Augmented hyperaemia and reduced tissue injury in response to ischaemia in subjects with the 34C > T variant of the AMPD1 gene

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Aims In patients with coronary artery disease, the 34C > T variant of the adenosine mono-phosphate deaminase gene (AMPD1), encoding a dysfunctional protein, predicts improved survival. We hypothesized that in subjects with this variant allele, ischaemia-induced intracellular adenosine formation is increased, augmenting reactive hyperaemia and ischaemia tolerance.

Methods and results We selected 10 healthy subjects with the CT genotype and 10 CC controls. The forearm vasodilator response to 2 and 5 min of ischaemia (venous occlusion plethysmography, expressed as percentage of maximum blood flow after 13 min of ischaemia) was higher in the CT group 56% (49–74%) and 77% (71–86%) vs. 49% (42–53%) and 60% (55–70%) in the CC group [median (interquartile range), P = 0.01]. Additionally, ischaemia–reperfusion injury was assessed in the thenar muscle using 99mTc-annexin A5 scintigraphy after forearm ischaemic exercise to detect externalized membrane phosphatidylserines. At reperfusion, 99mTc-annexin was administered intravenously. The change in annexin targeting between 1 and 4 h post-injection was −2.3% (interquartile range −2.4 to −1.6%) in the CT group vs. −0.3% (−0.6 to 1.3%) in controls (n = 7 in both groups, P = 0.03).

Conclusion The 34C > T variant of AMPD1 augments vasodilation and reduces tissue injury in response to forearm ischaemia. These mechanisms could contribute to the survival benefit of cardiovascular patients with this variant allele.

Introduction

In patients with coronary artery disease, the presence of the 34C > T polymorphism on at least one of the alleles encoding for the enzyme adenosine mono-phosphate deaminase (AMPD1) is associated with prolonged survival.1,2 AMPD1 is one of the enzymes involved in the intracellular metabolism of the purine nucleoside adenosine (Figure 1), catalysing the conversion of AMP to IMP.3 In humans, four AMPD isoforms have been described, named after the source from which they were initially purified: M (muscle), L (liver), E1, and E2 (erythrocytes), encoded by the different genes AMPD1, AMPD2, and AMPD3.4 Approximately 15–20% of Caucasian and African-American individuals are heterozygous or homozygous for the 34C > T variant of AMPD1, encoding a truncated protein with the loss of function.5

Under conditions of impending tissue danger, such as ischaemia, the extracellular adenosine concentration rapidly increases.6 Subsequent stimulation of adenosine receptors induces various effects such as vasodilation, ischaemia preconditioning, and inhibition of inflammation.7 On the basis of the observation that AMPD activity is significantly reduced in patients heterozygous for the 34C > T variant (CT genotype), it was suggested that in these patients during ischaemia, AMP is preferentially degraded into adenosine, which will increase ischaemic tolerance.1

We hypothesized that subjects with the 34C > T variant show augmented reactive hyperaemia and reduced ischaemia–reperfusion injury in response to ischaemia. To test this hypothesis, we selected healthy subjects with the CT genotype and controls homozygous for the common C allele (CC genotype), with otherwise similar parameters of adenosine transport and metabolism. The vasodilator response

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to 2, 5, and 13 min of forearm ischaemia was determined by venous occlusion plethysmography. During the last minute of the 13 min period of ischaemia, the subjects also performed rhythmic contractions of the hand, because this stimulus has previously been demonstrated to induce maximum vasodilation, which is dependent only on the structural characteristics of the vessel wall. We hypothesized that subjects with the 34C > T variant of AMPD1 have higher forearm blood flow (FFB) responses to 2 and 5 min of ischaemia, but not to 13 min of ischaemia. This experiment was repeated in the presence of dipyridamole, as a pharmacological tool to inhibit facilitated diffusion of adenosine through the equilibrative nucleoside transporter (ENT), to explore the role of increased intracellular adenosine formation. We hypothesized that dipyridamole potentiates reactive hyperaemia, as previous evidence suggests that during ischaemia, transport of adenosine over the cellular membrane is mainly from outside the cell inwards. This effect of dipyridamole, however, is expected to be less pronounced in subjects with the CT genotype, because in these subjects, impaired conversion of AMP to IMP will enhance the intracellular adenosine formation, thus decreasing the transmembranous adenosine concentration gradient, which is the driving force for adenosine transport.

