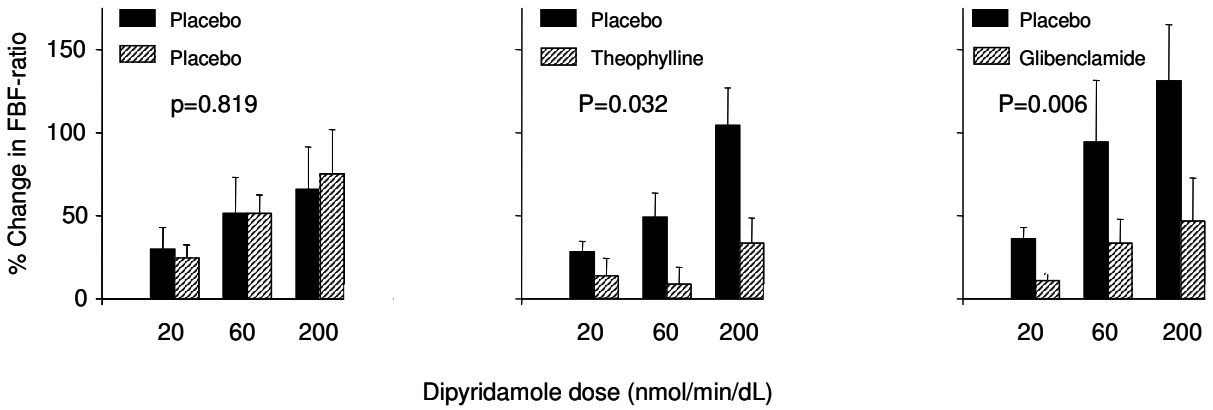

Figure 3. The effect of theophylline (middle panel) and glibenclamide (right panel) on the dipyridamole-induced vasodilation expressed as percentage changes in forearm bloodflow (FBF) ratio from baseline. The left panel shows the results of the time-control study (black bars: first set of dipyridamole infusions; grey bars: second set of dipyridamole infusions). P-values represent the level of significance for the effect of repeated infusion (left panel), theophylline (middle panel) and glibenclamide (right panel) on dipyridamole-induced vasodilation as analysed with ANOVA for repeated measurements.

Discussion

The main finding of this study is that glibenclamide inhibits the vasodilator response to dipyridamole but not to adenosine in the human forearm vascular bed. Furthermore, theophylline, an adenosine receptor antagonist, significantly inhibited the vasodilator response to dipyridamole. These observations support our working hypothesis that dipyridamole-induced inhibition of nucleoside transport results in increased levels of endogenous adenosine with subsequent stimulation of adenosine receptors and opening of vascular K_{ATP} channels. The inhibitory action of dipyridamole on nucleoside transport was confirmed *in vivo* by the augmenting effect of dipyridamole on adenosine-induced vasodilation. This discussion will focus on the observed difference in sensitivity to glibenclamide between adenosine- and dipyridamole-induced vasodilation.

Possible differences in local glibenclamide concentration

The last three adenosine doses induced more vasodilation than dipyridamole which may have resulted in more dilution of glibenclamide in this part of the adenosine experiment as compared with dipyridamole or the lower adenosine infusions. This could potentially have reduced the blockade of vascular K_{ATP} channels during the three highest adenosine infusion rates. However, glibenclamide could not inhibit the vasomotor response to adenosine doses that were equipotent to dipyridamole. Furthermore, we have previously shown in the same experimental set up that glibenclamide at similar local concentrations as observed during adenosine infusion in the present study, sufficiently blocked K_{ATP} channels in the forearm vascular bed [19]. Therefore, differences in forearm kinetics of glibenclamide between the two study protocols do not explain the divergent action of glibenclamide on the vasodilator effect of adenosine and dipyridamole. Nevertheless, we can not exclude the possibility that the infusion of a higher dose of glibenclamide would have inhibited the vasodilator response to adenosine.

Dipyridamole-induced vasodilation may not be related to stimulation of adenosine receptors

Apart from inhibition of adenosine transport, dipyridamole may have non-specific actions such as release of prostacycline [25] or inhibition of phosphodiesterases resulting in intracellular increases of cAMP and cGMP [26]. However, theophylline at a dose that did not affect basal tone and therefore did probably not inhibit intracellular phosphodiesterases, inhibited the vasodilator response to dipyridamole. This observation provides strong support for our notion that the vasomotor action of dipyridamole in the forearm is mediated by adenosine-receptor stimulation and that non-specific actions of dipyridamole are not involved in the observed interaction between dipyridamole and glibenclamide.
In isolated small subcutaneous arteries from humans, glibenclamide did not inhibit dipyridamole-induced vasodilation [27]. A difference in endogenous adenosine formation between our in vivo model and this in vitro preparation could explain this contrasting result. For example, sympathetic denervation may have reduced endogenous adenosine formation in the in vitro isolated blood vessel model [8;28].

Dipyridamole and adenosine may stimulate adenosine receptors on different cells

Adenosine receptors are found on both endothelium and vascular smooth muscle cells and stimulation by adenosine of either cell type results in vasodilation [29;30]. Apart from adenosine receptors, the endothelium expresses a dipyridamole-sensitive equilibrative nucleoside transport protein [18]. Because intracellular adenosine concentrations are low during normoxic conditions, the concentration gradient favors rapid endothelial uptake of luminally applied adenosine and subsequent metabolism of adenosine. Thus, the endothelium functions as a metabolic barrier that prevents intraluminally applied adenosine from reaching the vascular smooth muscle cells as documented in large arteries [18]. However, it is not known whether this barrier function also occurs at the level of arterioles. At high doses of intra-arterially infused adenosine, some adenosine may have reached vascular smooth muscle cells. Nevertheless, it is likely that at lower doses, the endothelial cell is completely responsible for the vasodilator response to intra-arterial adenosine [15] although the exact nature of the involved endothelium-derived relaxing factor is still a matter of debate in humans in vivo [31-34]. Therefore, the lack of effect of glibenclamide suggests that K_{ATP} channels are not involved in the endothelial mechanism of adenosine-induced vasodilation. In contrast to adenosine, dipyridamole-induced stimulation of adenosine receptors is dependent on endogenous formation of adenosine which occurs at both the adventitial and luminal side. Therefore, adenosine receptors on vascular smooth muscle cells are likely to be involved in the dipyridamole-induced vasodilator response. The observed difference between adenosine and dipyridamole in their susceptibility to glibenclamide suggests that ATP-dependent potassium channels in smooth muscle cells but not in endothelial cells are involved in the dipyridamole-induced vasodilation.
Based on this hypothesis, we predicted that dipyridamole-induced inhibition of endothelial nucleoside transport would enhance the availability of intraluminally applied adenosine for adenosine receptors on vascular smooth muscle cells. Therefore, we studied the effect of glibenclamide on adenosine-induced vasodilation in the presence of dipyridamole. Since dipyridamole-induced vasodilation would prevent a correct interpretation of an interaction between adenosine and glibenclamide, we used a low dose of dipyidamole that did not induce vasodilation itself. This dose potentiated the vasodilator response to adenosine, indicating functionally significant nucleoside transport inhibition. Nevertheless, this low dose of dipyridamole did not reveal an interaction between adenosine and glibenclamide. The most obvious explanation for this negative finding is that the low dose of dipyridamole augmented the vasodilator response to intra-arterial adenosine by significant inhibition of nucleoside transporters on erythrocytes but did not sufficiently block vascular nucleoside transporters to increase the availability of adenosine at the vascular smooth muscle cells. Alternatively, our primary hypothesis that glibenclamide reduces dipyridamole-induced vasodilation by inhibition of adenosine-induced opening of K_{ATP} channels may be false. Theoretically, glibenclamide may have reduced formation of endogenous adenosine which could have reduced the ability of dipyridamole to increase the concentration of endogenous adenosine. Since interstitial adenosine concentrations were not measured in this study, this possibility can not be excluded.

Clinical relevance

Regardless its mechanism, the observed interaction between glibenclamide and dipyridamole is of potential clinical relevance because adenosine and dipyridamole are clinically used as pharmacological tools to detect reversible cardiac ischemia [35]. Glibenclamide is often used in patients with type 2 diabetes who are at increased risk for the development of coronary atherosclerosis. Intra-arterially infused glibenclamide reached a local concentration of 43 ± 8 ng·ml^{-1} which is well within the minimally effective concentration range to reduce plasma glucose in patients with type 2 diabetes (30-50 ng·ml^{-1}). In these patients, the use of glibenclamide could negate dipyridamole thallium stress-tests as demonstrated previously for the adenosine receptor antagonist caffeine [36]. Therefore, our data suggest that adenosine in stead of dipyridamole should be used as a pharmacological tool to detect insufficient coronary blood supply in patients who use glibenclamide. However, our forearm data can not be extrapolated directly to the heart and therefore, this important implication needs further confirmation in the coronary circulation during dipyridamole-thallium stress testing.

In conclusion, like adenosine, the dipyridamole-induced forearm vasodilation is inhibited by theophylline which indicates that it is mediated by stimulation of adenosine receptors. In contrast to adenosine, the vasodilator response to equipotent doses of dipyridamole is inhibited by glibenclamide. Although these observations do not provide definite prove, they support our working hypothesis that dipyridamole and intraluminally applied adenosine act on different cells in the vascular wall. Furthermore, it indicates the involvement of ATP-sensitive potassium channels in the vasodilator response to dipyridamole.

Acknowledgments

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CHAPTER 4

Diadenosine pentaphosphate vasodilates the forearm vascular bed: Inhibition by theophylline and augmentation by dipyridamole.

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Abstract

In rats, diadenosine pentaphosphate (AP_5A) has been implicated in the pathogenesis of essential hypertension. This study describes for the first time the vasomotor action of AP_5A in humans, using the 'perfused forearm technique'.
Diadenosine pentaphosphate evoked a dose-dependent forearm vasodilator response equal to adenosine but less than adenosine-5`-triphosphate (ATP) at equimolar doses. The P_1-purinoceptor antagonist theophylline (0.28 $\mu mol \cdot min^{-1} \cdot dl^{-1}$) reduced the percentage decrease in forearm vascular resistance (FVR) to AP_5A (0.6, 6 and 20 $nmol \cdot min^{-1} \cdot dl^{-1}$): -8 ± 6, -50 ± 6, -68 ± 4% during saline versus -7 ± 4, -33 ± 5 and -45 ± 6% during theophylline (ANOVA for repeated measures; $p<0.05$ for the interaction between purine dose and theophylline; n=10). The inhibitor of equilibrative nucleoside transport dipyridamole (7.4 $\mu mol \cdot min^{-1} \cdot dl^{-1}$) augmented the AP_5A - (0.6 and 6 $nmol \cdot min^{-1} \cdot dl^{-1}$) induced decrease in FVR: -34 ± 6 and -67 ± 5 % during saline versus -49 ± 5 and -80 ± 3 % during dipyridamole ($p<0.05$ for the effect of dipyridamole; n=6). The bivalent cation chelator ethylene diamine tetra acetic acid (EDTA), inhibited the rapid degradation of AP_5A in vitro. In vivo, the highest tolerated intra-arterial EDTA dose (0.76 $\mu mol \cdot min^{-1} \cdot dl^{-1}$) was not sufficient to inhibit AP_5A-metabolism.
Thus, intra-arterial AP_5A reduces forearm vascular tone dose-dependently. This is, at least in part, mediated by its degradation product adenosine. Our data do not support an in vivo vasoconstrictor action of AP_5A, and as such AP_5A seems not likely to contribute to the pathogenesis of primary hypertension in man.

Introduction

Diadenosine polyphosphates (AP_nA, n=2-7) are newly recognized endogenous compounds, consisting of two adenosine molecules bridged by two to seven phosphate groups [1;2]. Diadenosine polyphosphates occur in thrombocyte dense granules [3], in chromaffin granules of the adrenal medulla and in nerve terminals, where they are co-stored with neurotransmitters like adenosine-5`-triphosphate (ATP) [4;5]. They could potentially be involved in modulation of vascular tone [3], thrombocyte aggregation [6] and neurotransmission [4]. In 1989, Zidek *et al.* cross-circulated spontaneously hypertensive and normotensive rats, which induced a significant rise in blood pressure in the normotensive rats [7]. They observed a vasoconstrictor action of plasma from hypertensive patients in isolated rat aortic strips [8]. Based on these findings, a circulating hypertensive factor was postulated. Agha *et el.* isolated substances from human platelets which increased the perfusion pressure of isolated rat kidneys [9]. These substances were present at higher concentrations in hypertensive subjects as compared to normotensive controls. Subsequently, Schlüter *et al*. identified these vasopressor agents as AP_5A and AP_6A [3]. Both AP_5A and AP_6A induced vasoconstriction in perfused rat kidneys and aortic rings, and an increase in blood pressure after intra-aortic injection in rats [3]. Thus, Schlüter and colleagues hypothesized that AP_5A and AP_6A may play a part in local vasoregulation and possibly in the pathogenesis of hypertension [3]. However, animal data are not unequivocal with respect to the vasomotor action of dinucleotide polyphosphates. Furthermore, important interspecies differences in purine pharmacology exist [10;11]. Therefore, we characterized the effect of AP_5A on forearm vascular tone in human volunteers. We chose for AP_5A , because this dinucleotide appeared to be the most potent vasoconstrictor *in vitro* [12-15].
The following questions were posed: [1] What is the effect of AP_5A on forearm vascular tone? and [2] What is the involvement of P_1-purinergic receptors in the vascular response to AP_5A?
Our results indicate that AP_5A dilates the forearm vascular bed, which involves adenosine-induced stimulation of P_1-purinoceptors. *In-vitro* experiments further support rapid degradation of AP_5A.

Methods

Subjects

After approval of this study by the local ethics committee, 49 normotensive nonsmoking healthy Caucasian male and female volunteers signed informed consent to participate. Two people took part in two studies. The volunteers had no history of hypertension, diabetes mellitus, or hyperlipidemia. Before participation, they underwent a physical examination, electrocardiagraphy, and laboratory investigation of cholesterol, triglycerides and glucose. Medication was not allowed except for oral contraceptives. Subjects did not use caffeine-containing beverages and alcohol for 24 hours prior to the study (caffeine acts as an adenosine-receptor antagonist), and participants abstained from food two hours before testing. To confirm compliance to the caffeine abstinence, blood was sampled for the measurement of plasma caffeine concentration before each experiment.

General outline of the procedure

The experiments were performed in the afternoon in a quiet, temperature-controlled room (23° C) and lasted approximately 4 hours. The vasomotor actions of AP_5A and other substances were studied with the 'perfused forearm technique' as described before [16;17]. Briefly, the brachial artery was cannulated for measurement of intra-arterial blood pressure and infusion of substances. Forearm blood flow was measured using ECG triggered mercury-in-silastic strain gauge plethysmography. Within each protocol the total infusion rate was kept constant at 50 $\mu L \cdot min^{-1} \cdot dl^{-1}$ of forearm tissue for the first protocol and 100 $\mu L \cdot min^{-1} \cdot dl^{-1}$ for the other protocols. Baseline recordings were always performed during saline (NaCl 0.9%) infusion for 5 minutes. Each purine dose was infused for 5 minutes.

Comparison of the vasomotor action of AP_5A with equimolar doses of adenosine and ATP.

In 6 subjects, the vasomotor action of equimolar doses of intra-arterially infused AP_5A, adenosine and ATP were compared. After baseline measurements, three increasing doses of each purine (0.6, 6 and 20 $nmol \cdot min^{-1} \cdot dl^{-1}$) were infused. The purines were infused in a fixed order: first AP_5A, followed by adenosine and ATP. The different purines were infused with a thirty minute interval to prevent any carry-over effect.

The effect of the P1-(adenosine) receptor antagonist theophylline on the AP_5A-induced forearm vasodilator response.

In 10 volunteers, the effect of intra-arterially infused theophylline, a competitive P_1-receptor antagonist of both A_1- and A_2- receptors [18;19], was studied on the vasodilator response to AP_5A and adenosine. After baseline measurements with saline-infusion, two increasing doses of adenosine (6 and 20 $nmol \cdot min^{-1} \cdot dl^{-1}$) were co-infused with saline into the brachial artery. After a 15 minute drug-free interval baseline recordings were repeated, followed by three doses of AP_5A (0.6, 6 and 20 $nmol \cdot min^{-1} \cdot dl^{-1}$) co-infused with saline. This scheme was repeated with theophylline (0.28 $\mu mol \cdot min^{-1} \cdot dl^{-1}$) instead of saline.

The effect of an inhibitor of equilibrative nucleoside transport, dipyridamole, on the forearm vasodilator response to AP_5A.

First, the vasomotor action of 7.4, 14.8 and 22.2 μmol dipyridamole $\cdot min^{-1} \cdot dl^{-1}$ was studied in 8 volunteers (dose-finding study). Each dose was infused for 15 minutes. The lowest dose appeared to lack a significant vasomotor action. This dose was used in two groups of volunteers to test its ability to potentiate the vasodilator action of adenosine (positive control; n=7) and AP_5A (n=6). Each purine was infused at a dose of 0.6 and 6 $nmol \cdot min^{-1} \cdot dl^{-1}$. In five volunteers AP_5A was measured in the venous effluent at the end of the infusion of the highest dose of AP_5A in the absence

and presence of dipyridamole. For this purpose, 3 ml blood was collected in prechilled tubes containing EDTA at a final concentration of 4 mmol·L^{-1} blood.

Effect of EDTA on AP_5A metabolism in human blood in vitro.

From two healthy volunteers, 30 ml blood was collected using heparin as anticoagulant. AP_5A was added to whole blood at a final concentration of approximately 3.5 nmol·ml^{-1} plasma in the absence or presence of EDTA (0.5 and 5 mM). This AP_5A concentration was based on the calculated AP_5A concentration in the forearm vascular bed, assuming no degradation. AP_5A was measured in plasma directly and after 30 and 180 minutes of incubation at 37°C. In addition the half-life of AP_5A added to plasma was measured in 2 healthy volunteers.

Characterization of EDTA as an inhibitor of AP_5A metabolism in human blood in vivo.

First we studied the tolerability and the vasomotor effect of increasing doses of intra-arterially infused ethylene diamine tetra acetic acid (EDTA): 0.19, 0.38, 0.76, 1.52 and 3.04 µmol·min^{-1}·dl^{-1}, resulting in calculated forearm concentrations of 0.1, 0.3, 0.5, 1 and 2 mmol EDTA·L^{-1} plasma (n=6). Each dose was infused for 15 minutes with 15 minute drug free intervals between subsequent doses. The dose of 0.76 µmol·min^{-1}·dl^{-1} EDTA was the highest tolerated dose: higher doses EDTA caused stinging pain at the infusion site, radiating to the forearm. This dose did not affect baseline tone itself, in contrast to the higher doses. Therefore, in the final experiment, AP_5A (0.6 and 6 nmol·min^{-1}·dl^{-1}) was infused with saline and repeated during EDTA (0.76 µmol·min^{-1}·dl^{-1}; n=8). Venous catheters were inserted in a deep antecubital vein of both arms to sample blood for analysis of ionized calcium.

Drugs and solutions

AP_5A solutions were prepared from vials containing 5.3 mg lyophilized powder (synthesized for this study by Clinalfa, Switzerland). Adenosine solutions were prepared from 2-ml ampoules containing 6 mg adenosine (*Adenocor*, Sanofi Winthrop, the Netherlands). ATP solutions were prepared from 2-ml ampoules containing 20 mg ATP (S*triadyne*, Wyeth, France). Theophylline solutions were prepared from 10-ml ampoules containing 175.7 mg theophylline (Byk bv, the Netherlands). Dipyridamole solutions were prepared from 10 ml ampoules containing 50 mg (Boehringer Ingelheim bv, Germany). A sterile EDTA stock solution was prepared at the hospital pharmacy and kept in cool storage until further dilution before the experiment. NaCl 0.9% was used as solvent for all drug solutions.

