

Coronary artery reperfusion: The ADP receptor P2Y₁ mediates early reactive hyperemia *in vivo* in pigs

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Abstract

The physiological mechanisms that regulate reactive hyperemia are not fully understood. We postulated that the endothelial P2Y₁ receptor that release vasodilatory factors in response to ADP might play a vital role in the regulation of coronary flow. Intracoronary flow was measured with a Doppler flow-wire in a porcine model. 2-MeSADP (10⁻⁵ M), ATP (10⁻⁴ M) or UTP (10⁻⁴ M) alone or as co-infusion with a selective P2Y₁ receptor blocker, MRS 2179 (10⁻³ M) was locally delivered through the tip of a coronary angioplasty balloon. In separate pigs the coronary artery was occluded with the balloon for 10 min. During the first and tenth minutes of coronary ischemia, 2.5 ml of MRS 2179 (10⁻³ M) was delivered distal to the occlusion in 8 pigs, 10 pigs were used as controls. MRS 2179 fully inhibited the 2-MeSADP-mediated coronary flow increase ($P < 0.05$) with no effect on UTP, indicating selective P2Y₁ inhibition. ATP-mediated flow increase was significantly inhibited by MRS 2179. During reactive hyperemia following coronary occlusion, flow increased by nearly sevenfold. MRS 2179, however, reduced the post-ischemic hyperemia by a mean of 46% during the period 1–2.5 min following balloon deflation ($P < 0.05$), which corresponds to peak velocity flow during reperfusion. In conclusion, MRS 2179, a selective P2Y₁ receptor blocker, significantly reduces the increased coronary flow caused both by 2-MeSADP and reactive hyperemia in coronary arteries. Thus, ADP acting on the endothelial P2Y₁ receptor may play a major role in coronary flow during post-ischemic hyperemia.

Abbreviations: ADP – adenosine diphosphate; ATP – adenosine triphosphate; EDHF – endothelium-derived hyperpolarizing factor; LAD – left anterior descending artery; NO – nitrous oxide; SMC – smooth muscle cells; UTP – uridine triphosphate

Introduction

During a coronary artery occlusion, the area of the heart supplied by the artery is deprived of its blood supply. Upon reperfusion there is a dramatic rise in coronary blood flow, far above the baseline flow prior to the occlusion [1]. The mechanism for this enormous increase in flow during reactive hyperemia is somewhat of an enigma because the incurred oxygen debt does not in itself justify the flow increase [2]. Several factors have been implicated, and the mechanism is now thought to be multifactorial in origin. Several substances (adrenalin, ADP/ATP, substance P, bradykinin and to some extent also adenosine) activate receptors on the endothelium of the coronary artery, stimulating release of nitrous oxide (NO), prostaglandins and endothelium-derived hyperpolarizing factor (EDHF), that in turn cause relaxation of the underlying smooth

muscle cells (SMC) [2–6]. Adenosine stimulates SMC relaxation directly by acting on specific receptors (mainly A_{2A}) on the SMC [3]. K_{ATP}⁺ channels are regulated by the cellular metabolic state and when they are activated the SMC is hyperpolarized, which causes relaxation. Thus, the K_{ATP}⁺ channels could provide a link between intracellular ischemia and vasorelaxation. Indeed, the K_{ATP}⁺ channel inhibitor glibenclamide has been shown to inhibit a part of the reactive hyperemia [7, 8]. The effect of adenosine, prostaglandins, and NO during reactive hyperemia in coronary vessels has been studied with use of specific receptor blockers (NOS inhibitors, adenosine receptor antagonists, and cyclo-oxygenase inhibitors), and it has been found that there still exists an unaccountable rise in blood flow during reperfusion [7, 9–12].

A role for P2 receptors in reactive hyperemia has been proposed by Burnstock and Rongen, but no direct experimental evidence has yet been published probably because of the previous lack of specific antagonists [13–15]. In the previous studies, it was demonstrated that adenosine and even more potently ATP administered through intracoronary injection and infusion can cause a pharmacologic

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reactive hyperemia nearly as prominent as reactive hyperemia [13–17].

The extracellular purine nucleotides ATP and ADP, which regulate vascular tone and blood pressure by stimulating P₂ receptors, are released in the heart during ischemia from cardiac myocytes, endothelial cells, red blood cells, platelets and sympathetic nerves [3, 13, 14, 18]. P₂Y₁ receptors are found in abundance on the endothelial cells of the coronary vessel wall and can promote hyperemia in response to selective P₂Y₁ agonists [3, 13, 14, 19]. Research has shown that P₂Y₁ receptors promote smooth muscle relaxation through both NO and EDHF [3–6]. Recently, a selective P₂Y₁ receptor inhibitor, MRS 2179, has become available [4, 5, 20], facilitating further exploration of these effects.

We therefore decided to test the hypothesis that a selective blockade of coronary P₂Y₁ receptors could diminish the post-ischemic hyperemia compared to controls in a porcine model.

Materials and methods

Animals

A total of 27 healthy domestic pigs of both sexes weighing 25 kg were fasted overnight with free access to water and were premedicated with azaperone (Stresnil Vet., Leo; Helsingborg, Sweden), 2 mg/kg i.m. 30 min before the procedure. After induction of anesthesia with thiopental 5–25 mg/kg (Pentothal; Abbott, Stockholm, Sweden), the animals were orally intubated with cuffed endotracheal tubes. A slow infusion of 1.25 µl/ml Fentanyl (Fentanyl; Pharmalink, Stockholm, Sweden) in Ringer's acetate solution was started at a rate of 1.5 ml/min and adjusted as needed. Mechanical ventilation was then established with a Siemens-Elema 300B ventilator in the volume-controlled mode. Initial settings were: Respiratory rate of 15 min, tidal volume of 10 ml/kg, and positive end-expiratory pressure of 5 cm H₂O. Minute volume was subsequently adjusted in order to obtain normocapnia (35–40 mm Hg). The animals were ventilated with a mixture of dinitrous oxide (70%) and oxygen (30%). Anesthesia was complemented with small intermittent doses of 5 mg meprobamat (Mebumal; DAK, Copenhagen, Denmark) and thiopental (Pentothal; Abbott, Stockholm, Sweden), if needed.