In an additional series of experiments, ischaemia-reperfusion injury was determined in the thenar muscle, using 99mTc-annexin A5 scintigraphy to detect phosphatidylserine exposure on cellular membranes of affected cells as early marker of ischaemic injury, as described previously.

**Methods**

**Subjects**

The study protocol was approved by the Institutional Review Board of the Radboud University Nijmegen Medical Centre. Ninety-six healthy subjects participated after signing informed consent. Blood was drawn for genotyping of the AMPD1 and adenosine A2A receptor genes (ADORA2A) and for the determination of the activities of the ENT, adenosine kinase, and adenosine deaminase. The ADORA2A 1976C > T genotype was determined, as the TT variant could potentially affect its function. From these volunteers, subjects were selected to participate in the current study or another study on the effect of the 1976C>T polymorphism in ADORA2A. Twenty subjects had the CT genotype and two the TT genotype for AMPD1. To study reactive hyperaemia, we selected 10 subjects with the CT genotype and 10 control subjects with the CC genotype, with comparable sex, age, and body mass index. To assess tolerance to ischaemia-reperfusion injury, we selected seven male subjects with the CT genotype and seven controls (Table 1).

**Post-occlusive reactive hyperaemia**

Experiments were performed in a temperature-controlled room (23°C) in the morning after an overnight fast and 24 h of caffeine abstinence. The brachial artery of the non-dominant arm was cannulated with a 20-gauge catheter for intra-arterial drug administration and blood pressure recording. FBF was recorded simultaneously on both arms by venous occlusion plethysmography using mercury-in-silastic strain gauges. Before each recording, a wrist cuff was inflated to 200 mmHg to exclude the hand circulation. Arterial occlusion of the non-dominant forearm was induced by inflation of an additional upper arm cuff to 200 mmHg.

Thirty minutes after insertion, FBF was recorded for 5 min during saline infusion (baseline FBF). Subsequently, the FBF response to 2, 5, and 13 min of forearm ischaemia was measured for 3, 5, and 5 min, respectively, as described previously. During the last minute of the 13 min period of ischaemia, the subjects performed rhythmic handgripping, as a stimulus for maximal forearm vasodilation.

After 40 min of equilibration, this experiment was repeated, but now by concomitant administration of dipyridamole (Persantin<sup>®</sup>, Boehringer Ingelheim, España SA, Spain) into the brachial artery (7.4 nmol/min/dL of forearm tissue). A previous study demonstrated that this dose does not affect baseline FBF, but significantly potentiates adenosine-induced vasodilation. Administration of dipyridamole was discontinued 10 s after initiation of forearm occlusion and restarted 10 s before reperfusion. After 30 min of equilibration, the FBF response to intrabrachial administration of sodium nitroprusside (SNP; 0.06 and 0.6 μg/min/dL; Pharmacy Radboud University Nijmegen Medical Centre) and acetylcysteine (ACH; 0.5 and 2.0 μg/min/dL; miochol<sup>®</sup>-E, Novartis, Bournonville Pharma BV, The Hague, The Netherlands) was recorded for 5 min per dose to study endothelium-independent and endothelium-dependent vasodilation, respectively.