Analytical methods

AP_5A: In vitro studies on AP_5A metabolism with/without EDTA were performed in freshly collected plasma. Venous plasma collected during intra-arterial AP_5A infusions was stored at -80 º. Sample pretreatment was as follows: 0.5 ml plasma

was mixed with 100 µL 0.01 M phosphate buffer and 100 µL 4 M $HCL0_4$. After standing for 5 min, the tube was centrifugated for 10 min on 2700 RPM. In the supernatant, 1.5 ml sodium phosphate buffer was added and AP_5A was measured using HPLC with spectophotometric detection. The chromatographic system consisted of a Spectra Physics (Breda, the Netherlands) P2000 binary gradient pump, a 5 µm ODS Hypersil guard column (20x2.1mm), a Hewlett Packard (Amsterdam, the Netherlands) 5 µm BDS Hypersil C_{18} analytical column (200x4.6 mm) and a Spectra Physics AS 3000 autosampler with a built-in column heater. The mobile phase, acetonitrile 0.02 M phosphate buffer, pH 6.0 was delivered with a flow-rate of 1 $ml \cdot min^{-1}$. The column effluent was monitored with a Spectra Physics UV1000 variable wavelength detector set at 258 nm. The signal was processed by a Spectra Physics SP4400 integrator. The column heater was set at 40ºC and the injection volume was 10 µL.
The retention time of AP_5A in the chromatogram was 8.8 min for AP_4A and 10.9 min for AP_5A. The limit of detection of the method was 0.1 µmol $AP_5A \cdot L^{-1}$. The inter-day coefficient of variation was 9% at a concentration of 3.6 nmol $AP_5A \cdot ml^{-1}$.
Caffeine: Samples were analyzed by HPLC [20].
Free ionised Calcium: this was measured with an ion-selective electrode on a Chiron bloodgas-analyzer.

Statistical analysis

Mean arterial BP (MAP) was measured continuously during each recording of FBF and averaged per FBF measurement. Forearm vascular resistance (FVR) was calculated as the quotient of simultaneously measured MAP and FBF and expressed in arbitrary units (AU). For saline infusion, data obtained during the last four minutes were averaged to one value, and for drug infusion, data from the last two minutes were averaged to ensure a steady state condition. The vascular response was expressed as percentage change from baseline in forearm vascular resistance. Analyses were performed on infused and non-infused arm separately, to detect possible systemic effects of the drug infusions on forearm vascular tone [17]. All results were expressed as mean ± SE. In the first study, the effects of the three purine receptor agonists were compared with an ANOVA for repeated measures, with agonist and agonist dose as within subject factors. Effects of interventions on purine receptor agonists were analyzed by an ANOVA for repeated measures with intervention and agonist dose as within subject factors. Two-sided P-values <0.05 were considered to indicate statistically significant differences.
Based on previous studies [16], a theophylline- or dipyridamole- induced difference of 16% or 23% in FVR- response to adenosine can be detected with 10 or 6 experiments respectively at a power of 0.8, acccepting an α-error of 0.05. These calculations are based on a paired-t-test on a single dose. In fact, we performed a repeated measures ANOVA which increases the power of the analysis.

Results

The demographic data of the participants are shown in table 1.

Plasma caffeine levels could not be detected in all but one of the participants, indicating excellent compliance to caffeine abstinence.

Table 1. Demographic characteristics

N= 49 (39 M / 10 F)	mean ± SD
Age (years)	23 ± 4
BMI ($kg \cdot m^{-2}$)	22.5 ± 2.1
SBP (mmHg)*	22 ± 8
DBP (mmHg)*	75 ± 7
HR ($beats \cdot min^{-1}$) ¶	62 ± 9
Cholesterol ($mmol \cdot L^{-1}$)	4.0 ± 0.6
Triglycerides ($mmol \cdot L^{-1}$)	0.8 ± 0.3
Glucose ($mmol \cdot L^{-1}$)	4.7 ± 0.4

* Auscultatory measurement after 5 minutes of rest in a supine position.
¶ Measured by pulse frequency counting after 5 minutes of supine rest

Comparison of the vasomotor action of AP_5A with equimolar doses of adenosine and ATP (n=6).

AP_5A reduced FVR from 30±2 AU at baseline to 26±3, 14±2 and 9±1 AU for three increasing doses, respectively ($p<0.05$). Likewise, equimolar doses of adenosine reduced FVR from 24±3 AU at baseline to 27±3, 16±2 AU and 9±1 AU. Similarly, ATP reduced FVR from 34±5 AU to 12±1, 7±1 and 6±1 AU. Baseline FVR did not significantly differ between the three purines. The vasodilator response to AP_5A significantly differed from ATP ($p<0.05$), but not from adenosine ($p>0.1$). Thus, the rank order of vasodilator potency was ATP>AP_5A = adenosine (figure 1). Purine infusions did not significantly affect FBF or FVR in the non-infused arm, blood pressure or heart rate (data not shown).

The effect of the P_1-(adenosine) receptor antagonist theophylline on the AP_5A-induced forearm vasodilator response (n=10).

During saline, AP_5A reduced FVR from 26±4 AU to 22±2, 12±1 and 8±1 AU. During theophylline, AP_5A reduced FVR from 21±2 AU to 20±2, 14±1 and 11±2 AU. Baseline vascular tone did not differ significantly between theophylline and saline. For both adenosine and AP_5A, the ANOVA for repeated measurements revealed a significant interaction between theophylline and purine-dose (figure 2). The effect of theophylline alone was not significant (for AP_5A: $p=0.075$, for adenosine: $p>0.1$). Thus, the effect of theophylline was dependent on the purine-dose. Purine infusions

did not significantly affect FBF or FVR in the control arm, blood pressure or heart rate (data not shown).

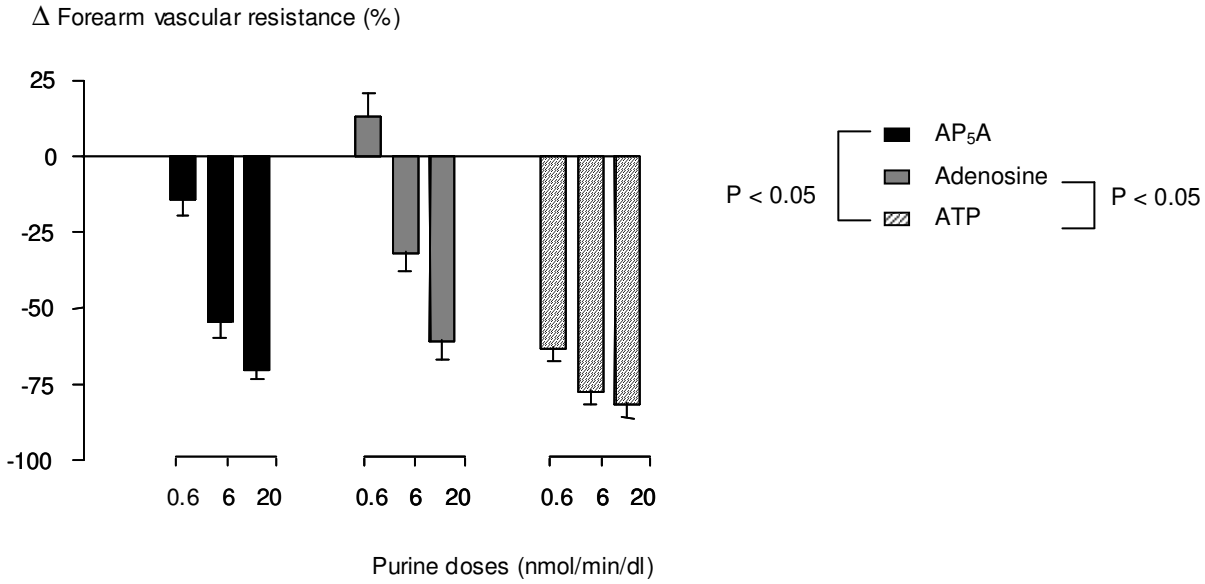

Figure 1. Comparison of forearm vasodilator response (percentage change in forearm vascular resistance, n = 6) to equimolar doses of diadenosine pentaphosphate (AP_5A, black bars), adenosine (gray bars), and adenosine triphosphate (ATP, hatched bars) (0.6, 6 and 20 $nmol \cdot min^{-1}$ per deciliter). Levels of significance for comparisons of percentage change in forearm vascular resistance (FVR) between purines are as follows: $p<0.05$, AP_5A versus ATP; $P<0.05$, adenosine versus ATP.

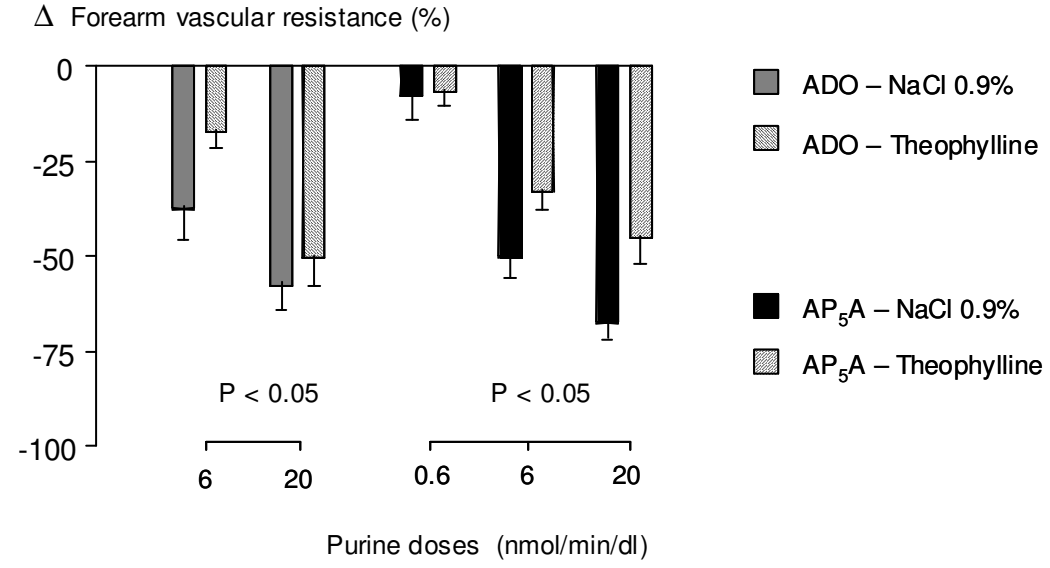

Figure 2. Effect of theophylline (0.28 $\mu mol \cdot min^{-1} \cdot dl^{-1}$) on the vasodilator response to adenosine and AP_5A. P-values indicate level of significance for the interaction between theophylline and purine-dose.

The effect of dipyridamole, an inhibitor of equilibrative nucleoside transport, on the forearm vasodilator response to AP_5A (n=6).

In a pilot study 22.2 µmol dipyridamole $\cdot min^{-1} \cdot dl^{-1}$ caused significant vasodilation: the FVR was reduced from 28±2.5 during saline to 23±2.6 AU during the last 5 minutes of dipyridamole infusion ($p<0.05$, n=8). At a dose of 7.4 $\mu mol \cdot min^{-1} \cdot dl^{-1}$ dipyridamole did not affect baseline tone but significantly potentiated the vasodilator response to both adenosine and AP_5A (figure 3). In the control arm, vascular tone was not significantly affected by any drug infusion. Likewise, blood pressure and heart rate were not affected by the drug infusions (data not shown).
At the end of the infusion of 6 nmol AP_5A 6 $\cdot min^{-1} \cdot dl^{-1}$, the AP_5A-concentration in the venous effluent was 0.36 ± 0.1 $\mu mol \cdot L^{-1}$ (n=5). Assuming a calculated forearm concentration of 3.75 $\mu mol \cdot L^{-1}$ plasma, the extraction and/or degradation of AP_5A during one transit time through the arm is 90%. In the presence of dipyridamole, the AP_5A-concentration in the venous effluent was 0.27±0.1 $\mu mol \cdot L^{-1}$ (n=5) indicating that the augmenting effect of dipyridamole on AP_5A-induced vasodilation did not result from changes in local AP_5A concentrations.

Characterization of EDTA as an inhibitor of AP_5A-metabolism in human blood in vitro.

Plasma half-life of AP_5A added to plasma was 3.3 min (95% confidence interval: 2.7-4). In the presence of 5 mM EDTA, degradation of AP_5A was completely blocked. However, at 0.5 mM, recovery of AP_5A was reduced, indicating preserved metabolism (table 2).

Table 2. Time course in recovery of diadenosine pentaphosphate (AP_5A, micrograms per milliliter) in plasma and whole blood in absence and presence of ethylene diaminetetra-acetic acid (EDTA) (mean ± SE, n=2)

Time (min)	Plasma	Whole Blood 0-mmol/L EDTA	0.5-mmol/L EDTA	5-mmol/L EDTA
0	3.7 ± 0.1	3.8 ± 0.3	3.8 ± 0.3	3.8 ± 0.3
5	1.3 ± 0.01			
10	0.5 ± 0.03	2.2 ± 0.2	1.1 ± 0.3	3.6 ± 0.3
15	0.1 ± 0.01			
20				
30				
40		0	0	3.3 ± 0.1
190		0	0	3.4 ± 0.1

Values at time zero indicate calculated concentrations of AP_5A, assuming absent metabolism

Characterization of EDTA as an inhibitor of AP_5A-metabolism in human blood in vivo (n=8).

In the pilot study, EDTA 0.76 $\mu mol \cdot min^{-1} \cdot dl^{-1}$ was tolerated by 5 out of 6 subjects. This dose had no influence on baseline vascular tone, but reduced ionized Ca^{2+}

concentration in the infused arm to 0.95 ± 0.1 mmol·L^{-1} during infusion of EDTA versus 1.24 ± 0.03 in the control arm, indicating a local reduction of 23% (n=3). EDTA in a dose of 1.52 µmol·min^{-1}·dl^{-1} was tolerated by 3 out of 6 volunteers and increased FBF from 2.1±0.27 (baseline) to 4.6±1.1 µmol·dl^{-1} forearm volume (FAV)·min^{-1}, although this difference was not statistically significant (p=0.07; Wilcoxon signed ranks test).
At the dose of 0.76 µmol·dl^{-1} FAV·min^{-1}, the AP_5A-induced reduction in FVR (0.6 and 6 nmol·dl^{-1} FAV·min^{-1}) was not affected (figure 4). During co-infusion of AP_5A 6 nmol·min^{-1}·dl^{-1} with EDTA the ionized calcium in the infused arm decreased from 1.24±0.01 at baseline to 1.13±0.04 mmol·L^{-1} (mean±SE; n=7), indicating 8.9 % reduction in Ca^{2+} (p<0.05 for relative change). In the control arm, ionized calcium did not change: 1.27±0.01 at baseline and 1.27±0.03 during AP_5A 6 nmol·dl^{-1} FAV·min^{-1} with EDTA (p>0.1).

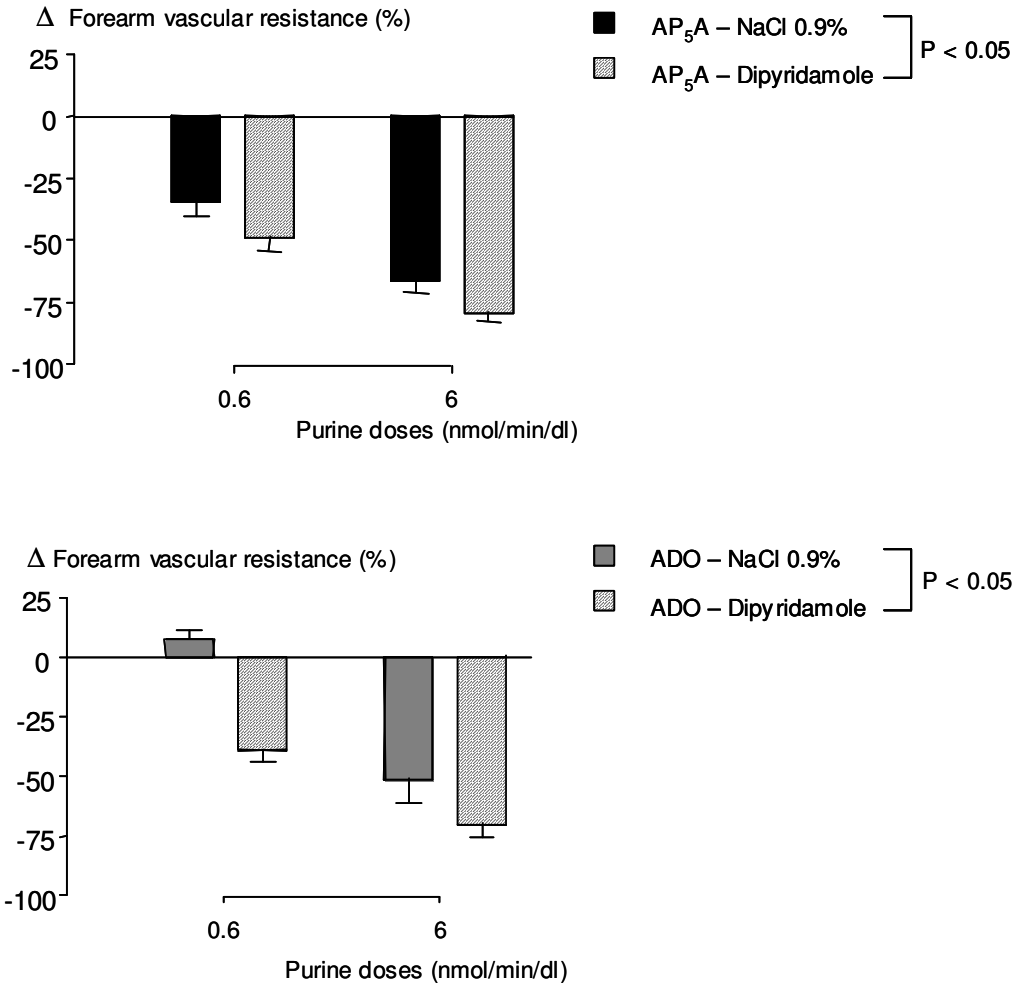

Figure 3. Effect of dipyridamole (7.4 µmol·min^{-1}·dl^{-1}) on AP_5A (upper panel)- and adenosine (lower panel)-induced forearm vasodilation. P-values indicate level of significance for effect of dipyridamole on purine-induced % change in FVR (ANOVA for repeated measurements). The interaction between dipyridamole and purine dose was not statistically significant (ANOVA for repeated measurements p>0.1 for AP_5A and p>0.05 for adenosine).

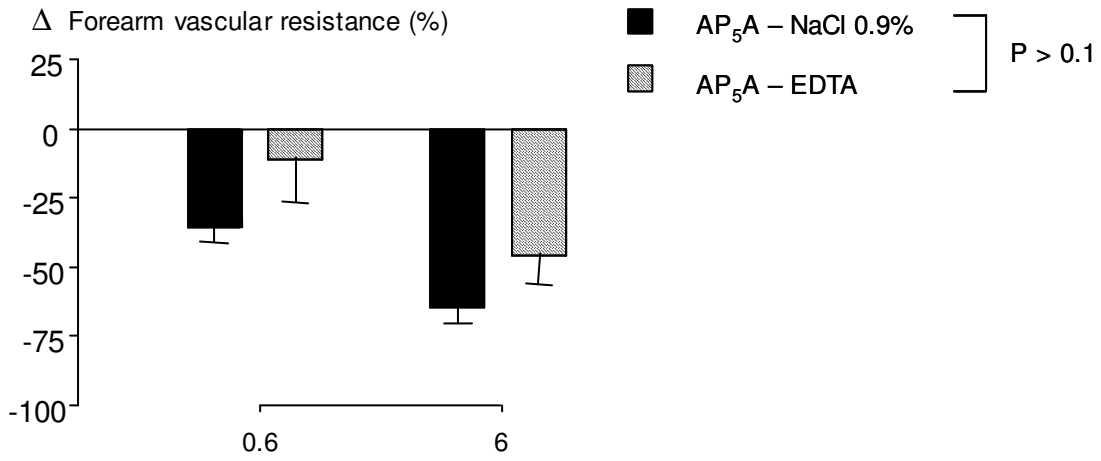

Figure 4. Effect of ethylene diamine tetra acetic acid (EDTA; 0.76 µmol·min^{-1}·dl^{-1}) on AP_5A-induced forearm vasodilation. P-value indicates level of significance for effect of EDTA on AP_5A-induced % change in FVR

Discussion

This study shows for the first time that intra-arterial infusion of AP_5A dilates the forearm vascular bed in humans *in vivo*. Theophylline inhibits the vasodilator effect of both adenosine and AP_5A, suggesting the involvement of P_1 purinoceptors. Therefore, we hypothesized that adenosine, one of the breakdown products of AP_5A [21;22], contributes to the AP_5A-induced vasodilation.

Metabolism of AP_5A in the human forearm

Diadenosine pentaphospate is degraded by hydrolases on endothelium yielding ATP, AMP and adenosine as vasodilating metabolites [1;23]. The vasoactive effect and degradation of the metabolite AP_4 is unknown [1].
To further support the hypothesis that AP_5A induces vasodilation through its breakdown products, we used three approaches. First, we used dipyridamole to inhibit equilibrative nucleoside transport (ENT) in the forearm [24]. Dipyridamole inhibits adenosine uptake without having a known effect on AP_5A metabolism. To prevent a possible vasomotor action of increased concentrations of endogenous adenosine [25], a dipyridamole dose was used that did not significantly affect baseline vascular tone. This dose augmented the vasodilator response to exogenous adenosine, indicating significant inhibition of ENT. Likewise, dipyridamole augmented AP_5A-induced vasodilation, indicating increased formation of adenosine during AP_5A infusion. This observation confirms rapid metabolism of AP_5A to adenosine in the human forearm *in vivo.*
Second, we studied the stability of AP_5A in human blood *in vitro* and in the forearm *in vivo.* Only a small portion of AP_5A added to human blood could be recovered, confirming rapid degradation. Furthermore, the measured concentration of AP_5A in venous plasma collected from the infused arm, during intra-arterial administration of AP_5A supports degradation of most of the infused AP_5A during one transit time.

EDTA, a known inhibitor of enzymes that are involved in AP_5A- and ATP breakdown [22;26;27], significantly inhibited the degradation of AP_5A *in vitro* and improved recovery, indicating the validity of our method to detect AP_5A.
Third, in an attempt to inhibit the degradation of AP_5A *in vivo*, we investigated the effect of EDTA on AP_5A-induced vasodilation in the forearm. EDTA reduced the free calcium concentration in the infused but not in the control arm, indicating local chelation of divalent cations. Furthermore, EDTA induced a small vasodilator response. As far as we know, this is the first description of a direct vasodilator action of EDTA. This observation probably reflects reduced availability of calcium to maintain adrenergic- and endotheline- mediated contraction of vascular smooth muscle cells. In patients with hyperparathyroidism, an increased forearm vascular tone has been reported [28]. Our observation suggests that an increased serum calcium concentration contributes to the altered forearm vasomotor state in these patients. This is supported by others showing that calcium infusion increases blood pressure and total vascular resistance in healthy volunteers [29].
Unfortunately, pain at the site of infusion prevented the use of a dose of EDTA that would be sufficient to inhibit ectonucleotidases, which explains why EDTA failed to affect AP_5A-induced vasodilation significantly. This is confirmed by the reduced recovery of AP_5A *in vitro* at an EDTA concentration of 0.5 mM, that was estimated to occur *in vivo* during infusion of the highest tolerated dose into the brachial artery.
Our experimental design does not directly allow us to draw conclusions about the contribution of nitric oxide (NO) in AP_5A induced forearm vasodilation, because we did not co-infuse AP_5A with a NO-antagonist. Adenosine but not ATP has been shown to stimulate NO release in the human forearm [30;11]. Since the vasodilator response on AP_5A at least partially depends on formation of adenosine, it is expected that NO is also involved in AP_5A-induced vasodilation.

Comparison of this study with previous observations in animals

Our observation that AP_5A reduces forearm vascular tone at physiologically relevant concentrations [31], is at variance with a human *in vitro* study which showed vasoconstriction of isolated umbilical arteries in response to AP_5A [32], and with animal *in vitro* studies which demonstrated vasoconstriction in isolated renal resistance arteries [2;3;9;12], isolated perfused mesenteric arteries [14] and isolated rat aortic strips [33]. However, more recently also a vasodilator response to AP_5A has been observed in animal preparations *in vitro* [15;34]. How can these discrepancies be explained?
In vitro, enzymatic degradation of AP_5A may be reduced for several reasons. First, the abundant amount of the substrate AP_5A relative to the small amount of enzymes in the isolated blood vessel wall contrasts with the *in vivo* situation. Second, in these *in vitro* preparations AP_5A can easily reach the vascular smooth muscle cells without the need to pass the endothelium, which is a rich source of ectonucleotidase activity [23;35]. Apart from differences in ectonucleotidase activity, differences between species or vascular beds may exist. For example, AP_5A gives rise to vasodilation in the isolated guinea pig heart [36], while it elicits a vasoconstrictor response in the isolated perfused rat kidney [12]. However, these results obtained in perfused organ preparations do not exclude degradation of AP_5A to adenosine and ATP, because adenosine is known to induce vasoconstriction in the afferent glomerular arteriole [37]. Furthermore, a P_{2x} purinoceptor was hold responsible for the vasoconstrictor

response to AP_5A in the perfused rat kidney [12]. Finally, methodological differences may explain some of the differences between *in vitro* studies. For example, Ralevic *et al.* observed vasoconstriction in isolated perfused mesenteric arteries of the rat [14], which differs from a report from Steinmetz *et al.* who studied precontracted isolated rat mesenteric resistance arteries in a Mulvany wire myograph and observed only a transient vasoconstriction, followed by sustained dilation. This discrepancy may find its origin in the use of bolus injections in the first study and steady state agonist concentrations in the second.
Results from *in vivo* studies on the pressor effect of AP_5A in intact rats are conflicting: both a reduction in blood pressure [38;39] as well as an increase in blood pressure [3] have been reported.

Implications of this study for the role of AP_5A in the pathogenesis of hypertension

Schlüter *et al.* originally hypothesized that thrombocyte- or plasma- derived AP_5A and AP_6A are involved in the pathogenesis of hypertension [3]. However, our observation that luminal AP_5A is rapidly metabolized and, through its degradation products, elicits a vasodilator response in the human forearm, does not support a pathogenic role of plasma- or thrombocyte-derived AP_5A in the development of hypertension in humans. Furthermore, our observations provide an explanation for some contrasting results from in-vitro and animal studies.
Three limitations of our study should be mentioned. First, we studied AP_5A in the forearm vascular bed. An increased forearm vascular resistance has been observed in patients with essential hypertension, supporting the contribution of this vascular bed in the pathogenesis of hypertension [40]. However, regional differences in the vasomotor action of AP_5A may exist. Our study does not exclude constriction of renal resistance arteries in response to AP_5A which could cause hypertension. Second, we studied AP_5A in a healthy vascular bed with normal endothelial function. Therefore, our results do not exclude a vasoconstrictor action in atherosclerotic vessels where the metabolism of this purine could possibly be reduced, and where it may have direct access to the vascular smooth muscle cells. However, if AP_5A causes hypertension as suggested by the cross circulation experiments in rats, it should be able to increase vasoconstriction in an otherwise healthy vascular bed. Third, our results do not exclude a vasoconstrictor action of adventitially released AP_5A as may occur during activation of the sympathetic nervous system.

In conclusion, our results indicate that intra-arterial infusion of AP_5A induces forearm vasodilation, which at least in part is mediated by its breakdown product adenosine. Our observations do not support a role for luminal AP_5A in the pathogenesis of hypertension.

Acknowledgments

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CHAPTER 5

The influence of diazepam and midazolam on adenosine-induced forearm vasodilation in humans.

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Abstract

Adenosine is an endogenous purine with vasodilating and cardioprotective properties. Animal experiments have shown that some benzodiazepine-induced effects can be explained by potentiation of adenosine effects, via inhibition of the nucleoside transport system. The objective of this study was to determine whether the frequently used benzodiazepines diazepam and midazolam increase adenosine-induced vasodilation in the human forearm vascular bed, measured by venous occlusion plethysmography.

Adenosine (0.6, 6, 20 and 60 $nmol \cdot min^{-1} \cdot dl^{-1}$ Fore Arm Volume) was infused into the brachial artery with and without concomitant separate infusion of diazepam (21 $nmol \cdot min^{-1} \cdot dl^{-1}$, n=9) and midazolam (23 $nmol \cdot min^{-1} \cdot dl^{-1}$, n=8). Plasma concentrations of diazepam resp. midazolam at the end of the infusion protocol averaged 0.5 ± 0.2 $\mu g \cdot ml^{-1}$ plasma (1.6 µM) for diazepam versus 1.2 ± 0.4 $\mu g \cdot ml^{-1}$ plasma (3 µM) for midazolam. Intra-arterial infusion of the benzodiazepines did not alter baseline vascular tone, and had no significant influence on the forearm vasodilator response to adenosine. The adenosine-induced relative change in Forearm Vascular Resistance (FVR) was -3 ± 7, -48 ± 8, -75 ± 6 and -85 ± 3% in the absence and 3.5 ± 11, -54 ± 5, -74 ± 5 and -82 ± 3% res. in the presence of diazepam ($p>0.1$, repeated measures ANOVA, n=9). Likewise, in the absence resp. presence of midazolam FVR fell by 1 ± 6, 55 ± 5, 74 ± 3 and 84 ± 2% resp. 11 ± 11, 59 ± 2, 80 ± 3 and 87 ± 2% ($p>0.1$, n=7).

Intra-brachial infusion of diazepam- and midazolam resulting in forearm concentrations in the high-therapeutic range does not augment adenosine-induced forearm vasodilation. A possible interaction at supra-therapeutic levels of the benzodiazepines can not be excluded from the present study, but lacks clinical significance.

Introduction

Adenosine is involved in autoregulation of cerebral- and coronary blood flow and is a mediator in the process of ischemic preconditioning [1]. Adenosine-induced vasodilation is mediated by specific membane receptors, the so-called adenosine A_2 -receptors [2]. The A_1-receptor is responsible for the central depressant action of adenosine, and for its antiarrhythmic and anti-ischemic properties. Adenosine has a half-life of a few seconds [3]. Inhibition of its metabolism offers a unique possibility to harness the tissue -protective properties of adenosine in a site- and event specific manner.
Benzodiazepines are widely prescribed for their anxiolytic, anticonvulsant and sedative effects. These properties of benzodiazepines are often used in patients with acute cardiac events. Most of these clinical effects can be explained by benzodiazepine-induced enhancement of synaptic transmission of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) [4]. An intriguing alternative mechanism for its therapeutic actions is the capacity of benzodiazepines to inhibit adenosine uptake. This results in increased extracellular adenosine levels and potentiation of adenosine-effects [5]. Phillis initially observed that diazepam potentiates the depressant action on cerebral neurons evoked by adenosine [6]. Moreover, the adenosine receptor antagonists theophylline and caffeine have been shown to antagonize several central actions of diazepam, while adenosine and its analogues elicit many effects corresponding to those of benzodiazepines [5]. Although most studies on the adenosine-benzodiazepine interaction focused on the central effects of adenosine, several studies on peripheral tissues have confirmed the potentiation of adenosine by micromolar concentrations of diazepam. For example, diazepam potentiates adenosine responses by inhibiting adenosine uptake in rat vas deferens, guinea pig hearts, trachea and taenia coli [7-10], rat coecum [11], rat ventricle [12], and mouse diaphragm [13]. To our knowledge, human in-vivo studies about the adenosine-benzodiazepine interaction are lacking so far. We hypothesized that diazepam and midazolam in high-therapeutic forearm concentrations might augment adenosine-mediated vasodilation in the forearm skeletal muscle vascular bed in healthy human volunteers. Studies were performed with the perfused forearm technique. This experimental set up has been validated previously to demonstrate adenosine uptake inhibition in humans in vivo [14;15].

Methods

Subjects

Studies were performed in 24 healthy, non-smoking volunteers. Demographic data are shown in table 1. Participants did not use concomitant medication except for oral contraceptives, and all subjects were normotensive. All participants underwent a physical examination, laboratory screening (total cholesterol, triglycerides and glucose) and electrocardiography before entering the study. The study protocol was approved by the hospital ethics comittee, and all participants signed written informed consent before their participation. Participants were asked to abstain from caffeine-containing beverages and alcohol for 24 hours before the experiment, and to abstain from food-intake 2 hours prior to the study.

Table 1. Demographic characteristics

N = 24 (8M / 16 F)	mean ± SD
Age (years)	21 ± 2
BMI ($kg \cdot m^{-2}$)	22 ± 2
SBP (mmHg)*	118 ± 9
DBP (mmHg)*	73 ± 7
HR (bpm) ¶	64 ± 9
Cholesterol ($mmol \cdot L^{-1}$)	4.1 ± 0.6
Triglycerides ($mmol \cdot L^{-1}$)	0.7 ± 0.2
Glucose ($mmol \cdot L^{-1}$)	4.6 ± 0.3

* Auscultatory measurement after 5 minutes of rest in supine position
¶ Measured by pulse frequency counting after 5 minutes of supine rest

General outline of the procedure

The experiments were performed in the morning with the subjects supine in a quiet temperature -controlled room (23-24°C). After local anesthesia (xylocaine 2%), the brachial artery of the non-dominant arm was cannulated (Angiocath, 20 gauge, Deseret Medical, Becton Dickinson Sandy, UT, USA) for drug infusion (syringe infusion pump, type STC-521, Terumo Corp., Tokyo, Japan) and intra-arterial blood pressure measurement (Hewlett Packard monitor, type 78353B, Hewlett Packard GmbH, Böblingen, Germany). A deep antecubital vein was cannulated bilaterally for blood sampling to determine plasma benzodiazepine-concentrations. Drug- and volume infusion rates were calculated per deciliter of forearm tissue, which was measured by the water displacement method. Bilateral Forearm Blood Flow (FBF) was measured by electrocardiogram triggered mercury-in-silastic strain gauge plethysmography, as described before [15], while the hand circulation was occluded using wrist cuffs [16]. After intra-arterial cannulation, at least 30 minutes of equilibration were included to obtain a steady state before baseline measurements. Pilot studies with intra-brachial infusions of diazepam (n=3) and midazolam (n=4) alone were performed first, for safety and dose-finding. Baseline values were recorded during infusion of saline (NaCl 0.9%). Diazepam (*Diazemuls)* was infused ino the brachial artery in three increasing doses: 1.2, 3.5 (each during ten minutes) and 10.5 (during thirty minutes) $nmol \cdot min^{-1} \cdot dl^{-1}$ (n=3). Each dose was preceded by a five-minutes measurement of baseline values during infusion of NaCl 0.9%. During the last minute of diazepam infusion, venous blood samples were drawn for measurement of local plasma diazepam concentrations. Midazolam pilot experiments with 0.8, 2.5 and 7.5 $nmol \cdot min^{-1} \cdot dl^{-1}$ were performed using the same infusion schedule (n=4).

The final experiments started with infusion of saline (NaCl 0.9%) to obtain baseline recordings (see figure 1 for the infusion schedule). Thereafter, four increasing doses of adenosine (0.6, 6, 20 and 60 nmol·min^{-1}·dl^{-1}) were co-infused with saline during 5 minutes per dose. The succeeding adenosine-doses were interrupted once by a 5 minutes drug free interval during which the wrist cuffs were deflated to allow recovery of the hand circulation. After a subsequent equilibration period of 45 minutes to allow parameters to return to baseline levels, baseline recordings were repeated during infusion of saline followed by separate infusion of diazepam (21 nmol·min^{-1}·dl^{-1}) resp. midazolam (23 nmol·min^{-1}·dl^{-1}). After 15 minutes of benzodiazepine-infusion, subsequent infusions of adenosine were started. Diazepam and midazolam administration was continued during the adenosine infusions. The rate of infused volume and the amount of connected syringes was kept constant throughout each experiment. Venous benzodiazepine plasma concentrations from both arms were measured at baseline, after the second and after the last adenosine dose.

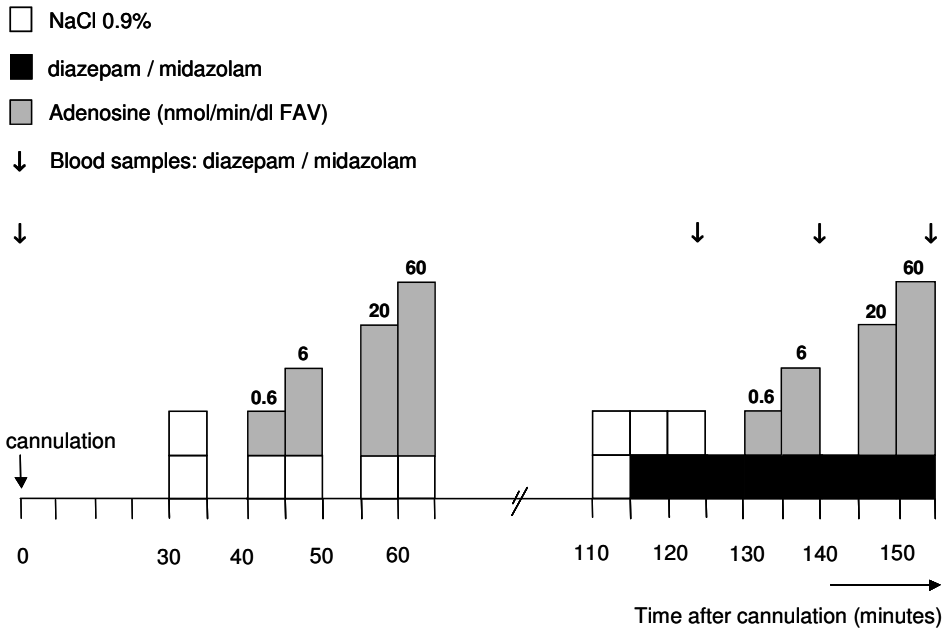

Figure 1. Infusion schedule

Drugs and solutions

Adenosine was prepared for each experiment by diluting Adenocor (6 mg/2ml, Sanofi Winthrop) vials in NaCl 0.9% to reach the necessary concentrations. Diazemuls (Dumex, Baarn) and midazolam (Dormicum, Roche, Mijdrecht, The Netherlands) were diluted in saline to reach maximal syringe-concentrations of 0.06 and 0.09 mg·ml^{-1} respectively. We used the emulsion preparation *Diazemuls* for diazepam administration because it proved to be not arterio-toxic in animal experiments [17] in contrast to the regular preparation. All solutions were freshly prepared.

Analytical procedures

Plasma levels of midazolam were determined using a specific HPLC method with UV detection at 220 nm as described elsewhere [18]. In brief, the method included a liquid-liquid extraction of alkalized plasma with cyclohexane-dichloromethane (55-45 v/v) followed by evaporation of the organic layer. Separation was achieved on a Inertsil ODS-3 C-18 column (15 X 0.46 cm) with an isocratic mobile phase of 0.1M phosphate buffer (pH7.0) -acetonitrile (65-35% v/v). The inter- and intra-day coefficients of variation of the assay were less than 7% over the range till 0.4 $\mu g \cdot ml^{-1}$. A good linearity for midazolam was obtained till a concentration of 10 $\mu g \cdot ml^{-1}$ (R^2=0.9999). The limit of quantification was 0.5 $ng \cdot ml^{-1}$.
Plasma levels of diazepam were determined with a routine High Pressure Liquid Chromatography method for benzodiazepines with UV detection at 313 nm. This modified method of Meijer [19] included an automatic solid phase extraction with an ASPEC (Gilson) on Isolute C18 (100 mg) SPE columns. After conditioning of the SPE columns with 2 ml of methanol and 2 ml of ultrapure water the system added 1.0 ml of plasma with 0.2 ml internal standard of 10 $\mu g \cdot ml^{-1}$ chlorodesmethyldiazepam onto the SPE columns. After washing the columns with 2.0 ml of 0.05M K_2HPO_4 and 1 ml of ultrapure water, the benzodiazepines were eluted with 0.35 ml acetonitrile. The eluate was mixed with 0.75 ml water before injection of 0.2 ml into the chromatograph. Analyses were performed on a Sperisorb 3ODS2 column (12.5 X 0.46 cm) with an isocratic mobile phase of 0.02M phosphate buffer (pH 7.0) - acetonitrile (62-38% v/v) at a flow of 1 $ml \cdot min^{-1}$. The inter- and intra-day coefficients of variation for diazepam and the desmethyldiazepam were less than 7% at a concentration of 0.1 $\mu g \cdot ml^{-1}$. The limit of quantification was less than 0.02 $\mu g \cdot ml^{-1}$.