**Ischaemia–reperfusion injury**

Circulation to the non-dominant forearm was interrupted for 10 min by inflation of an upper arm cuff to 200 mmHg. Concomitantly, subjects performed rhythmic isometric handgripping with a dynamometer at 50% of maximum force, alternating 5 s of contraction and 5 s of relaxation until exhaustion. Immediately on reperfusion, 99mTc-annexin A5 was administered intravenously into the brachial artery (7.4 nmol/min/dL of forearm tissue) and a gamma camera equipped with a low-energy high-resolution collimator (Siemens Orbiter, Hoffman Estates, IL, USA) connected to a Hermes Gold image processing system (Nuclear Diagnostics, Stockholm, Sweden) as previously described.

**Genetic analysis**

Blood was drawn in EDTA-containing vacutainers and stored at −70°C until DNA isolation. Genomic DNA isolation was performed using a standard desalting protocol. Genotyping was performed by pyrosequencing according to the protocol of the manufacturer (Pyrosequencing AB, Uppsala, Sweden).

For genotyping of the AMPD1 34C > T variant, the following reaction was carried out: primers FW8076 (5'-gcaacctacagtgcttac-3', 10 pmol) and RV8077 (5'-agcgcgtctcgggtcatatagtatatagcc-3', 10 pmol).
HClO₄ was added, and the solution was placed on ice. After centrifugation, 100, 200, and 300 mg samples of the annexin-HYNIC conjugate in 500 μl distilled water, 20 μl 50 mM Tris·HCl, 2 μl 100 mM DTT, 2 μl 50 mM GTP, 0.7 μL 3 mM ethyleneglycol-2-(2-hydroxy-3-yl)-adine (EHNA), and 100 μL Tris-buffer in a final concentration of 0.1, 0.2, 0.5, 1, and 2 μmol/L at 37°C. After 3.5 min, 50 μL 1.5 M HClO₄ was added, and the solution was placed on ice. After centrifugation, 100 μL 0.5 M triocytamine in chloroform was added to 150 μl supernatant. Finally, after centrifugation, the AMP concentration in the supernatant was measured with reversed-phase HPLC using tetraethylammonium hydrogen sulfate (10 mM) as the ion-pair forming agent (pH 6.0) with UV detection set at 254 nm. The increase in AMP was used to calculate V_max and K_m values. Plasma caffeine concentration was determined by reversed-phase HPLC with UV detection set at 273 nm. All measurements were performed in duplicate.

Preparation of ⁹⁹ᵐTc-HYNIC-annexin A5

Briefly, recombinant human annexin A5 (kindly provided by Dr Van der Heijden, Theuse Imaging Corporation, Boston, MA, USA) was conjugated with succinimidyl-hydrazinonicotinic acid (HYNIC). About 100 μg samples of the annexin-HYNIC conjugate in 500 μL 113 mM tricine, pH 6.8, were stored at −20°C. Before each experiment, ⁹⁹ᵐTc-Pertechnetate (500 MBq) was added to the 100 μg sample of the annexin-HYNIC conjugate in the presence of 50 μg stannous sulfate. About 400 MBq ⁹⁹ᵐTc-annexin A5 was administered to each subject.

Statistical analysis

As not all variables showed a Gaussian distribution, we present the data as median and interquartile range, and we used Wilcoxon and Kruskal tests to evaluate differences. All tests were two-sided and a result with P < 0.05 was considered statistically significant. The primary tests in two different studies were on the difference in vasodilator response and in annexin targeting, respectively. The effect of dipryramide on reactive hyperaemia in the first study was a secondary endpoint, which only served to support the main findings, and the P-values of this analysis should be interpreted.
with some caution, as we applied no correction for multiple testing. Analysis of the FBF responses was performed off-line blinded for the specific $AMPD1$ genotypes. For each post-occlusive period, the highest FBF was selected. The FBF response to 2 and 5 min of ischaemia was expressed as percentage of maximum FBF after 13 min of ischaemia. A Koziot test was used to compare these responses between both genotype groups. The effect of dipyridamole on FBF responses to 2 and 5 min of ischaemia was expressed as percentage increase, and a Koziot test was used to compare both genotype groups.