Statistical analysis

All results are mean ± SE, unless indicated otherwise. $P<0.05$ (two sided) was considered statistically significant. Mean arterial blood pressure (MAP) was calculated from the electronically integrated area under the brachial arterial pulse-wave curve and averaged per FBF measurement. Forearm vascular resistance (FVR) was calculated from simultaneously measured MAP and FBF (MAP/FBF) and expressed as arbitrary units (AU). The mean calculated FVRs and hemodynamic parameters obtained during the last 4 minutes of saline infusion or during the last 2 minutes of each drug infusion were taken as the response and used for further analysis. Benzodiazepine-induced effects on vascular tone were expressed as absolute difference from baseline and analyzed using a paired student t-test. The vasodilator response to adenosine was expressed as the percentage change in FVR from the preceding saline infusion or benzodiazepine infusion. To avoid multiple comparison, the effect of the benzodiazepines on adenosine-induced vasodilation were assessed with repeated measures ANOVA. The presence of benzodiazepine and adenosine doses were used as within subject factors. The statistical analyses were performed using the SPSS personal computer software package (SPSS Corp., Gorinchem, The Netherlands).

Results

Pilot studies:

The pilot experiments did not reveal any signs of possible benzodiazepine induced arterio-toxicity, and none of the participants experienced drowsiness. Baseline vascular tone was not influenced by infusion of diazepam nor midazolam. The mean plasma-concentrations at the end of the thirty minutes infusion of the highest doses in the pilot experiments were 1.1 mg diazepam·L^{-1} (n=3) and 0.7 mg midazolam·L^{-1} (n=4). As we aimed to reach high therapeutic plasma concentrations of about 2 mg diazepam ·L^{-1} and 1 mg·L^{-1} midazolam, the infused benzodiazepine doses were adjusted to 21 nmol·dl^{-1}·min^{-1} diazepam and 23 nmol·dl^{-1}·min^{-1} midazolam for the final experiment.

Adenosine-benzodiazepine studies:

In the absence of diazepam, adenosine increased forearm blood flow (FBF) from 2.5±0.6 at baseline to 2.9±0.8, 5.5±1.3, 11.1±1.8 and 18.3±3.1 ml·min^{-1}·dl^{-1} during the four incremental adenosine doses respectively. Recontrol values for FBF or FVR did not significantly differ from baseline (2.5±0.6 vs 3.2±0.7 ml·min^{-1}·dl^{-1}; 45.4±6.5 vs 38±6.2 AU; p>0.1; n=9). Subsequent infusion of diazepam did not significantly affect FBF (3.2±0.8 ml·min^{-1}·dl^{-1}) or FVR (42.3±9.3 AU, n=9, p>0.1 vs recontrol). In the presence of diazepam, adenosine increased FBF from 3.2±0.8 at baseline to 3.4±0.8, 6.8±1.3, 12.9±1.8 and 18.4±3.3 ml·min^{-1}·dl^{-1} during the four increasing adenosine doses respectively (p>0.1 versus adenosine response in absence of diazepam, n=9). Similar results were obtained when expressed as percentage change in FVR (see figure 2). The mean cumulative diazepam dose was 2.1±0.3 mg (n=9). Diazepam plasma concentrations were obtained in 7 from 9 participants. Diazepam concentrations amounted 1.1±0.2 at baseline, 0.9±0.3 after the second and 0.5±0.2 µg·ml^{-1} after the last adenosine dose respectively. This decrease in concentration is due to adenosine-induced vasodilation; at a constant infusion-rate of diazepam, an adenosine-induced increase in forearm blood flow results in increased dilution of diazepam. During the last minute of the experiment, diazepam could be detected in the control arm in 5 of 7 participants, with a mean value of 0.04±0.01 µg·ml^{-1}. None of the volunteers in this experiment with diazepam experienced drowsiness.
Likewise, in the absence of midazolam, adenosine increased FBF from 2.1±0.4 at baseline to 2.2±0.5, 4.8±0.9, 7.9±1 and 13±1.1 ml·min^{-1}·dl^{-1} during the four incremental adenosine doses respectively. Recontrol values for FBF or FVR did not significantly differ from baseline (2.1±0.4 vs 1.9±0.3 ml·min^{-1}·dl^{-1}; 51.4±8.2 vs 57.5±10.1 AU; p>0.1; n=7). Subsequent infusion of midazolam did not significantly affect FBF (2±0.3 vs 1.9±0.3 ml·min^{-1}·dl^{-1}) or FVR (57.5±10.1 vs 64.7±13.4 AU, n=7, p>0.1 vs recontrol). In the presence of midazolam, adenosine increased FBF from 2.1±0.4 at baseline to 2.2±0.5, 4.8±0.9, 7.9±1 and 13±1.1 ml·min^{-1}·dl^{-1} during the four increasing adenosine doses respectively (p>0.1 versus adenosine response in absence of midazolam, n=7). Similar results were obtained when expressed as percentage change in FVR (see figure 2). All but two participants in the midazolam-

adenosine study experienced drowsiness. In the experimental arm, midazolam plasma concentrations changed from 2.5±0.2 at baseline to 2.7±0.4 and 1.2±0.4 $mg \cdot L^{-1}$ during the second and last adenosine dose. The mean cumulative midazolam dose that was infused was 3.2±0.8 mg (n=8). During the last minute of the experiment, midazolam could be detected in the control arm of all volunteers, with a mean value of 0.04 $mg \cdot L^{-1}$. Infusion of midazolam 23 $nmol \cdot min^{-1} \cdot dl^{-1}$ changed FVR from 57±10 at recontrol to 65±13 AU (n=7, p>0.1, paired samles t-test). In the absence resp. presence of midazolam, the percentage decrease in FVR was 1±6, 55±5, 74±3 and 84±2% resp.11±11, 59±2, 80±3 and 87±2% (p>0.1, n=7). Diazepam nor midazolam-infusion induced significant changes in blood pressure or heart rate. The course of FVR in the control arm did not change significantly during the studies.

Discussion

This study showed that adenosine-induced vasodilation in the human forearm can not be augmented by concomitant intra-arterial infusion of diazepam or midazolam in a dose that results in high therapeutic forearm concentrations. We were interested in a possible benzodiazepine-induced augmentation of adenosine effects because such an interaction could be exploited in cardiovascular diseases and during anesthesia. In a previous study, we showed that the dose-response curve for adenosine-induced vasodilation in the human forearm is reproducible [15]. The explanation for the discrepancy between our data and previous reports by others is that benzodiazepine concentrations in the forearm have been too low to induce significant inhibition of adenosine transport. Indeed, benzodiazepines have been shown to have a relative low affinity (IC_{50}>1µM) for nucleoside transport in for example human erythrocytes, contrasting with the nanomolar affinity of the prototypical transport blocker dipyridamole [20]. We actually reached micromolar plasma concentrations of benzodiazepines, which does not rule out a concentration problem however, since we measured total plasma concentrations. Diazepam and midazolam have a high plasma protein binding (>90%). The study of the influence of higher doses of diazepam in our study was hampered by potential toxic effects on the vascular wall [17;21;22]. For midazolam, drowsiness in our volunteers was the dose limiting side effect in these studies. We can not rule out a possible interaction at supra-therapeutic levels of the benzodiazepines from the present study, but this lacks clinical significance. Finally, we performed experiments on peripheral tissues which does not rule out a possible benzodiazepine-induced inhibition of adenosine transport in the central nervous system.
We did not observe any vasodilator action of the tested benzodiazepines, indicating that a possible blood pressure lowering action of these drugs as observed during anaesthesia [23] can not be explained by a direct action on peripheral vascular tone. We conclude that adenosine-induced vasodilation in the forearm vascular bed of human volunteers can not be augmented by diazepam or midazalam concentrations at the upper end of the clinical relevant range.

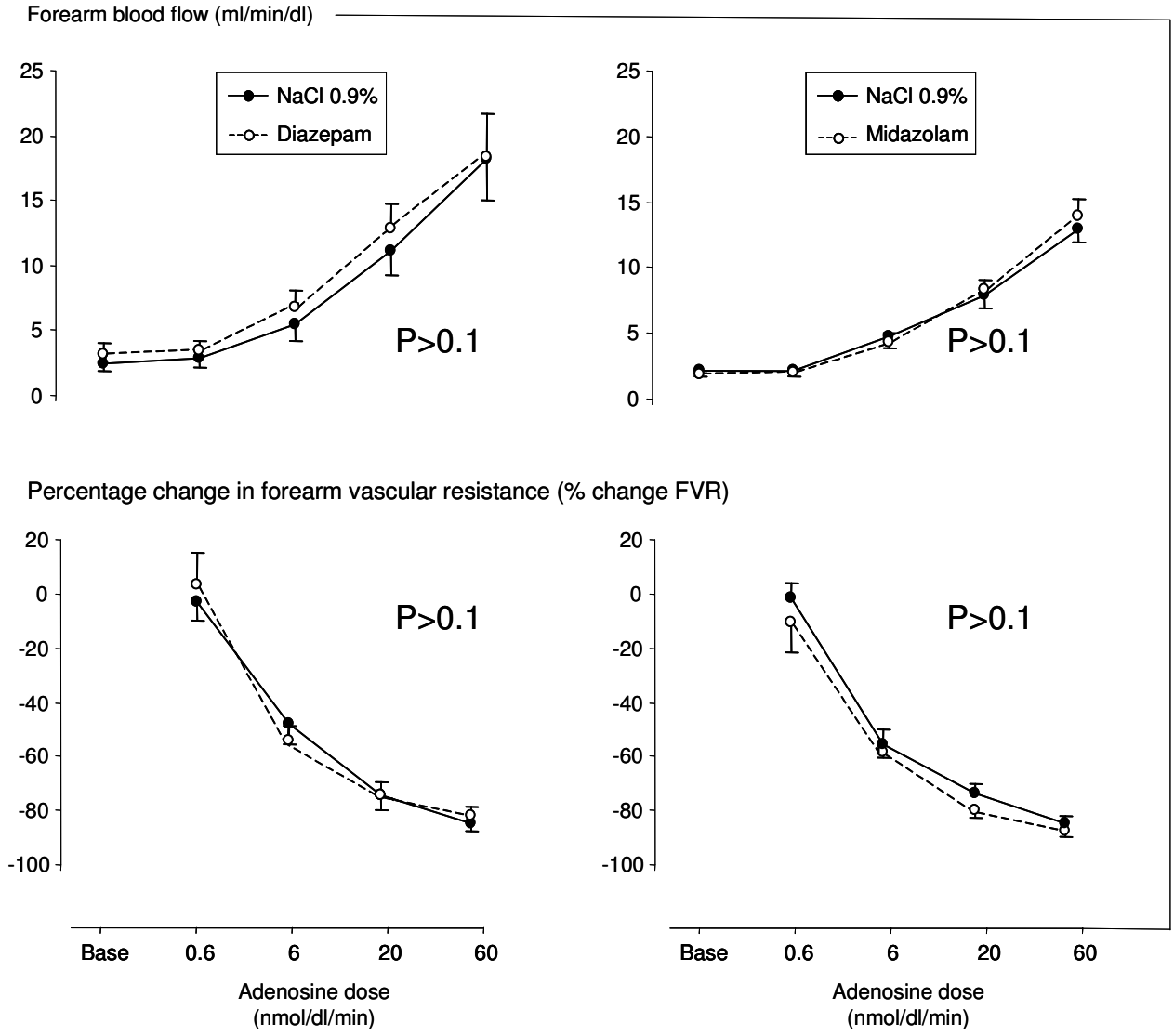

Figure 2. Effect of diazepam (left) and midazolam (right) infusions on adenosine- (0.6, 6, 20 and 60 nmol·min^{-1}·dl^{-1}) induced change in forearm blood flow (FBF, ml·dl^{-1} FAV·min^{-1}, mean ± SE), and expressed as percentage change (mean±SE) in forearm vascular resistance (FVR) from preceding baseline (saline or saline with benzodiazepine). Solid line: with concomitant saline infusion; dashed line: with concomitant benzodiazepine infusion. P-value indicates level of significance between the curves with and without concomitant benzodiazepine infusion (ANOVA for repeated measures).

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CHAPTER 6

ATP-induced vasodilation in human skeletal muscle

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Abstract

The purine nucleotide adenosine-5`-triphosphate (ATP) exerts pronounced effects on the cardiovascular system. The mechanism of action of the vasodilator response to ATP in humans has not been elucidated yet. Proposed endothelium derived relaxing factors (EDRFs) were studied in a series of experiments, using the perfused forearm technique.

Adenosine 5`-triphosphate (0.2, 0.6, 6 and 20 nmol·dl^{-1} forearm volume·min^{-1}) evoked a dose-dependent forearm vasodilator response which could not be inhibited by the separate infusion of the non-selective COX inhibitor indomethacin (5 µg·dl^{-1}·min^{-1}, n=10), the blocker of Na^+/K^+-ATPase ouabain (0.2 µg·dl^{-1}·min^{-1}, n=8), the blocker of K_{Ca}-channels tetraethylammonium-chloride (TEA, 0.1 mg·dl^{-1}·min^{-1}, n=10), nor by the K_{ATP}-channel blocker glibenclamide (2 µg·dl^{-1}·min^{-1}, n=10). All blockers, except glibenclamide, caused a significant increase in baseline vascular tone. The obtained results might be due to compensatory actions of unblocked EDRFs. Combined infusion of TEA, indomethacin and L-NMMA (n=6) significantly increased baseline forearm vascular resistance. The ATP-induced relative decrease in Forearm Vascular Resistance was 48 ± 5, 67 ± 3, 88 ± 2 and 92 ± 2 % in the absence and 23 ± 7, 62 ± 4, 89 ± 2 and 93 ± 1 % in the presence of the combination of TEA, indomethacin and L-NMMA ($p<0.05$, repeated measures ANOVA, n=6). A similar inhibition was obtained for sodium nitroprusside (SNP, $p<0.05$ repeated measures ANOVA, n=6), indicating a non-specific interaction due to the blocker induced vasoconstriction.

ATP-induced vasodilation in the human forearm can not be inhibited by separate infusion of indomethacin, ouabain, glibenclamide or TEA, or by a combined infusion of TEA, indomethacin and L-NMMA. Endothelium-independent mechanisms and involvement of unblocked EDRFs, such as CO, might play a role, and call for further studies.

Introduction

Adenosine-5`-triphosphate (ATP) is an endogenous purine nucleotide consisting of a purine base (adenine), ribose and 3 phosphate groups. ATP is released from aggregating thrombocytes [1;2], endothelium [3], sympathetic nerve endings [4;5], and ischemic muscle cells [6]. Extracellular ATP exerts potent and diverse effects on the cardiovascular system via activation of P2 receptors [6;7]. In general, P2x and P2y receptors on vascular smooth muscle cells (VSMCs) mediate vasoconstriction, while stimulation of P2y receptors on endothelial cells causes vasodilation. This dual action of ATP on vascular tone may have important clinical consequences: during thrombocyte aggregation at sites of severe atherosclerosis, locally released ATP might induce vasoconstriction mediated by P2x-receptors located on VSMCs, which is unopposed by P2y-receptors mediated vasodilation because of endothelial damage [8]. Local vasoconstriction will then further aggrevate ischemia. Therefore, better understanding of ATP-induced vasodilation may reveal new targets for pharmacological intervention to reduce or prevent vasospasm, thrombus formation, and ischemia.

The exact mechanism of ATP-induced endothelium-dependent vasodilation in humans is still a matter of debate. In vitro studies that explored the vasomotor effect of ATP in the presence and absence of an intact endothelium revealed an important role of the endothelium in ATP-induced vasodilation.

The proposed Endothelium Derived Relaxing Factors (EDRFs) are NO, prostacyclin and Endothelium Derived Hyperpolarizing Factors (EDHFs) [9-12].

The exact nature of EDHF is stil uncertain, although its mechanism of action through opening of potassium channels and/or activation of Na^+/K^+-ATPase has been well established [13-16]. A study in isolated mesenteric rat-arteries showed that the prolonged phase of vasorelaxation to ATP was attenuated by ouabain and by glibenclamide, indicating direct or indirect involvement of Na^+/K^+-ATPase and K_{ATP} -channels [17]. *In-vivo* studies on ATP-induced vasodilation are rare. Former experiments by our group revealed that the ATP-induced vasodilation in the human forearm exceeds the vasodilation induced by equimolar adenosine infusion, which is the degradation product of ATP with the highest P1-purinergic receptor agonist activity [18]. This demonstrates that the metabolite adenosine hardly contributes to the ATP-induced vasodilation in the human forearm. This is further supported by the fact that the P1-purinoceptor antagonist theophylline did not affect the vasodilator response to ATP [18]. It was shown previously that ATP-induced vasodilation in the human forearm can not be inhibited by the competitive NO-synthase antagonist N^G-monomethyl-L-arginine (L-NMMA) [18;19]. Finally, previous experiments by our group have demonstrated that the vasodilator response to intra-arterial ATP in the forearm is not limited by any vasoconstrictor action, including vasoconstriction that could theoretically have resulted from P2x receptor stimulation on vascular smooth muscle cells [18].

The aim of this study was to identify a possible role for cyclo-oxygenase products and endothelium derived hyperpolarizing factor(s) (EDHF) in ATP-induced vasodilation. Cyclo-oxygenase mediates the formation of the intermediate compound prostaglandin H2 (PGH_2), the precursor for several prostaglandins, such as prostacyclin (PGI_2) and thromboxane A2 (TXA_2 , an endothelium derived contracting factor). Prostacyclin acts on receptors on VSMCs mediating vasodilation by increase of intracellular cAMP via stimulation of adenylate cyclase [20]. In this study, cyclooxygenase activity was blocked with indomethacin.

Other arachidonic acid metabolites that might be partly responsible for EDHF activity are epoxides (EET`s, formed by cytochrome-P-450) and hydroxyeicosatraenoic acid (HETE, formed by lipoxygenases). EET`s and HETE mediate relaxation of VSMCs by opening calcium-dependent potassium channels [21;22]. Another compound that mediates vasodilation by opening calcium-dependent potassium channels (K_{Ca} -channels) in VSMCs is endothelium derived hydrogen peroxide (H_2O_2) [23]. In this study, K_{Ca} -channels were blocked with tetraethylammonium (TEA). ATP-sensitive potassium channels (K_{ATP} -channels) also play a role in mediating vasodilation by hyperpolarizing VSMCs [24]. Glibenclamide was used to block K_{ATP} -channels. Finally, potassium itself acts as EDHF by inducing hyperpolarisation of VSMCs by activation of Na/K-ATPase [25]. The role of Na^+/K^+-ATPase was studied by concomitant infusion with ouabain, a compound that inhibits Na^+/K^+-ATPase and has been shown to block the relaxation and hyperpolarization caused by EDHF [26]. *In vitro* [27;28] and *in vivo* studies [29] have shown that EDRFs can compensate for inhibition of formation or function of a single EDRF. The same might be true for ATP-induced vasodilation in the human forearm, which was studied in an additional experiment, by combined infusion with TEA, indomethacin and L-NMMA.

Methods

Subjects

The study protocol was approved by the local ethics comittee, and all participants signed written informed consent before their participation. The investigation conforms with the principles outlined in the declaration of Helsinki. Demographic data are shown in table 1. The experiments were performed in healthy, normotensive male and female volunteers. They did not use concomitant medication except for oral contraceptive drugs. All participants underwent a physical examination, laboratory screening (total cholesterol, triglycerides and glucose) and electrocardiography before entering the study. Participants were asked to abstain from caffeine-containing beverages and alcohol for 24 hours before the experiment, and to abstain from food-intake 2 hours prior to the study.

General outline of the procedure

The experiments were performed in the morning in a quiet room with stable temperature (23°C), with the subjects in supine position. After local anesthesia (xylocaine 2%), the brachial artery of the non-dominant arm was cannulated (Angiocath, 20 gauge, Deseret Medical, Becton Dickinson Sandy, UT, USA) for drug infusion (syringe infusion pump, type STC-521, Terumo Corp., Tokyo, Japan) and intra-arterial blood pressure measurement (Hewlett Packard monitor, type 78353B, Hewlett Packard GmbH, Böblingen, Germany). Drug- and volume infusion rates were calculated per deciliter of forearm tissue, which was measured for each person by water displacement.
In protocols involving glibenclamide, a deep antecubital vein of the infused arm was cannulated for blood sampling. Bilateral forearm blood flow (FBF) was measured by ECG triggered mercury-in-silastic strain gauge plethysmography, as described before

[30], while the hand circulation was occluded using wrist cuffs [31]. All experiments started 30 minutes after intra-arterial cannulation with the measurement of baseline blood flow, obtained during infusion of saline (NaCl 0.9%). Thereafter, increasing doses of ATP were co-infused with saline. Each ATP dose was infused for 5 minutes, together with saline or a blocker. The succeeding ATP-doses were interrupted once by a 10 min drug free interval. This was done because prolonged occlusion of the hand circulation can cause discomfort leading to changes in blood pressure and heart rate. The rate of infused volume and the amount of connected syringes was kept constant throughout each experiment.

The effect of indomethacin on ATP-induced forearm vasodilaton (n=12)

In this study we used three increasing doses of ATP (0.6, 6 and 20 nmol·dl^{-1} FAV·min^{-1}). Forty-five minutes after infusion of the highest dose, baseline recordings were repeated during infusion of saline folllowed by indomethacin (5 µg·dl^{-1}·min^{-1}) respectively. Subsequently, ATP infusions were repeated in the presence of indomethacin.

The effect of TEA on ATP-induced forearm-vasodilation (n=10)

After baseline measurements, ATP 0.2, 0.6, 6 and 20 nmol·dl^{-1} FAV·min^{-1} were infused. Baseline recordings were repeated after a 30 minute drug-free interval. TEA (0.1 mg·dl^{-1}·min^{-1}) was infused for thirty minutes, followed by co-infusion with the increasing ATP-doses.

The effect of glibenclamide on ATP-induced forearm-vasodilation (n=16)

In the first glibenclamide study (n=10), we infused ATP 0.2, 0.6 , 6 and 20 nmol·dl^{-1}·min^{-1}. After 30 minutes, recontrol values were obtained and glibenclamide (2 µg·dl^{-1}·min^{-1}) was subsequently infused. Ten minutes after the start of glibenclamide, ATP-infusions were repeated. Venous blood samples were collected from the experimental arm to measure the effect of glibenclamide on glucose-, insulin-, and C-peptide concentrations and to determine the concentration of glibenclamide during the course of the study. To check the validity of an observed small effect of glibenclamide on ATP-induced vasodilation, the protocol was repeated but now with ATP 0.1, 0.2, 0.4 and 0.8 nmol·dl^{-1}·min^{-1} (n=6).

The effect of ouabain on ATP-induced forearm-vasodilation (n=8)

The outline of this study is similar to the glibenclamide study. Ouabain was infused instead of glibenclamide, in a concentration of 0.2 µg·dl^{-1}·min^{-1}.

The influence of combined infusion of TEA, indomethacin and L-NMMA on ATP-induced forearm-vasodilation (n=6)

After baseline measurements, four increasing doses of ATP were infused (0.2, 2, 6 and 20 nmol·dl^{-1}·min^{-1}). The second half of the experiment started 30 minutes after cessation of the highest ATP-dose. Saline infusions were subsequently replaced by infusion of TEA (0.1 mg·dl^{-1}·min^{-1}), indomethacin (5 µg·dl^{-1}·min^{-1}), and L-NMMA (0.2 mg·dl^{-1}·min^{-1}). A graphic presentation of the protocol is provided in figure 1.
Since this combination of antagonists increased FVR significantly and reduced the % change in FVR in response to ATP, a similar protocol was used in a separate group of 6 volunteers but now ATP was replaced by nitroprusside as a vasodilative control (SNP; 0.02, 0.1, 0.2, and 0.6 µg·dl^{-1}·min^{-1}).
Blood samples were collected in three of the six participants during the second half of the infusion schedule to determine oxygen consumption before and during the combined antagonist infusion. A venous and arterial blood sample were taken at the end of placebo infusion (t =100, see figure 1) and during antagonist infusion (t =130) for determination of oxygen saturation and hemoglobin. Oxygen consumption was calculated by measuring the arterio-venous difference in the product of saturation (%), blood flow (ml·dl^{-1} FAV·min^{-1}) and hemoglobin (mM), expressed in Arbitrary Units.

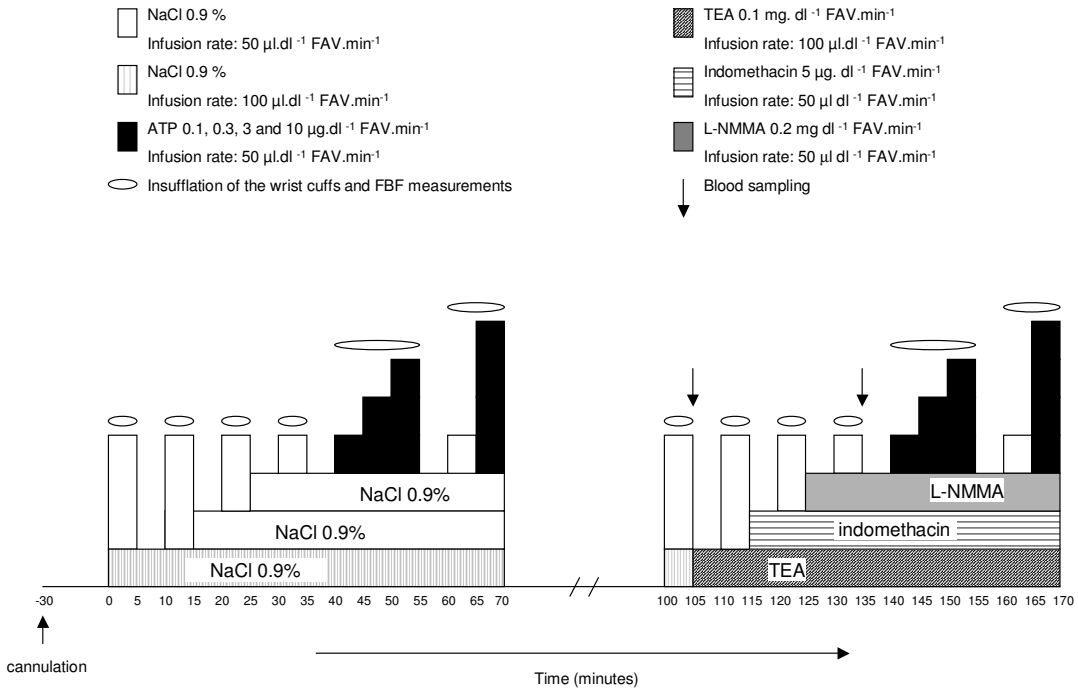

Figure 1. Infusion protocol of the ATP study with combined infusion of TEA, indomethacin and L-NMMA.

Drugs and solutions

All solutions were freshly prepared. ATP (*Striadyne*, Wyeth Laboratories) was diluted to reach the necessary concentrations. Indomethacin (GenRX-Mosby Inc., St. Louis, MO, USA), TEA (Sigma Chemical Co, St. Louis, MO, USA), glibenclamide (Hoechst AG, Frankfurt, Germany), L-NMMA (Sigma Chemical Co, St. Louis, MO, USA) and ouabain (Pharmachemie, Haarlem, the Netherlands) were diluted in NaCl 0.9% to reach final syringe concentrations of 5 µg (indomethacin), 50 µg (TEA), 2 µg (glibenclamide), 0.2 mg (L-NMMA) and 0.2 µg (ouabain) per 50 µL respectively. Lyophilized SNP (*Nipride*, Roche Nederland, Mijdrecht, the Netherlands) was diluted in glucose 5% and protected against light.

Analytical procedures

Insulin and C-peptide concentrations were determined in our laboratories using specific radioimmunoassays. In the insulin assay standard and tracer insulin was prepared from mono-component human insulin (Novo, Zoeterwoude, the Netherlands). Insulin concentrations below 5.0 $mE \cdot L^{-1}$ remained undetected. C-peptide was measured with a standard kit (D.P.C., Los Angeles, Calif., USA). The detection limit for glibenclamide was below 5.0 $ng \cdot ml^{-1}$. Plasma glucose concentrations were assessed in our laboratories with a Hitachi 747 (Roche diagnostics, Indianapolis, IN, USA). Glibenclamide was measured by high performance liquid chromatography (HPLC) [32]. Hemoglobin concentrations were assessed with the Advia 1650 (Bayer diagnostics, Leverkusen, Germany). Oxygen saturation was determined with the Rapidlab 248 (Bayer diagnostics).

Statistical analysis

Mean arterial blood pressure (MAP) was measured continuously during each recording of FBF and averaged per FBF measurement. Forearm vascular resistance (FVR) was calculated from simultaneously measured MAP and FBF (MAP/FBF) and expressed as arbitrary units (AU). The calculated FVRs and hemodynamic parameters obtained during the last 4 minutes of saline infusion or during the last 2 minutes of each drug infusion were averaged to one value. Drug-induced effects were expressed absolute (t-tests for the effect of an antagonist on baseline values) or as the percentage change from preceding saline infusion or antagonist infusion. All results are mean ± SE, unless indicated otherwise. Based on reproducibility data from a previous study by our group [18], it can be estimated that for a dose of 6 $nmol \cdot dl^{-1}$ forearm volume, a minimal difference in percentage change in forearm vascular resistance from baseline can be detected of 21 % (N=10) or 31% (N=6) with a power of 0.9 and an alpha of 0.05 (paired t-test). To avoid multiple comparison, the effect of antagonists on ATP and SNP-induced vasodilation were assessed with repeated measures ANOVA. The presence of antagonists and vasodilator doses were used as within subject factors. To explore the effect of previous vasodilator treatment on the vasoconstrictive effect of combined infusion of TEA, indomethacin and L-NMMA, the vasodilator was used as between group factor. T-tests were

applied as post hoc tests when applicable. P<0.05 (two sided) was considered statistically significant.

Results

The demographic data of the participants are shown in table 1. The course of FVR in the infused arm is shown in figure 2 for each experiment. The course of FVR in the control arm was not significantly affected by any of the blockers or vasodilators used. Recontrol values for FVR did not differ from baseline.

	Indomethacin	TEA	Glibenclamide	Glibenclamide + low dose ATP	Ouabain	TEA + Indomethacin + L-NMMA	SNP control study
N	12	10	10	6	8	6	6
M/F	12 / 0	4 / 6	6 / 4	4 / 2	4 / 4	1/5	1/5
Age (year)	22.9 ± 6.0	22.6 ± 3.2	22.5 ± 2.2	21.8 ± 2.5	22.3 ± 2.1	20.8 ± 0.4	21.2 ± 3.6
BMI (kg m^{-2})	21 ± 1.6	22.7 ± 2.1	23.1 ± 2.1	21.5 ± 1.4	23.0 ± 2.4	21.6 ± 2.9	22.1 ± 1.5
SBP (mmHg)	129.3 ± 9.0	122.5 ± 9.3	122.8 ± 7.7	123.5 ± 9.5	120.1 ± 8.5	129 ± 10.2	115.3 ± 9.3
DBP (mmHg)	70.9 ± 8.2	69.4 ± 6.5	76.2 ± 8.9	72.2 ± 7.8	76.4 ± 4.4	76.3 ± 5.7	67.0 ± 6.3
HR (bpm)	69.1 ± 12.3	60.7 ± 11.4	60.8 ± 9.5	62.7 ± 15.2	61.5 ± 8.0	68.3 ± 7.0	59.8 ± 6.3
Glc (mmol L^{-1})	-	4.4 ± 0.2	4.5 ± 0.4	4.4 ± 0.6	4.5 ± 0.3	4.1 ± 0.5	4.8 ± 0.7
Chol (mmol L^{-1})	-	4.0 ± 0.6	4.0 ± 0.4	3.9 ± 0.6	3.8 ± 0.5	4.0 ± 0.4	4.1 ± 0.8
TG (mmol L^{-1})	-	1.0 ± 0.5	0.8 ± 0.5	0.8 ± 0.2	0.9 ± 0.4	1.1 ± 0.5	0.8 ± 0.1

Table 1: Baseline characteristics of the study groups (mean ± SD)

M/F; Male/Female, BMI; Body Mass Index; SBP; Systolic Blood Pressure, DBP; Diastolic Blood Pressure, HR; Heart Rate, Glc; glucose, Chol; cholesterol, TG; triglycerides.

The effect of the antagonists on baseline FVR

Apart from glibenclamide, all used blockers induced a vasoconstrictor response. This response was most pronounced for the combined infusion of TEA, indomethacin, and L-NMMA (see table 2). The vasoconstrictor action of TEA was only significant after previous infusion of ATP but not after SNP (see table 3) and significantly differed between the ATP and SNP pretreated group. In the ATP-study with combined infusion of TEA, indomethacin and L-NMMA calculated values for oxygen consumption were 2.9, 7.1 and 7.2 versus 6.4, 6.1 and 6.5 AU in the absence and presence of antagonists respectively. Forearm oxygen consumption was not affected by simultaneous infusion of TEA, L-NMMA and indomethacin, which argues against vasoconstriction-induced ischemia.

Table 2. Effect of the antagonists on baseline vascualr tone (FVR, absolute value, mean ± SE):

mean ± SE	Saline	Antagonist
Indomethacin*	53 ± 9	61 ± 9
TEA*	30 ± 2	62 ± 13
Glibenclamide	56 ± 11	47 ± 6
Glibenclamide (low dose ATP)	44 ± 5	49 ± 9
Ouabain*	49 ± 6	63 ± 9
T + I + L*	34 ± 8	92 ± 10
T + I + L* (SNP)	48 ± 3	85 ± 10

ATP-and SNP doses are expressed in nmol·min^{-1}·dl^{-1} Forearm Volume.
T = TEA; I = Indomethacin; L = L-NMMA
*: $p < 0.05$ for baseline versus antagonist (paired t-test)

Table 3. FVR (AU, mean ± SE) and % change during subsequent combination of antagonists:

FVR, AU (% change in FVR)		Baseline	Recontrol	T	T+I	T+I+L
ATP (N = 6)		37 ± 7	34 ± 8	46 ± 8* (39 ± 6$^{\#}$)	77 ± 5† (109 ± 57)	92 ± 10 (21 ± 13)
SNP (N = 6)	41 ± 4	48 ± 3	54 ± 4 (12 ± 5)	63 ± 5† (19 ± 7)	85 ± 10¶ (35 ± 12)	

T = TEA; I = Indomethacin; L = L-NMMA
$^{\#}$: $p<0.05$ for between group comparison of relative responses (ATP group with SNP group)
*: $p<0.05$ vs recontrol (paired t-test)
†: $p<0.05$ vs T (paired t-test)
¶: $p<0.05$ vs T + I (paired t-test)

Influence of the antagonists on ATP-induced forearm vasodilation

Indomethacin, TEA and ouabain (infused seperately) did not reduce ATP-induced forearm vasodilation (see figure 2). ATP- (0.2, 0.6 , 6 and 20 nmol·dl^{-1}·min^{-1}) induced vasodilation was significantly reduced by glibenclamide ($p<0.05$ for the interaction

between ATP and glibenclamide). This effect was solely due to the lowest ATP-dose, and could not be reproduced in an additional study with ATP infused in a lower dose range: ATP 0.1, 0.2, 0.4 and 0.8 nmol·dl^{-1}·min^{-1} reduced FVR by 31.1±5.9, 52.9±6.9, 62.7±5.6 and 72.2±2.6 % versus 27.6±10.8, 52.1±6.8, 65.8±5.9 and 70.9±2.9 % in the absence and presence of glibenclamide respectively ($p>0.1$, $n=6$).
Glibenclamide concentrations were measured before start of the glibenclamide infusion, after the third ATP dose and after the last ATP dose. Concentrations were 1.5±0.3, 0.2±0.03 and 0.2±0.02 (ATP 0.2, 0.6 , 6 and 20 nmol·dl^{-1}·min^{-1}) and 1.2±0.1, 0.5±0.03 and 1.2±0.1 µg·mL^{-1} (ATP 0.1, 0.2 , 0.4 and 0.8 nmol·dl^{-1}·min^{-1}) respectively.
Glucose concentrations did not alter significantly : 4.5±0.1 vs 4.3±0.1 (samples taken before resp. during glibenclamide infusion) versus 4.4±0.1 vs 3.6±0.1 mmol·L^{-1} (higher vs lower ATP dose range). Plasma insulin concentration increased significantly from 6.4±0.5 to 11.9±1.4 (higher ATP dose range, $p<0.05$) and 7.2±0.7 to 13.8±0.5 mE·L^{-1} (lower dose range, $p<0.05$). C-peptide also increased significantly during the course of the study: from 0.4±0.1 to 0.6±0.1(higher ATP doses, $p<0.05$) and 0.3±0.02 to 0.6±0.01 (lower ATP dose range, $p<0.05$).
Combined infusion of TEA, indomethacin and L-NMMA inhibited ATP- as well as SNP induced forearm vasodilation to a similar extent (figure 2). This inhibition is therefore considered as a non-specific effect due to the vasoconstrictive response to the infused antagonists.

Discussion

This study showed that all blockers, except glibenclamide, caused a significant increase in baseline vascular tone. ATP-induced vasodilation in the human forearm could not be inhibited by concomitant infusion of indomethacin, TEA, glibenclamide or ouabain alone, or by a combined infusion of TEA, indomethacin and L-NMMA.

The effect of antagonists on baseline vascular tone.

Human data on the influence of the used blockers on baseline vascular tone are very scarce. This is remarkable, because they have nevertheless become established and widely used compounds in pharmacological research. Data on animal experiments vary depending on species and vascular bed as will be indicated hereafter.
Indomethacin-induced increase of FVR suggests that continuous release of prostacyclin plays a role in the maintenance of resting forearm blood flow. Wilson and Kapoor [33] and Duffy *et al.* [34] previously detected that inhibition of cyclooxygenase with aspirin or indomethacin decreased resting forearm blood flow by 20-30%. Prostacyclin also contributes to metabolic vasodilation [35;36] as well as to resting and metabolic vasodilation in coronary arteries [37].
The effect of K_{Ca} channel inhibition on basal vascular tone differs depending on the experimental setting. Increase in baseline tone has been reported in cerebral arteries [38]. In guinea-pig resistance arteries no change was found [39;40]. In the current study, TEA increased the FVR of the forearm vascular bed 30 minutes after infusing ATP and this vasoconstrictor response significantly differed from the effect of TEA after pretreatment with SNP. Pickers *et al.* also found that TEA had no significant effect on baseline vascular tone after SNP infusion [41]. TEA also had no influence on baseline vascular tone after infusion of hydrochlorothiazide [42] and C-type

natriuretic peptide [43]. FVR at recontrol, just before start of TEA-infusion, did not differ from baseline values, which makes it unlikely that the observed vasocontrictor action of TEA is due to vanishing ATP-induced vasodilation by a carry over effect, but can not be excluded. Although the difference in TEA-response between SNP and ATP pretreated groups was small and should not be overemphasized, this observation may indicate a pharmacodynamic carry over effect of the previous ATP infusions on the maintenance of vascular tone, possibly by inducing the release of an alternative EDRF that could affect K_{Ca} channels at baseline.
Our finding that glibenclamide had no influence on basal vascular tone in the human forearm vascular bed is consistent with previous findings [44-46]. Glibenclamide had no influence on basal vascular tone of carotid, femoral and mesenteric endothelium-denuded strips from rats [47]. However, infusion of glibenclamide into the coronary vasculature of anesthetized dogs and in isolated rabbit hearts resultated in significant increase in coronary resistance.
Ouabain infusion alone induced vasoconstriction, which has been reported before [48-50]. Baseline activity of Na^+K^+-ATPase apparently contributes to resting vascular tone, probably by maintaining membrane polarity.

Why was ATP-induced vasodilation not inhibited by any of the antagonists we used ?