In the second study, all digital scintigraphic images were analysed off-line by the same blinded investigator (W.J.G.O.), using Hermes Gold software. Region-of-interest analysis was performed for the thenar muscle region, as described previously. Radioactivity was expressed as counts per pixel. To correct for background activity, the targeting of annexin was expressed as the percentage difference between the experimental and control hand, and the results were expressed as the change in targeting between 1 and 4 h post-reperfusion, as described previously.

Results

$V_{\text{max}}$ and $K_{\text{m}}$ values of adenosine deaminase, adenosine kinase, and the ENT did not differ between both groups, and $ADORA2A$ genotype frequencies were equally distributed (Table 1).

Post-occlusive reactive hyperaemia

Baseline FBF did not significantly differ between both groups [2.1 (1.7–2.6) mL/min/dL of forearm tissue in the CT genotype group and 2.2 (1.7–2.9) mL/min/dL in the CC group; $P = 0.8$]. In addition, maximum vasodilation in response to 13 min of ischaemia did not differ between the groups [38.5 (31.9–46.0) mL/min/dL in the CT group vs. 40.5 (32.4–51.3) mL/min/dL in the CC group; $P = 0.5$]. FBF in response to the 2 and 5 min period of ischaemia was higher in the CT genotype group when compared with the controls (Figure 2, $P = 0.01$). There were no differences in FBF in the control arm and mean arterial pressure between both groups.

The effect of administration of dipyridamole on baseline FBF was not different between both groups [from 2.1 (1.4–2.7) to 2.3 (1.4–3.0) mL/min/dL in the CT genotype group to 2.0 (1.4–2.5) to 2.4 (2.0–3.1) mL/min/dL in the CC group; $P = 0.4$]. Dipyridamole significantly potentiated FBF responses to 2 and 5 min of ischaemia in the control group [FBF 25.2 (19.9–32.4) and 35.5 (31.2–42.1) mL/min/dL during dipyridamole infusion after 2 and 5 min of ischaemia, respectively, $P = 0.02$ and 0.005 vs. FBF without dipyridamole], but not in the CT genotype group [FBF 27.4 (21.5–32.3) and 31.9 (29.5–40.0) mL/min/dL during dipyridamole administration after 2 and 5 min of ischaemia, respectively]. The effect of dipyridamole was more pronounced in the CC genotype group than in the CT group (Figure 3, $P = 0.01$).

Finally, SNP- and ACh-induced vasodilation did not differ between both groups. Administration of SNP increased FBF from 2.8 (1.7–3.3) mL/min/dL at baseline to 7.3 (4.6–8.0) and 14.6 (11.5–19.8) mL/min/dL in subjects with the CT genotype and from 2.5 (2.0–3.2) mL/min/dL to 6.4 (5.2–8.7) and 11.6 (8.9–21.7) mL/min/dL in the CC control group ($P = 0.8$). Administration of ACh increased FBF from 3.1 (2.4–4.9) mL/min/dL at baseline to 6.8 (3.6–9.1) and 9.9 (5.2–12.3) mL/min/dL in subjects with the CT genotype and from 3.2 (2.7–3.9) mL/min/dL to 6.8 (5.2–11.4) and 8.2 (6.2–15.3) mL/min/dL in the CC control group ($P = 1.0$).

Ischaemia–reperfusion injury

There was no difference in the duration of ischaemic exercise [140 (130–190) s in the CT genotype group vs. 170 (125–180) s in the controls, $P = 0.7$] and maximum contractile force [50 (46–72) kg in the CT genotype group vs. 48 (40–52) kg in controls, $P = 0.3$] between both groups.

Annexin targeting was less in the CT genotype group than in CC controls (Figure 4; $P = 0.03$).

Discussion

The results of the present study demonstrate that in subjects with the 34C $>$ T variant of $AMPD1$, reactive hyperaemia in response to forearm ischaemia is enhanced and tissue injury is reduced. These mechanisms could well contribute to the reduced cardiovascular mortality in patients with genotypes containing the variant allele.
Augmented hyperaemia and reduced tissue injury

Previous studies have shown that the 34C>T variant of AMPD1 is associated with improved survival in patients with heart failure and in those with coronary artery disease. Also in cardiac donors, the 34C>T variant appears to protect against acute heart failure. It is unknown which mechanism is responsible for these beneficial cardiovascular effects. The C→T transition results in a nonsense mutation encoding a truncated AMPD protein. AMPD activity in human muscle biopsies is 30–40% of normal in subjects with the CT genotype and <2% in subjects homozygous for the variant allele. Although predominantly expressed in skeletal muscle, AMPD1 is also expressed in cardiomyocytes and aorta. Indeed, also in cardiac tissue, AMPD activity is ~50% reduced in patients with the CT genotype. Considering the various beneficial cardiovascular effects of adenosine receptor stimulation, it is logical to assume that in subjects with AMPD deficiency, ischaemia-induced adenosine formation and subsequent adenosine receptor stimulation are potentiated, provided that alternative routes of adenosine metabolism are unchanged (Figure 1) and that this mechanism is responsible for the improved cardiovascular outcome.

In the present study, we focused on reactive hyperaemia, because previous studies demonstrated that reactive hyperaemia is, at least partially, mediated by adenosine receptor stimulation. We postulated that a higher reactive hyperaemia in subjects with the 34C>T variant of AMPD1 indicates increased adenosine receptor stimulation. In addition, the enhanced reactive hyperaemia itself could also have beneficial effects on recovery after an ischaemic insult. This mechanism was already suggested by recent preliminary data in healthy volunteers with the 34C>T variant, which demonstrated augmented reactive hyperaemia in the forearm, but only in the second minute after reperfusion, and after correction for per cent body fat. In line with these observations, we showed that reactive hyperaemia in response to 2 and 5 min of ischaemia is augmented in subjects with the CT genotype, compared with a control group, with otherwise similar activities of adenosine deaminase, adenosine kinase, and the ENT and with similar ADORA2A genotype frequencies. The groups did not differ in endothelial function. The observation that reactive hyperaemia in response to a stimulus for maximum forearm vasodilation was similar between both groups excludes that structural changes of the vessel wall account for the observed differences. Moreover, we corrected for possible structural differences by expressing the FBF responses as percentage of maximum FBF in each subject.

We assume that this augmented reactive hyperaemia is mediated by increased adenosine receptor stimulation. Unfortunately, it is extremely difficult to reliably measure adenosine concentrations in humans in vivo, because of the short half-life of adenosine in blood (<1 s) and because the endothelium acts as a highly active metabolic barrier for adenosine. Therefore, the concentration of adenosine in blood does not reflect that of adenosine in the vicinity of adenosine receptors on several cell types, such as endothelial cells, cardiomyocytes, and vascular smooth muscle cells. Several studies have demonstrated that during strenuous exercise, the tissue adenosine concentration in skeletal muscle biopsies is increased in subjects with the CT genotype when compared with controls, but there are no data on the extracellular adenosine concentration during exercise or ischaemia in these subjects. Because of these difficulties in measuring adenosine concentration, we previously validated dipyridamole as a pharmacological tool to inhibit the ENT. We demonstrated that systemic administration of dipyridamole (administered orally) significantly inhibits ex vivo adenosine uptake in isolated erythrocytes. Second, during concomitant administration of adenosine into the brachial artery, administration of dipyridamole increased the adenosine concentration in the venous effluent (as measured with microdialysis). Thirdly, in the same dose as in the present study, dipyridamole did not affect baseline vascular tone, but significantly potentiated the vasodilator response to the intrabrachial administration of adenosine. Finally, the vasodilator response to intrabrachial administration of dipyridamole and the haemodynamic responses to the systemic administration of dipyridamole could significantly be reduced by adenosine receptor antagonists. In the present study, we used this property of dipyridamole to explore the contribution of facilitated adenosine transport to the observed difference in reactive hyperaemia between subjects with the CT genotype and controls.