First, in vivo, ATP might induce its vasodilation via an endothelium independent, instead of the proposed endothelium dependent, mechanism. Few *in vitro* studies have already shown that ATP exerts vasodilation partially via endothelium independent mechanisms [51-54]. Vascular smooth muscle cells express P2y receptors which may mediate vasodilation [55].
Second, the infused concentrations of the antagonists might have been insufficient to inhibit the actions of EDRFs that are released in response to ATP. This is unlikely for any of the blockers used, however. Previously in our laboratory, Pickkers and de Hoon showed that indomethacin at a concentration of 5 $\mu g \cdot dl^{-1}$ FAV$\cdot min^{-1}$ was able to inhibit cyclooxygenase: in a set of experiments they confirmed adequate cyclooxygenase inhibition by the absence of thromboxane-B2 formation in blood drawn from an antecubital vein of the indomethacin infused forearm, determined by RIA (unpublished results). The observed vasoconstrictive effect of indomethacin and its clinical use as treatment for patent ductus arteriosus in preterm infants further support blockade of vascular cyclo-oxygenase. Likewise, TEA 0.1 $mg \cdot dl^{-1}$ FAV$\cdot min^{-1}$ has been shown to inhibit vasodilation in the human forearm to the endothelium dependent vasodilator bradykinin [56], which acts via EDHFs. TEA was also able to inhibit the C-type natriuretic peptide (CNP)-induced vasodilation (43) and the vasodilation induced by acetazolamide [41] in the human forearm; both openers of K_{Ca} channels. The infused concentration of 2.0 µg glibenclamide $\cdot dl^{-1}$ FAV$\cdot min^{-1}$ was based on a study previously performed in our laboratory that showed that a concentration seven times lower was capable of effectively blocking K_{ATP} channels [45]. The significant rise in insulin and C-peptide during the course of the studies with glibenclamide indicates systemic spill of glibenclamide with subsequent stimulation of insulin secretion. However, the course in FVR in the control arm was not significantly affected by the glibenclamide infusion, indicating that the systemic changes of humoral parameters did not interfere with forearm vascular tone. Ouabain was infused at a concentration of 0.2 $\mu g \cdot dl^{-1}$ FAV$\cdot min^{-1}$, based on studies from our research group demonstrating that this concentration effectively blocked Na^+K^+

ATPase [50;57]. We have previously confirmed that L-NMMA 0.1 mg·dl^{-1} FAV·min^{-1} significantly inhibits acetylcholine induced vasodilation [18]. As we used a dose of L-NMMA twice as high it is unlikely that NO-synthase was insufficiently inhibited. The studies regarding these references were all done under the same conditions as the currently reported experiments. We conclude that the lack of effect of the used antagonists on ATP-induced vasodilation was not due to insufficient doses.
Third, we may not have blocked the action of all EDHFs. Several EDHFs have been suggested, like metabolites of arachidonic acid produced through the cytochrome P450 (CYP 450) monooxygenase pathway and reactive oxygen species (ROS) [58;59]. Their mechanism of action has in all cases been directly or indirectly linked to potassium channels and Na^+K^+ATPase. In the current study, we only blocked the K_{Ca} channels by TEA. TEA antagonizes various types of potassium channels with different degrees of potency, but the compound has been shown to block K_{Ca} channels selectively at concentrations below 1 mM [60]. TEA at an infusion rate of 0.1 mg·dl^{-1} FAV·min^{-1} results in a local plasma concentration of approximately 0.5 mmol·L^{-1}. ATP sensitive potassium channels (K_{ATP} channels) and voltage-dependent potassium (K_V) channels can be blocked by TEA at a concentration of respectively 7 and 10 mM [60]. Thus the concentration we used was not sufficient to block these channels. EDHFs might, however, also exert their effects via K_{ATP}, K_v and K_{IR} (inwardly rectifying potassium) channels, although their role in EDHF-induced vasodilation is uncertain. Furthermore, glibenclamide did not inhibit ATP-induced vasodilation. Fourth, a redundancy of vasodilator mechanisms of ATP could potentially have prevented inhibition of ATP- induced vasodilation by interruption of a single vasodilator pathway. However, use of a combination of substances that affect NO, COX, and EDHF still did not reveal a reduction in ATP-induced vasodilation. It is of interest for further experiments to combine infusion of bariumchloride (blocker of K_{IR}-channels) and ouabain, which might have a greater inhibitory effect on ATP-induced vasodilation than ouabain alone. Finally, there might be a different mechanism of vasodilation/EDRF besides NO, prostacyclin and EDHFs that contributes to the ATP-induced vasodilation *in-vivo*. For instance, evidence is accumulating that carbon monoxide (CO) can be an important vascular paracrine factor [61].
In conclusion, the present findings do not support a role for NO, prostacyclin or EDHFs that act by opening K_{ATP} channels, K_{Ca} channels, or by activation of Na/K-ATPase in ATP-induced vasodilation. In humans, the role of endothelium-independent mechanisms and involvement of unblocked EDRFs remains to be explored.

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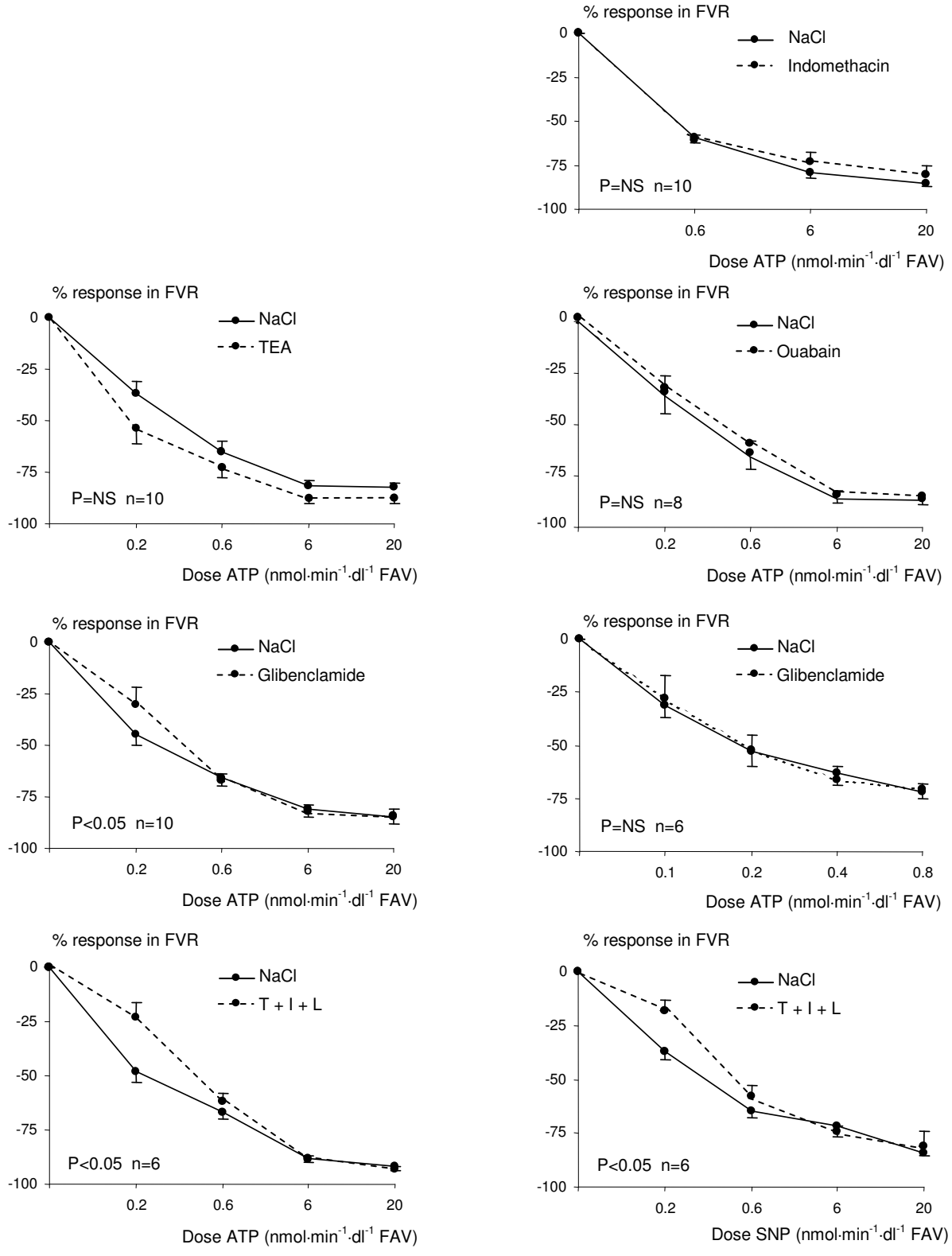

Figure 2. Relative response in FVR (infused arm) during infusion of ATP and (last graph) of SNP with and without antagonists as indicated. T = TEA, I = Indomethacin, L = L-NMMA. For doses: see text. P-values indicate ANOVA for repeated measures for the effect of the antagonist(s) on the vasodilator response curve.

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CHAPTER 7

Preserved vasodilator response to adenosine in insulin-dependent diabetes mellitus

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Abstract

Experimental data derived from animal models suggest that the endogenous nucleoside adenosine has important cardioprotective properties. The potent vasodilator effects of adenosine may contribute to this cardioprotection as ischemia-induced release of endogenous adenosine has been suggested to adjust local blood flow to the metabolic demands of the tissue. Interestingly, the vascular effects of adenosine appeared to be impaired in animal models for diabetes mellitus. This observation may be of importance with respect to the increased cardiovascular mortality in diabetes. Therefore, we investigated the in vivo vasodilator effects of adenosine in insulin-dependent diabetic patients.
In twelve uncomplicated insulin-dependent male diabetic patients and twelve healthy male age-matched subjects, the brachial artery was cannulated for infusion of adenosine (0.15, 0.5, 1.5, 5, 15 and 50 μg·100 ml^{-1}·min^{-1}) and for measurement of mean arterial pressure (MAP). Forearm blood flow (FBF) was measured by venous occlusion mercury-in-silastic strain gauge plethysmography. Maximal vasodilation was assessed by standardized post occlusive reactive hyperaemia (PORH). Baseline forearm blood flow was 2.7 ± 0.4 and 1.8 ± 0.2 ml·100 ml^{-1}·min^{-1} for the diabetic patients and control group respectively. In the diabetic patients, adenosine infusion raised forearm blood flow to 2.4 ± 0.4, 2.6 ± 0.4, 4.4 ± 0.7, 6.3 ± 1.0, 9.8 ± 1.5 and 14.2 ± 2.1 ml·100 ml^{-1}·min^{-1} for the respective dosages. In the control group these values were 1.7 ± 0.2, 1.9 ± 0.3, 3.2 ± 0.8, 6.0 ± 1.2, 10.9 ± 2.1 and 17.1 ± 3.4 ml·100 ml^{-1}·min^{-1} respectively ($p>0.1$ for between group comparison). Forearm blood flow at the contralateral side was not significantly affected by the placebo and adenosine infusions. Comparable results were obtained when results were expressed as changes in forearm vascular resistance or forearm blood flow ratio (FBF infused arm/FBF control arm). Maximal vasodilation did not differ between the two groups. We conclude that the forearm vasodilator response to adenosine is preserved in uncomplicated insulin-dependent diabetic patients. This observation argues against a primary role of a reduced adenosine responsiveness in the cardiovascular sequelae of diabetes.

Introduction

Adenosine has potentially important cardioprotective properties like inhibition of neutrophil activation with subsequent reduced free radical formation, inhibition of thrombocyte aggregation, vasodilation, presynaptic inhibition of norepinephrine release, and opening of potassium channels [1]. These effects are mediated by adenosine receptors, located on the outer cell membrane. In animals, myocardial infarct size is reduced when adenosine is infused either before ischaemia or during the reperfusion period [2-4]. In addition, adenosine reduces the incidence of ischaemia-induced arrhythmias [5]. Infusion of a selective adenosine receptor antagonist increases infarct size, indicating a role for endogenous adenosine as a cardioprotective autacoid [6]. Since the vasodilator action of adenosine is thought to play a role in the local adjustment of oxygen demand to oxygen supply [7;8], this may contribute to the cardioprotective properties of adenosine.
Interestingly, an impaired responsiveness to the vasodilator effect of adenosine has been observed in animal models for diabetes mellitus [9;10]. Several mechanisms may be responsible for this reduced responsiveness to adenosine. Animal and human data have indicated both fascilitating as well as inhibiting interactions between adenosine and the sympathetic nervous system [11-16]. Furthermore, human and animal studies show that adenosine-induced vasodilation is at least partially mediated by the endothelium [17-19]. Since insulin alters sympathetic nervous system activity [20;21] and diabetes mellitus has been associated with reduced endothelium-dependent vasodilation [22-25], both neural and endothelial mechanisms may be involved in the reduced reactivity to adenosine in these descriptive animal studies. Additionally, direct actions of adenosine on cardiac and vascular muscle cells may be reduced in patients with diabetes mellitus.
Although vascular reactivity in human diabetes has been studied extensively over the past few years [22-25], no human data are available on the responsiveness to the endogenous nucleoside adenosine. Since diabetes is an independent risk factor for developing cardiovascular disease [26], and is often associated with concomitant hypercholesterolaemia and hypertension which further attributes to an increased risk of ischaemic heart disease [27;28], an impairment in adenosine responsiveness may be of clinical interest. Pharmacological compounds are currently being developed to potentiate the action of endogenous adenosine at sites of ischemia [13]. In this context it is valuable to know if diabetic patients exhibit decreased responses to adenosine. Furthermore, a reduced vascular responsiveness to adenosine may also be of importance in the metabolic control of patients with diabetes mellitus since adenosine enhances glucose uptake in some animal models [29;30]. This metabolic effect of adenosine may in part be due to its effect on blood flow [31]. Therefore, we evaluated the vasodilator response to adenosine in patients with uncomplicated insulin dependent diabetes mellitus and compared these observations with a carefully matched control group.

Patients and methods

Patients

After approval of the local ethics committee, twelve normotensive nonsmoking caucasian male patients with insulin-dependent diabetes mellitus were selected from our outpatient population. Diabetes mellitus was diagnosed at least 5 years before participation to this study. Patients with evidence of macro- or microvascular disease were excluded from the study because these vascular complications would result in a nonspecific impairment of the reactivity to any vasodilator substance. Macrovascular disease was assessed by taking their history (no coronary artery disease, heart failure, cerebrovascular disease, peripheral vascular disease or foot ulcers), physical examination and a twelve lead electrocardiogram. Microvascular disease was excluded by demonstrating the absence of orthostatic hypotension and peripheral loss of sensibility, by a normal fundoscopy, and by an albumin excretion ratio less than 20 $\mu g \cdot min^{-1}$. None of the patients used medication other than subcutaneous insulin injection. Only patients with a glycosylated haemoglobin concentration (HbA1c) between 7 and 10 % as measured during insulin treatment were included.

Table 1. Demographic characteristics of the study groups (mean±SD)

	Diabetic subjects	Healthy subjects
N	12	12
Male/female	12/0	12/0
Age (years)	34.6 ± 5.9	34.8 ± 6.0
Body Mass Index ($kg \cdot m^{-2}$)	23.6 ± 2.4	23.3 ± 2.4
Systolic blood pressure (mmHg)*	123 ± 10.4	118 ± 8.2
Diastolic blood pressure (mmHg)*	63.2 ± 8.6	62.4 ± 4.9
Mean arterial pressure (mmHg)*	85.2 ± 9.8	82.4 ± 6.8
Heart rate (beats per minute)#	67.3 ± 16.8	57.4 ± 9.6
HbA1c (%)	8.5 ± 0.9	
Time after diagnosis (years)	16.2 ± 8.3	
Glucose ($mmol \cdot L^{-1}$)@	13.0 ± 5.1	

*: Intra-arterially measured during placebo infusion; #: Measured by electrocardiographic recordings during placebo infusion; @: Determined during the experiment; for each subject the 6 determinations were averaged to one value.

The control group consisted of twelve male non-smoking healthy caucasian volunteers.These subjects were carefully matched for age, blood pressure and body weight.They had no history of diabetes mellitus and did not use concomitant medication. Physical examination and twelve lead electrocardiography did not reveal any abnormalities. Demographic data of the study groups are shown in table 1.

Methods

Before the start of the study, the subjects were asked to abstain from caffeine-containing products for at least 24 hours, because caffeine is a potent adenosine receptor antagonist [32]. In all participating subjects, the plasma caffeine concentration was below the limit of detection as measured in a sample that was collected immediately before starting the experiments (reversed phase HPLC; minimal level of detection: 0.2 $\mu g \cdot ml^{-1}$ [33]. All tests were performed in a temperature controlled laboratory (22-23 ºC), with participants in the supine position, after an overnight fast, starting at 8.00 am.
From a methodological point of view, the level of plasma insulin and glucose concentrations throughout the study is a very important issue. Recent studies have convincingly shown that baseline skeletal muscle flow in humans is not affected by hyperglycemia [34], whereas hyperinsulinemia induces an obvious increase in baseline skeletal muscle flow as well as in forearm vascular reactivity [35-38]. To avoid confounding of our results by insulin-mediated vasodilation, we instructed the diabetic subjects to skip their morning dose of insulin and not to use a breakfast. This was done in order to achieve low and steady state plasma insulin levels during the time of experiments. Although even lower insulin levels would have been reached by also skipping the long-acting insulin injection of the evening before the experiment, this would have introduced the risk of the development of ketosis or keto-acidosis, a factor which would certainly have affected the results. Since no insulin was administered in the morning hours, we had to accept the varying fasting glucose levels throughout the experiments, since correcting the glucose levels would inevitably have increased insulin levels. As stated above, recent data have convincingly shown that plasma glucose levels up to 15 $mmol \cdot l^{-1}$ do neither affect baseline forearm blood flow nor vascular reactivity of the forearm vascular bed [34].
After local anaesthesia (Xylocaïne, 2%), the left brachial artery was cannulated with a 20-gauge catheter (Angiocath, Deseret Medical, Inc., Becton Dickinson and Co, Sandy, UT, U.S.A.) for both intra-arterial adenosine infusion (automatic syringe infusion pump, type STC-521, Terumo Corporation, Tokyo, Japan) and blood pressure recording (Hewlett Packard GmbH, Böblingen, Germany). Forearm blood flow was registered simultaneously on both forearms by electrocardiography-triggered venous occlusion plethysmography using mercury-in-silastic strain gauges (Hokanson EC4, D.E. Hokanson, Inc., Washington, U.S.A). The upper arm collecting cuff was inflated using a rapid cuff inflator (Hokanson E-20, D.E. Hokanson, Inc., Washington, U.S.A.). At least one minute before the FBF measurements, the circulation of the left hand was occluded by inflation of a wrist cuff to 200 mmHg. Forearm blood flow was recorded three times per minute during the four minute placebo infusion and during the last two minutes of each adenosine infusion.
The experiment started with the measurement of baseline forearm blood flow during placebo infusion (NaCl, 0.9%). Apart from the course in the forearm blood flow, figure 1 shows the schedule of the several drug infusions. The effect of six increasing

dosages of adenosine (Sigma Chemical Co., St Louis, MO, U.S.A.; 0.15, 0.5, 1.5, 5, 15 and 50 µg·100 ml^{-1} forearm·min^{-1}) were compared with placebo (NaCl 0.9%). Prolonged occlusion of the hand circulation can cause discomfort with subsequent effects on blood pressure and heart rate. Therefore, a 5-minute rest period with desufflation of the wrist cuffs was allowed between the placebo infusion and the first adenosine dose and between the third and fourth adenosine dose.

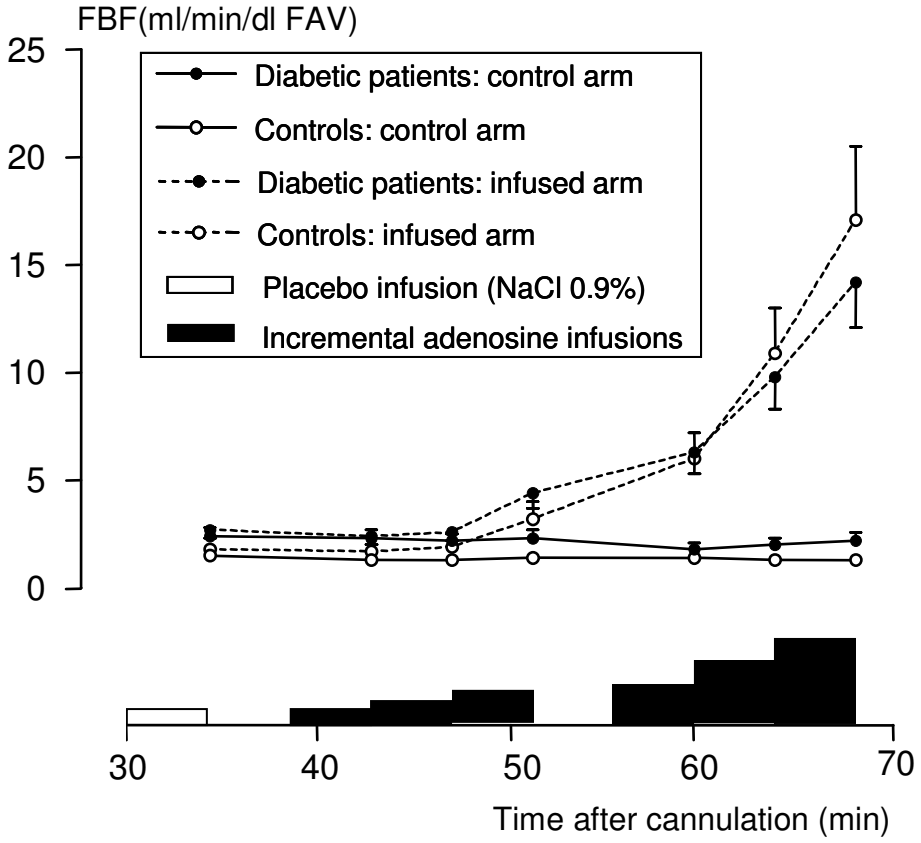

Figure 1. The course in forearm blood flow (FBF) before and during the intra-arterial infusion of adenosine is presented for the diabetic patients and the control group. There was no statistically significant difference between the groups (ANOVA for repeated measurements: P=1.0).