It is well known that during ischaemia, the extracellular adenosine concentration rapidly increases. In the present study, dipyridamole potentiated reactive hyperaemia in both groups (although this was not significant for the CT genotype group). This finding indicates that during ischaemia and early reperfusion, flux of adenosine over the cellular membrane is mainly directed inwards. In subjects with the CT genotype, the effect of dipyridamole on maximum reactive FBF was less than in controls. This observation suggests that in these subjects, during ischaemia and early reperfusion, the transmembranous adenosine gradient is less than in control subjects. This is compatible with the view that during ischaemia in subjects with decreased activity of the cytosolic AMPD enzyme, intracellular–and not extracellular–formation of adenosine from AMP is increased.

Adenosine receptor stimulation increases resistance to ischaemia–reperfusion by several mechanisms. We hypothesized that in patients with the 34C>T AMPD1 variant, augmented adenosine formation during ischaemia not only augments reactive hyperaemia, but also reduces ischaemia–reperfusion injury. To test this hypothesis, we used 99mTc-annexin A5 scintigraphy to determine...
ischaemia–reperfusion injury in thenar muscle. We demonstrated that ischaemia–reperfusion injury is attenuated in subjects with the 34C > T genotype. 99mTc-annexin A5 scintigraphy is based on the high-affinity binding of annexin A5 to phosphatidylserine residues on cellular membranes. It is well documented that loss of membrane asymmetry is an early general feature of apoptosis, resulting in externalization of phosphatidylserine residues in affected cells, thus providing binding sites for annexin A5.37 Alternatively, some previous studies have demonstrated that phosphatidylserine exposure could be a reversible event, preceding commitment to apoptotic cell death.38 By labelling recombinant annexin A5 with 99mTc-technetium, it is possible to detect these cellular changes in vivo with a gamma camera.39 In previous studies, we validated 99mTc-annexin A5 scintigraphy as a sensitive model to detect ischaemia–reperfusion injury in skeletal muscle and demonstrated that this injury could be reduced by the administration of adenosine and dipyridamole prior to the ischaemic insult.12,19 Additionally, we demonstrated that the adenosine receptor antagonist caffeine abolishes protection by ischaemic preconditioning.21 Several potential limitations of our study should be addressed. First, we only studied subjects who were heterozygous for the 34C > T variant. In subjects with the TT genotype, reactive hyperaemia and ischaemic tolerance could be augmented even more. Secondly, for technical reasons, we did not measure plasma adenosine concentrations. However, the observation that the effect of dipyridamole on reactive hyperaemia was less in subjects with the CT genotype provides evidence that indeed intracellular adenosine formation during ischaemia is enhanced in these subjects. Thirdly, we did not determine activities of endo- and ecto-5′-nucleotidase, which is also important in the formation of adenosine (Figure 1). Finally, we studied the response to ischaemia in the forearm and not in the heart. However, the 34C > T variant of AMPD1 also reduces AMPD activity in cardiac tissue.24

In conclusion, we demonstrated that the 34C > T variant of the AMPD1 gene augments post-occlusive reactive hyperaemia and tolerance to ischaemia–reperfusion. The reduced potentiating effect of dipyridamole on reactive hyperaemia in subjects with the CT genotype indicates that the AMPD1 gene determines the effect of dipyridamole and supports the concept that the transmembranous adenosine concentration gradient during ischaemia is diminished in these subjects, which is compatible with increased intracellular formation of adenosine during ischaemia. Our observations predict that in trials with ENT inhibitors designed to explore the protective effect of endogenous adenosine against ischaemia–reperfusion injury, most benefit is expected in patients without the 34C > T variant allele. The augmented reactive hyperaemia and increased ischaemic tolerance could contribute to the previously observed improved survival in cardiovascular patients with this variant allele and bridge the gap between the rational theoretical concept of adenosinergic cardiovasoactive protection and previous epidemiological observations.

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