During all procedures, total volume infusion was adjusted to forearm volume as measured by water displacement and kept at a constant rate of 100 µl·100 ml^{-1} forearm·min^{-1}. Placebo and each adenosine dosage were infused during 4 minutes. To exclude structural vascular changes in the diabetic patients, maximal vasodilation was measured during post occlusive reactive hyperaemia (PORH) according to the well-established method of Pedrinelli et al [39;40] twenty minutes after the end of the final adenosine infusion. A cuff applied to the left upper arm was inflated to 300 mm Hg for 13 minutes. During the last minute of ischaemia the subjects were asked to perform repeated hand contractions. Immediately after desufflation of the upperarm cuff, FBF measurements were started for at least 2 minutes with occluded hand circulation.

The lowest forearm vascular resistance (MAP/FBF) was considered to represent maximal vasodilation.
In the diabetic group, blood glucose concentrations were determined 6 times: immediately after arterial cannulation, after infusion of placebo, after the third and after the sixth adenosine dose, just before the test of maximal vasodilation and just before decannulation (Accutrend, type 1284851, Boehringer, Mannheim, Germany). Prior to the intra-arterial adenosine infusions, 10 ml arterial blood was collected with Li-heparin as anti-coagulant in 9 diabetic patients and in 4 control subjects for the determination of plasma insulin and detection of insulin antibodies. Plasma insulin was measured by radioimmunoassay using a specific antiserum raised in a guinea pig against human insulin. A second antibody was used to separate the antibody-bound and free fractions. Insulin antibodies were detected by incubation of the samples with 125I-insulin and subsequent precipitation with polyethyleneglycol [41].

Statistics

Mean arterial pressure (MAP) was measured continuously during each recording of forearm blood flow (FBF) and averaged per FBF registration. Forearm vascular resistance (FVR) was calculated from simultaneously measured MAP and FBF (MAP/FBF) and expressed as arbitrary units (AU). Additionally, the ratio of each simultaneously measured FBF (FBF infused arm/FBF control arm) was calculated. Forearm blood flows, the calculated flow ratios and FVR's obtained during each four minutes of placebo infusion or during the last two minutes of each drug infusion were averaged to one value. Adenosine-induced effects were expressed both as absolute and percentage change from preceding placebo infusion. Differences in responses to adenosine between the two study groups were analysed with an ANOVA for repeated measurements with the adenosine dosage as within-subject factor and the presence of diabetes mellitus as between-subject factor. Differences in baseline values were assessed with the unpaired Student t-test. Correlations were performed using the Pearson correlation coefficient. Since plasma insulin concentrations did not show a Gaussian distribution, group differences in insulin levels were analyzed by the Mann-Whitney U test. All results are expressed as mean±SE unless indicated otherwise; $P<0.05$ (two sided) was considered to indicate statistical significance.

Results

Baseline FBFs in the infused arm were 2.7 ± 0.4 and 1.8 ± 0.2 ml·100 ml^{-1} forearm·min^{-1} for the diabetic patients and control group respectively (P=0.07). In the control arm these values were 2.4 ± 0.4 and 1.5 ± 0.1 ml·100 ml^{-1} forearm·min^{-1} (P=0.05). During the adenosine infusions, FBF in the infused arm of the diabetic patients amounted 2.4 ± 0.4, 2.6 ± 0.4, 4.4 ± 0.7, 6.3 ± 1.0, 9.8 ± 1.5 and 14.2 ± 2.1 ml·100 ml^{-1} forearm·min^{-1} for the respective adenosine dosages of 0.15, 0.5, 1.5, 5, 15 and 50 µg adenosine·100 ml^{-1} forearm·min^{-1}. In the control group these values were 1.7 ± 0.2, 1.9 ± 0.3, 3.2 ± 0.8, 6.0 ± 1.2, 10.9 ± 2.1 and 17.1 ± 3.4 ml·100 ml^{-1} forearm·min^{-1}, respectively (see figure 1). In both groups, FBF in the control arm was not significantly affected during the placebo and adenosine infusions. Overall, repeated measures ANOVA did not reveal a significant difference between the two

groups (between subject effect: P=1.0). Comparable results were obtained when results were expressed as absolute or relative changes in FBF from baseline. Baseline FVRs in the infused arm were 39.6 ± 5.2 and 57.5 ± 7.6 AU for the diabetic patients and controls, respectively (P=0.07). In the control arm these values were 45.7 ± 6.3 and 63.7 ± 5.9 AU, respectively (P<0.05). During adenosine infusion, FVRs in the infused arm of the diabetic patients were 44.6 ± 6.6, 41.9 ± 5.4, 29.9 ± 7.1, 20.5 ± 4.6, 16.6 ± 5.7 and 12.2 ± 5.7 AU for the six increasing adenosine dosages, respectively. In the controls, these values were 57.5 ± 7.6, 58.5 ± 7.2, 56.1 ± 7.7, 45.8 ± 9.0, 24.6 ± 5.5, 16.0 ± 4.6 and 13.2 ± 5.2 AU, respectively. Overall, the course of FVR in the infused arm did not significantly differ between the two groups (between-subject effect: P=0.2). In both groups, FVR in the control arm was not affected. Comparable results were obtained when adenosine-induced changes in FVR were expressed as absolute or relative changes from baseline (see figure 2).

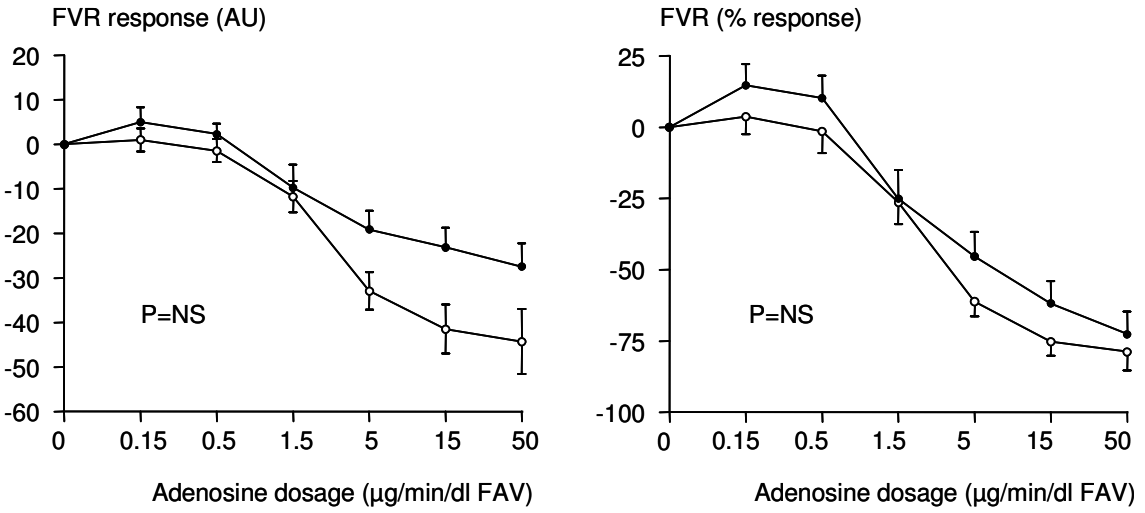

Figure 2. Adenosine-induced forearm vasodilation in controls (open symbols) and diabetic patients (closed symbols) expressed as absolute and percentage change in forearm vascular resistance (FVR) from baseline. P values indicate the level of significance for between group effects (ANOVA for repeated measurements).

Because we observed slight differences in baseline FBF- and FVR-values between the diabetic patients and the control group, we also analyzed the results of the FBF-ratio (FBFinfused arm divided by FBFcontralateral arm). Assessment of the percent changes of this ratio has been shown to be an appropriate method to analyze dose response curves [22;42]. During placebo infusion, the FBF-ratio was equal in both groups and numbered 1.2 ± 0.1.
In the diabetic patients, the FBF-ratios increased to 1.0 ± 0.1, 1.2 ± 0.1, 2.1 ± 0.4, 4.4 ± 1.0, 5.9 ± 1.2 and 9.2 ± 2.5 during the six increasing adenosine dosages, respectively. In the controls, these values were 1.3 ± 0.1, 1.5 ± 0.2, 2.2 ± 0.4, 4.2 ± 0.8, 8.9 ± 1.5 and 14.7 ± 2.1, respectively. Overall, the course of the ratio did not significantly differ between the two groups (between-subject effect: P=0.2). Comparable results were obtained when adenosine-induced changes in the ratio were expressed as absolute or relative changes from baseline.

The minimal FVR that occurred during post-occlusive reactive hyperaemia did not differ between diabetic patients and controls: 3.2 ± 0.2 versus 3.4 ± 0.3 AU (P=0.6). The individual courses of blood glucose concentration are shown in figure 3. Within each individual, glucose levels remained reasonably stable. However, between the patients a high variation in averaged glucose level existed ranging from 3.4 to 20.6 $mmol \cdot L^{-1}$. There was no correlation between the individual plasma glucose concentration and the vascular responsiveness to adenosine in the diabetic patients (r=-0.2, P=0.5). Plasma insulin concentration was 21.8 ± 2.5 $mE \cdot L^{-1}$ (N=9) in the diabetic patients versus 6.5 ± 1.3 $mE \cdot L^{-1}$ (N=4) in the controls (P<0.01). In four diabetic patients, insulin antibodies could be detected. After exclusion of these patients, plasma insulin concentration was still significantly higher in the diabetic patients as compared with the control subjects (20.8 ± 3.8 versus 6.5 ± 0.9 $mE \cdot L^{-1}$; P<0.05).

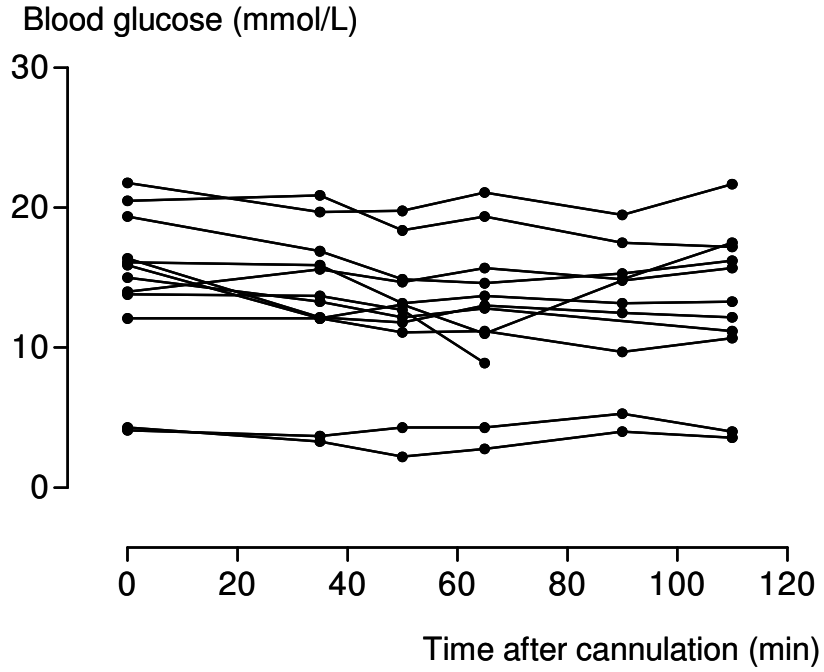

Figure 3. Individual courses of blood glucose concentrations, demonstrating the large inter-individual variability as well as the small intra-individual variability during the experiments.

Discussion

This study was performed to investigate whether the forearm vasodilator response to adenosine is affected in patients with insulin-dependent diabetes mellitus. Normotensive non-smoking diabetic patients without evidence of macro- or microvascular complications were selected to prevent possible confounding by structural arteriolar or microvascular changes. Maximal forearm vasodilation in the diabetic patients appeared to be equal to that of the age-matched control group confirming the absence of structural abnormalities in the forearm vascular bed of the diabetic subjects. In these carefully selected patients, we observed a preserved

vasodilator response to adenosine in the forearm vascular bed. This observation argues against a primary role of reduced adenosine responsiveness in the cardiovascular sequellae of diabetes.

The current results are in contrast with several observations in animal models for diabetes [9;10] that studied the responsiveness of the heart and coronary vasculature to adenosine. We can not exclude the possibility that diabetes mellitus differentially affects the coronary and forearm vascular bed in humans. However, other possible explanations should be discussed as well.

It has to be emphasized that animal models for diabetes represent a true insulinopenic state. In contrast, as a result of subcutaneous administration of insulin as opposed to the physiological release of insulin into the portal vein, the levels of insulin are elevated in treated patients with insulin-dependent diabetes mellitus. As such, treated patients with insulin-dependent diabetes do not represent an insulinopenic state, not even in the fasting state when plasma insulin concentrations reach their trough level. This may well explain the preserved adenosine responsiveness in our patients, because treatment of diabetic animals with insulin also restored the impaired responses to adenosine [9]. Since we did not study the effects of adenosine in a true insulinopenic state, our data do not exclude an interaction between insulin and adenosine. Nonetheless, our present results do allow the conclusion that the vascular responsiveness to adenosine is preserved in patients with diabetes mellitus who are regularly treated with insulin.

In the diabetic patients, baseline forearm blood flow and forearm vascular resistance were slightly different from that of the control subjects. This interesting phenomenon has been described before [22] and is not only confined to the forearm but has also been shown for the retinal, renal and cutaneous circulation [43-45]. It already exists in the early course of the disease before diabetic complications have developed [22;45]. Although the exact mechanism of this 'hyperdynamic circulation' in diabetic patients is not known, it may be related to the development of complications like diabetic nephropathy and diabetic microangiopathy [45]. Especially because of this difference in baseline forearm blood flow, we also included the results on the FBF-ratio (see method section). For the FBF-ratio, the diabetic patients and the control group had exactly the same baseline values. Using this parameter, statistical analysis of the adenosine responses revealed no difference between the two groups.

The main observation of the present study is that adenosine-mediated forearm vasodilation is not significantly affected in insulin-dependent diabetic patients. The response to the two highest adenosine dosages tended to be slightly reduced in the diabetic patients, but this difference did not reach statistical significance. In contrast to the two highest dosages, the responses to the lower dosages were very similar between the two groups. We regard these lower dosages more representative for the local physiological increases in adenosine concentration which are probably needed for the small adjustments of local flow in order to constantly balance tissue oxygen demand and supply.

The effect of intra-arterially supplied adenosine is determined by the adenosine concentration, adenosine receptor density and receptor function. Adenosine concentrations depend on the rate of cellular adenosine uptake. In theory, diabetes mellitus may impair this cellular uptake of adenosine [46]. Therefore, a reduced adenosine receptor density or function could have been masked by differences in adenosine kinetics between the two groups. However, the clinical significance of a possible receptor dysfunction is limited when the overall vasodilator effect of adenosine is not reduced in vivo as shown in the present study.

In conclusion, the vasodilator response to adenosine is preserved in patients with insulin-dependent diabetes mellitus who are regularly treated with insulin. This observation argues against a primary role of impaired adenosine responsiveness in the cardiovascular sequelae of diabetes.

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CHAPTER 8

Summary

The studies described in this thesis were meant to study adenosine kinetics in humans in-vivo, and dealt with the following questions:

1. What is the effect of intra-arterially infused dipyridamole and adenosine on interstitial and intravascular adenosine concentrations (chapter 2).

From the observations described in chapter 2, we conclude that intra-arterially infused adenosine does not enter the interstitium of skeletal muscle in humans in vivo. This observation is in agreement with in-vitro studies that have shown that the endothelium serves as a metabolic barrier for adenosine to reach the interstitial space. This finding supports the concept that the local actions of intra-arterially administered adenosine, such as vasodilation, presynaptic inhibition of norepinephrine release from forearm sympathetic nerve endings and stimulation of sympathetic afferents which elicit generalized sympatho-excitation, are mediated by adenosine-induced release of endothelial factors.
Dipyridamole reduced clearance of both intravascular and interstitial adenosine, providing direct biochemical evidence that dipyridamole inhibits cellular uptake of adenosine from these compartments. To our surprise however, interstitial access of intra-arterial adenosine was not increased by simultaneous inhibition of nucleoside transporters with dipyridamole. As yet, we do not have a clear explanation for this observation. Possibly, dipyridamole insensitive nucleoside transporters on endothelial cells are responsible for the endothelial metabolic barrier for adenosine. Another observation that is difficult to explain is why dipyridamole did not increase baseline interstitial and/or intravascular adenosine levels, despite our finding that dipyridamole-induced vasodilation is inhibited by adenosine receptor blockade (see below). Most likely, baseline adenosine formation occurs within the vascular wall (i.e. endothelial cells) at a site that is not accessible for interstitial or intravascular microdialysis catheters. Alternatively, a small rise in interstitial adenosine during infusion of dipyridamole remains below the limit of detection with the used microdialysis technique but is sufficient to induce vasodilation. Our finding that the endothelium in the human forearm constitutes a barrier impeding the delivery of intravascular adenosine into the underlying interstitium was described previously in a recent publication from Gamboa et al. [1]. In contrast with our results, intravenous dipyridamole-infusion enhanced the delivery of adenosine into the interstitium during intra-arterial infusion of adenosine. Differences in experimental set up may explain these contrasting results. Gamboa et al administered dipyridamole intravenously at a dose that is likely to result in a generalized sympatho-excitation, which may have resulted in increased perivascular release of the co-transmitter ATP and subsequent increased formation of interstital adenosine. Alternatively, differences in the technique to quantify dialysate adenosine could in theory have resulted in the contrasting results.

2. What is the role of adenosine in the pressor response to exercise in healthy volunteers (chapter 2).

Infusion of dipyridamole into the brachial artery did not augment the pressor response to hand grip exercise. This lack of potentiation of the exercise-pressor reflex was observed despite a proven inhibition of clearance of extracellular endogenous adenosine from the skeletal muscle interstitial compartment. Taken together, these findings argue against a role for endogenous adenosine in the blood pressure response to non-ischemic exercise in healthy volunteers. This study does not exclude a role for adenosine in the pressor response to ischemic exercise in

healthy volunteers or in the pressor response to exercise in patients with reduced perfusion of exercising muscle such as patients with heart failure.

3. Is there a difference in the mechanism of vasodilation between interstitial and intravascular adenosine (chapter 2 and 3).

After the effect of dipyridamole on adenosine kinetics was described biochemically in chapter 2, the vasodilator action of dipyridamole was further characterized and compared with the vasodilator action of adenosine in chapter 3. First, further evidence for the inhibitory action of dipyridamole on cellular uptake of extracellular adenosine was provided by showing a potentiating effect of dipyridamole on adenosine-induced vasodilation. We hypothesized that dipyridamole induces vasodilation by increasing the extracellular concentration of adenosine, resulting in adenosine receptor stimulation at sites of endogenous adenosine formation.
This concept was supported by an experiment in which the forearm vasodilator response to both adenosine and dipyridamole was antagonized by the adenosine receptor antagonist theophylline. We speculated that endogenous adenosine formation primarily occurs within the vascular wall and interstitial space, a compartment that is not well accessible for intravascular adenosine (see paragraph 1). Therefore, we further reasoned that infusion of dipyridamole into the brachial artery may stimulate adenosine receptors on vascular smooth muscle cells whereas infusion of adenosine stimulates these receptors on the endothelium. The post-adenosine-receptor pathways that are involved in vasodilation may differ between endothelium and vascular smooth muscle. In particular the role of ATP-sensitive potassium channels could differ between the two cell types. Therefore, we studied the effect of glibenclamide, a blocker of ATP-sensitive potassium channels, on the forearm vasodilator response to adenosine and dipyridamole infusion into the brachial artery of healthy volunteers. Our findings support this concept by demonstrating that glibenclamide inhibits the vasodilator response to dipyridamole but not to adenosine. This observation has potentially clinical relevance in patients who use glibenclamide. When these patients are evaluated for cardiac ischemia with Thallium scintigraphy, our results suggest that adenosine should be preferred over dipyridamole to prevent underestimation of ischemia. The forearm-studies described in this thesis do not enable to draw a conclusion on this issue. Studies on the effects of chronic glibenclamide treatment on adenosine- and dipyridamole induced coronary vasodilation in humans are scarse and conflicting. Farouque et al. [2] found a dose-dependent reduction of basal coronary flow and adenosine-induced hyperemia, whereas coronary flow reserve was unaffected. Reffelmann et al. [3] found no effect of glibenclamide on basal coronary flow, adenosine-stimulated hyperemia or flow reserve. In the work by Reffelmann, hyperinsulinemia was induced by the administration of intravenous glibenclamide wich may have blurred the results by inducing a vasodilatory action.
Finally, results from glibenclamide-studies can not be translated to newer sulfonylurea drugs with a lower affinity for to the vascular K_{ATP} channels without further evidence [4;5] .

4. What is the vasomotor action of AP_5A in humans, and what is the role of adenosine in AP_5A-induced changes in vascular tone (chapter 4).

In contrast to animal studies, our data clearly show that intra-arterial infusion of AP_5A does not evoke vasoconstriction. The observed vasodilator response to this diadenosine is explained by degradation to adenosine as evidenced by interaction

studies with dipyridamole and theophylline. Our study disproves the concept that the vasomotor action of thrombocyte-derived AP_5A plays a causal role in hypertension. Over the last years, research on diadenosine polyphosphates has not yielded clinically relevant results. Holla et al [6] described that thrombocyte concentrations of AP_5A and AP_6A are elevated in patients with essential hypertension. He hypothesizes that vasoconstriction caused by release of AP_5A and AP_6A from thrombocytes may contribute to the increase of vascular resistance in hypertensive patients. A causal relationship is lacking however. Recently, AP_4 was isolated from human ventricular cells [7]. AP_4 is a possible metabolite of AP_5A. AP_4 exerted vasodilation via endothelial P2Y1 receptors and vasoconstriction via P2X receptors on vascular smooth muscle cells. The role of AP_4 in the forearm vasodilator response to AP_5A is currently not known.

5. Do benzodiazepines inhibit cellular adenosine uptake in the human forearm (chapter 5).

In contrast to dipyridamole, clinically relevant concentrations of diazepam or midazolam did not augment the forearm vasodilator response to adenosine. This observation excludes an important interaction of these benzodiazepines with the nucleoside transporter. The apparent discrepancy with in-vitro data in the literature is most likely explained by the used concentrations of benzodiazepines which is higher in the reported in-vitro studies. Interestingly, we did not observe a vasodilator response to locally infused benzodiazepines, suggesting that a decrease in blood pressure that is often observed after intravenous infusion of benzodiazepines results from actions of these drugs in the central nervous system, unrelated to inhibition of nucleoside transport.

6. What is the mechanism of ATP-induced vasodilation in the human forearm (chapter 6).

The forearm vasodilator response to intra-arterial infusion of ATP appeared to be remarkably resistant to our pharmacological attempts to inhibit NO-synthase, cyclo-oxygenase, Na/K-ATPase, ATP sensitive potassium channels and Ca^{2+}-sensitive potassium channels. Therefore, the mechanism of vasodilation of this ubiquitous endogenous purine remains enigmatic in humans in vivo. We speculate that a currently unknown endothelium-derived relaxing factor is involved in this powerful vasodilator response to ATP. Further studies are needed to reveal this substance.

7. Is the vasodilator response to adenosine reduced in patients with uncomplicated insulin dependent diabetes mellitus (chapter 7).

Our study did not reveal a significant impairment of the vasodilator response to adenosine in patients with type 1 diabetes. Therefore, A_2-adenosine receptor responsiveness seems to be intact in these patients. The main drawback of our study is that the experiments were performed in a hyperinsulinemic state, but we can still conclude that the vascular responsiveness to adenosine is preserved in patients with diabetes mellitus who are regularly treated with insulin. Whether A_1-adenosine receptor-mediated protection against ischemia-reperfusion injury is reduced in these patients remains an unanswered question that needs to be addressed in further studies.

In summary, the experiments that are described in this thesis provide three independent lines of evidence that dipyridamole inhibits cellular uptake of extracellular adenosine in humans in vivo: (1) dipyridamole reduces the clearance of interstitial and intravascular adenosine as assessed by microdialysis, (2) dipyridamole-induced vasodilation is inhibited by the adenosine receptor antagonist theophylline and (3) dipyridamole augments the vasodilator response to exogenous adenosine.
Based on these results, dipyridamole was used as a pharmacological tool to study the role of adenosine in the exercise-pressor reflex and in the vasodilator response to AP_5A. Our observations indicate that adenosine does not play an important role in the blood pressure response to non-ischemic handgrip exercise in healthy volunteers. Dipyridamole augmented the vasodilator response to AP_5A, indicating important involvement of adenosine, a degradation product of AP_5A. The latter conclusion was further substantiated by an inhibitory action of theophylline on AP_5A-induced vasodilation and in-vitro data supporting rapid degradation of AP_5A to adenosine.
The observed difference between dipyridamole and adenosine-induced vasodilation with respect to their interaction with glibenclamide can be explained by differences in distribution between endogenous and exogenous adenosine and may have clinical importance in patients with type 2 diabetes who are treated with glibenclamide and are scheduled for a stress-Thallium scan of the heart.

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CHAPTER 9

Samenvatting

De experimenten die in dit proefschrift worden beschreven zijn opgezet om de kinetiek van adenosine in de humane in-vivo situatie te bestuderen, en zijn toegespitst op de volgende vragen:

1. Wat is het effect van intra-arteriëel geinfundeerd dipyridamol en adenosine op de interstitiële en intravasculaire adenosine-concentraties (Hoofdstuk 2).

Gebaseerd op de resultaten beschreven in hoofdstuk 2, concluderen we dat intra-arteriëel geïnfundeerd adenosine in de humane in-vivo situatie het interstitium van skeletspierweefsel niet bereikt. Deze bevinding komt overeen met in-vitro studies waaruit bleek dat het endotheel een metabole barrière vormt die adenosine belemmert om het interstitium te bereiken. Deze bevinding ondersteunt het concept dat de lokale effecten van intra-arteriëel toegediend adenosine, zoals vasodilatatie, presynaptische remming van noradrenaline-afgifte uit sympathische zenuweinden in de onderarm, en stimulatie van sympathische afferenten leidend tot gegeneraliseerde sympathicus-activatie, gemedieerd worden door adenosine-geinduceerde afgifte van endotheliale factoren.
Dipyridamol verminderde de klaring van zowel intravasculair als interstitiëel adenosine, hetgeen direct biochemisch bewijs vormt dat dipyridamol de cellulaire uptake van adenosine uit deze compartimenten remt. Verrassend was echter dat de interstitiële toegang van intra-arteriëel adenosine niet vergroot werd door gelijktijdige remming van nucleosidetransporters met dipyridamol. Vooralsnog hebben wij geen goede verklaring voor deze bevinding. Mogelijk zijn dipyridamol-ongevoelige nucleosidetransporters op endotheel-cellen verantwoordelijk voor de endotheliale metabole barrière voor adenosine.
Een andere bevinding die moeilijk verklaarbaar is, is waarom dipyridamol geen toename gaf van de basale interstitiële en/of intravasculaire adenosine-concentraties, ondanks de observatie dat dipyridamol-geïnduceerde vaatverwijding geremd wordt door adenosinereceptorblokkade (zie onder). Waarschijnlijk vindt basale adenosineproduktie plaats in de bloedvatwand (in het endotheel) op een plaats die niet toegankelijk is voor interstitiële of intravasculaire microdialyse catheters. Een alternatieve verklaring is dat een geringe toename van interstitiëel adenosine tijdens infusie van dipyridamol onder de detectie grens blijft van de toegepaste microdialyse-techniek, maar wel toereikend is om vasodilatatie te induceren. Onze bevinding dat het endotheel in de humane onderarm een barrière vormt voor de toegang van intravasculair adenosine tot het interstitium is reeds beschreven in een recente publicatie door Gamboa et al. [1]. In tegenstelling tot onze resultaten, induceerde intraveneuze dipyridamol-infusie een toename van de interstitiële adenosineconcentratie tijdens intra-arteriële infusie van adenosine. Deze contrasterende resultaten kunnen verklaard worden door een verschil in experimentele set-up. Gamboa et el. dienden dipyridamol intraveneus toe in een dosering waarvan aannemelijk is dat het geleid heeft tot gegeneraliseerde sympatho-excitatie, hetgeen geresulteerd kan hebben in toegenomen perivasculaire release van de co-transmitter ATP, en vervolgens toegenomen vorming van interstitiëel adenosine. Een alternatieve verklaring kan gezocht worden in verschil in techniek ter kwantificering van het dialysaat-adenosine, wat in theorie geleid kan hebben tot de contrasterende resultaten.

2. Wat is de rol van adenosine in de “pressor respons” op inspanning bij gezonde vrijwilligers (Hoofdstuk 2).

Infusie van dipyridamol in de arteria brachialis gaf géén toename van de bloeddrukstijging o.i.v. inspanning. Het onbreken van potentiëring van deze bloeddrukstijging werd waargenomen ondanks een bewezen remming van de klaring van extracellulair endogeen adenosine uit het interstitiële skeletspiercompartiment. Samen pleiten deze bevindingen tegen een rol voor endogeen adenosine in de bloeddrukrespons op niet-ischemische inspanning bij gezonde vrijwilligers. De huidige bevindingen sluiten een rol voor adenosine in de “pressor respons” op ischemische inspanning bij gezonde vrijwilligers of bij patiënten met verminderde perfusie van contraherende skeletspieren (zoals patiënten met hartfalen) echter niet uit.

3. Is er verschil in het vaatverwijdende mechanisme tussen interstitieel en intravasculair adenosine (Hoofdstuk 2 en 3).

Nadat het effect van dipyridamol op adenosinekinetiek biochemisch is beschreven in hoofdstuk 2, wordt het vaatverwijdende effect van dipyridamol nader onderzocht in hoofdstuk 3, en vergeleken met het vaatverwijdende effect van adenosine. Op de eerste plaats werd aanvullend bewijs verkregen voor het remmende effect van dipyridamol op de cellulaire uptake van extracellulair adenosine, doordat werd aangetoond dat dipyridamol het vaatverwijdende effect van adenosine versterkt. De veronderstelling was dat dipyridamol vaatverwijding induceert doordat het de extracellulaire adenosine-concentratie verhoogt, resulterend in stimulatie van de adenosinereceptor op plaatsen waar endogeen adenosine gevormd wordt. Deze veronderstelling werd ondersteund door een test waarbij de vaatverwijdende respons in de onderarm op zowel adenosine als dipyridamol geantagoneerd werd door de adenosinereceptorantagonist theophylline. Mogelijk vindt endogene adenosinevorming voornamelijk plaats binnenin de vaatwand en in de interstitiële ruimte, een compartiment dat niet goed toegankelijk is voor intravasculair adenosine (zie paragraaf 1). In dat geval zou infusie van dipyridamol in de arteria brachialis adenosinereceptoren op gladde spiercellen stimuleren terwijl infusie van adenosine deze receptoren op het endotheel stimuleert. De signaaltransductie voor vasodilatatie zou verschillend kunnen zijn voor endotheel en voor vasculair glad spierweefsel. In het bijzonder zou de rol van K_{ATP}-kanalen verschillend kunnen zijn voor de twee celtypen. Daarom werd het effect van glibenclamide bestudeerd (een blokker van K_{ATP}-kanalen) op de vaatverwijdende respons in de onderarm op adenosine en dipyridamol-infusie in de arteria brachialis van gezonde vrijwilligers. De resultaten steunen de veronderstelling omdat bleek dat glibenclamide wel de vaatverwijdende respons op dipyridamol maar niet die op adenosine remt. Deze bevinding is van potentieel klinisch belang voor patiënten die glibenclamide gebruiken. Wanneer deze patiënten geëvalueerd worden onder verdenking van cardiale ischemie door middel van Thallium scintigrafie, suggereren onze resultaten dat adenosine de voorkeur heeft boven dipyridamol om onderschatting van de ernst van de ischemie te voorkomen. Uit de studies uitgevoerd met het `onderarmsmodel`, beschreven in Hoofdstuk 3 van dit proefschrift, kan hierover echter geen conclusie getrokken worden. Onderzoek naar de effecten van chronische glibenclamidebehandeling op adenosine- en dipyridamol geinduceerde coronaire vasodilatatie bij mensen zijn spaarzaam en de resultaten zijn niet eensluidend. Farouque et al. [2] vonden een dosis-afhankelijke reductie van de basale coronaire flow en van adenosine-geinduceerde hyperemie, terwijl de coronaire flow reserve onveranderd bleef. Reffelmann et al. [3] vonden geen effect van glibenclamide op de basale coronaire flow, op adenosine-gestimuleerde hyperemie of flow reserve. In het

onderzoek door Reffelmann, werd hyperinsulinemie geinduceerd door de toediening van intraveneus glibenclamide hetgeen de resultaten vertekend kan hebben doordat hyperinsulinemie vaatverwijding induceert.
Tot slot kunnen resultaten van onderzoek uitgevoerd met glibenclamide niet zonder meer vertaald worden naar nieuwere sulfonylureum-derivaten met een lagere affiniteit voor de vasculaire K_{ATP}-kanalen [4;5] .

4. Wat is het vasoactieve effect van AP_5A bij de mens, en wat is de rol van adenosine bij AP_5A-geïnduceerde veranderingen in vaattonus (Hoofdstuk 4).

In tegenstelling tot dierexperimentele studies, laten onze data duidelijk zien dat intra-arteriële infusie van AP_5A geen vasoconstrictie veroorzaakt. De waargenomen vaatverwijdende respons op dit diadenosine-polyfosfaat wordt verklaard door degradatie tot adenosine, zoals blijkt uit interactie-onderzoek met dipyridamol en theofylline. Ons onderzoek pleit tegen het concept dat het vasomotore effect van AP_5A afkomstig uit thrombocyten, een causale rol speelt bij hypertensie.
Onderzoek op het gebied van de diadenosinepolyfosfaten heeft de afgelopen jaren geen klinisch relevante resultaten opgeleverd. Hollah et al. [6] beschreven dat thrombocyten-concentraties van AP_5A en AP_6A verhoogd zijn bij patiënten met essentiële hypertensie. Zijn hypothese is dat vasoconstrictie veroorzaakt door release van AP_5A en AP_6A uit thrombocyten kan bijdragen aan de toename in vaatweerstand bij patiënten met hypertensie. Een causale relatie is echter niet aangetoond. Recent werd AP_4 geisoleerd uit humane ventrikulaire cellen [7]. AP_4 is een mogelijke metaboliet van AP_5A. AP_4 induceerde vaatverwijding via endotheliale P2Y1 receptoren en vasoconstrictie via P2x receptoren op vasculaire gladde spiercellen. De rol van AP_4 in de AP_5A-gemedieerde vaatverwijdende respons in het skeletspiervaatbed van de onderarm is nog niet bekend.

5. Hebben benzodiazepine-derivaten een remmend effect op de cellulaire adenosine uptake in de onderarm in de humane in-vivo situatie (Hoofdstuk 5).

In tegenstelling tot dipyridamol, veroorzaakten diazepam en midazolam in klinisch relevante concentraties geen toename van adenosine-gemedieerde vaatverwijding in de onderarm. Deze waarneming pleit tegen een relevante interactie van deze benzodiazepinen met de nucleoside-transporter. De ogenschijnlijke discrepantie met in-vitro data in de literatuur wordt waarschijnlijk verklaard door de gebruikte benzodiazepineconcentraties, die hoger liggen in de in-vitro studies. We zagen geen vaatverwijdende respons op lokaal geïnfundeerde benzodiazepinen, hetgeen suggereert dat de bloeddrukdaling die vaak waargenomen wordt na intraveneuze infusie van benzodiazepinen veroorzaakt wordt via het centrale zenuwstelsel, en geen verband heeft met remming van de nucleosidetransporter.

6. Wat is het mechanisme van ATP-geinduceerde vaatverwijding in de onderarm bij gezonde vrijwilligers (Hoofdstuk 6).

De vaatverwijdende respons in de onderarm op intra-arteriële infusie van ATP bleek opvallend resistent tegen farmacologische pogingen tot remming van NO-synthase, cyclo-oxygenase, Na/K-ATPase, K_{ATP}-kanalen en K_{Ca2}^{+}-kanalen. Het vaatverwijdende mechanisme van dit endogene purine in het door ons gebruikte model blijft dus nog onopgehelderd. Mogelijk is een nu nog onbekende “endothelium-derived relaxing factor” betrokken bij de krachtige vaatverwijdende respons op ATP. Verder onderzoek is nodig om deze stof op te sporen.

7. Is de vaatverwijdende respons op adenosine verminderd bij patiënten met ongecompliceerde insuline-afhankelijke diabetes mellitus (Hoofdstuk 7).

Ons onderzoek liet geen significante afname zien van de vaatverwijdende respons op adenosine bij patiënten met type 1 diabetes. De A_2-adenosine receptor gevoeligheid lijkt daarom intact te zijn bij deze patiënten. Het grootste bezwaar van ons onderzoek is dat de experimenten werden uitgevoerd in een hyperinsulinemische toestand. In elk geval kan geconcludeerd worden dat de vasculaire gevoeligheid voor adenosine bewaard gebleven is bij patienten met diabetes mellitus die behandeld worden met insuline. Of A_1-adenosine receptor-gemedieerde bescherming tegen ischemie-reperfusie schade afgenomen is bij deze patiënten is door ons niet onderzocht.

Samengevat biedt het onderzoek dat beschreven staat in dit proefschrift drie onafhankelijke bewijzen dat dipyridamol de cellulaire uptake van extracellulair adenosine bij gezonde vrijwiligers in-vivo remt. (1) Dipyridamol vermindert de klaring van interstitiëel en intravasculair adenosine zoals gemeten met microdialyse. (2) Dipyridamol-geinduceerde vaatverwijding wordt geremd door de adenosinereceptorantagonist theophylline en (3) dipyridamol versterkt de vaatverwijdende respons op exogeen adenosine.
Gebaseerd op deze bevindingen, werd dipyridamol als farmacologisch middel gebruikt om de rol van adenosine te bestuderen in de “exercise-pressor reflex” en bij de vaatverwijdende respons op AP_5A. Onze resultaten wijzen erop dat adenosine geen belangrijke rol speelt in de bloeddrukrespons op niet-ischemische inspanning bij gezonde vrijwilligers. Dipyridamol versterkte de vaatverwijdende respons op AP_5A, wat wijst op substantiële betrokkenheid van adenosine, een degradatie produkt van AP_5A. Deze laatste conclusie is bovendien aannemelijk omdat theofylline AP_5A-geinduceerde vaatverwijding remt en omdat in-vitro data een snelle degradatie van AP_5A tot adenosine ondersteunen.
Het waargenomen verschil tussen dipyridamol en adenosine-geïnduceerde vaatverwijding met betrekking tot hun interactie met glibenclamide kan verklaard worden door verschil in distributie tussen endogeen en exogeen adenosine en zou klinisch van belang kunnen zijn bij patiënten met type 2 diabetes die behandeld worden met glibenclamide en een stress-Thallium scan van het hart moeten ondergaan.

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Curriculum Vitae

De schrijfster van dit proefschrift werd op 9 maart 1968 geboren in Roosendaal. Na het behalen van het Atheneum-diploma aan het Norbertus-college in Roosendaal, begon zij in 1987 aan de studie Geneeskunde aan de Katholieke Universiteit in Nijmegen. In 1994 behaalde ze het arts-examen, en startte met de opleiding tot internist, die ze volgde in het Bosch Medicentrum (Dr.J.Burghouts en Dr. P.Netten) en aan het UMC St Radboud (Prof. dr. J.van der Meer). In september 1998 begon ze aan het onderzoek dat geleid heeft tot dit proefschrift, afgewisseld met het vervolg van de opleiding. In 2001 werd zij geregistreerd als internist, in maart 2004 als endocrinoloog (opleider: Prof. dr. A.Hermus). Sinds september 2004 is zij werkzaam als internist-endocrinoloog in ziekenhuis het Spittaal te Zutphen.