A 6 F introducer sheath (Onset; Cordis, Miami, Florida, USA) was inserted into the surgically exposed left femoral artery. The side port of the introducer was connected to a pressure transducer and balanced to atmospheric pressure with zero reference at the mid-axillary level for continuously monitoring of the arterial pressure. A three-lead ECG was displayed on the same monitor as the pressure curve (78342 A, Hewlett and Packard, Boeblingen, Germany).

A 6 F introducer sheath (Onset; Cordis, Miami, Florida, USA) was inserted into the surgically exposed left carotid artery upon which a 6F JL 3.5 Wiseguide™ (Boston Scientific Scimed, Maple Grove, Minnesota, USA) was inserted into the left main coronary artery and 10,000 IU of

Heparin was administered. An angiogram was obtained using 8–10 ml of the contrast medium Omnipaque™ 300 mg I⁻/ml (Nycomed, Oslo, Norway) to ensure correct positioning of the catheter. The catheter was used to place a 0.014-inch, 12-MHz pulsed Doppler flow velocity transducer (Jometrics Flowire, Jomed NV) into the mid-portion of the left anterior descending artery (LAD) and a 0.014-inch PT choice™ guidewire (Boston Scientific Scimed, Maple Grove, Minnesota, USA) into the distal portion of the LAD. A 3.0 × 20 mm over-the-wire Maverick™ angioplasty balloon (Boston Scientific Scimed, Maple Grove, Minnesota, USA) was then positioned in the mid-portion of the LAD but proximal to the flow velocity transducer followed by the withdrawal of the PT choice guidewire. Continuous coronary velocity flow profiles were displayed and recorded using the Doppler flow wire connected to a FloMap monitor (Cardiometrics, Mountain View, California, USA). Flow was measured in units of average peak velocity (APV) in centimetres per second. All radiological procedures were performed in an experimental catheterization laboratory (Shimadzu, Kyoto, Japan).

The lumen of the angioplasty balloon was connected to an infusion pump, Asena CC (Alavis Medical, Bristol, UK). The infusion pump was initially used to infuse Ringer's acetate solution and NaCl (9%) at rates of 0.5, 1, 2, 4 and 6 ml/min in the LAD through the inner lumen of the angioplasty balloon catheter. At an infusion rate of 2 ml/min or less, there were no effects on blood flow in the LAD.

In five pigs, 2-MeSADP (10⁻⁵ M) at 1 ml/min was infused and Flomap measurements were performed. 2-MeSADP (10⁻⁵ M) was then infused with MRS 2179 (10⁻³ M) at a rate of 1 ml/min. To test the effect of MRS 2179 on ATP, 5 ml of ATP (10⁻⁴ M) was delivered into the LAD (*n*=9). Following the 30-min washout period, 5 ml of ATP (10⁻⁴ M) together with 5 ml of MRS 2179 (10⁻³ M) was delivered into the LAD. The order of the ATP and the combination of ATP + MRS2179 infusions was altered randomly. To test the effect of MRS 2179 on P₂Y_{2/4} receptors, 5 ml of UTP (10⁻⁴ M) was delivered into the LAD (*n*=3). Following the 30-min washout period, 5 ml of UTP (10⁻⁴ M) together with 5 ml of MRS 2179 (10⁻³ M) was delivered into the LAD. The 2-MeSADP infusions were delivered for 2 min while the ATP and UTP infusions were delivered for only 1 min.

To test the effect of MRS 2179 on reactive hyperemia, an occlusion of the LAD was achieved with inflation of the angioplasty balloon for a period of 10 min. During the first and tenth minutes of coronary ischemia, 2.5 ml of MRS2179 (10⁻³ M) was delivered distal to the occlusion in the LAD in eight pigs. A total of 10 pigs were used as controls. Reactive hyperemia was only measured once in each pig. Blood gas analysis was performed at baseline and at 1 and 10 min following reperfusion in the 18 pigs treated with balloon inflation.

Protocol

At baseline, measurements of blood pressure, pulse and APV were performed. Blood pressure and pulse were

measured continuously with coronary blood flow and APV analyzed once every 10 s. A blood gas analysis was performed at baseline and at 1 and 5 min post-reperfusion.

Reagents

Unless otherwise stated, drugs were purchased from Sigma (USA).

Ethics

The Ethics Committee of Lund University approved the project.

Calculation and statistics

Calculations and statistics were performed using the GraphPad Prism 3.02 software. Values are presented as mean ± S.E.M. Statistical significance was accepted when $P < 0.05$ (two-tailed test). One-way analysis of variance (ANOVA) test followed by Dunnett's multiple comparison test was used.

Results

During infusion with isotonic crystalloid (Ringer's acetate solution) and NaCl (9%) in the LAD there was a slight flow increase with infusion rates at or above 3 ml/min, but not at flow < 2 ml/min. In Figure 1, Ringer's acetate solution was infused at 1 ml/min.

When the ADP analogue 2-MeSADP (10^{-5} M) was infused at a rate of 1 ml/min, flow in the LAD increased significantly ($P < 0.05$; Figure 1). However, the effects of 2-MeSADP (10^{-5} M) on blood flow in the LAD was fully inhibited when infused together with the P2Y₁ receptor antagonist MRS 2179 (10^{-3} M) at a rate of 1 ml/min,

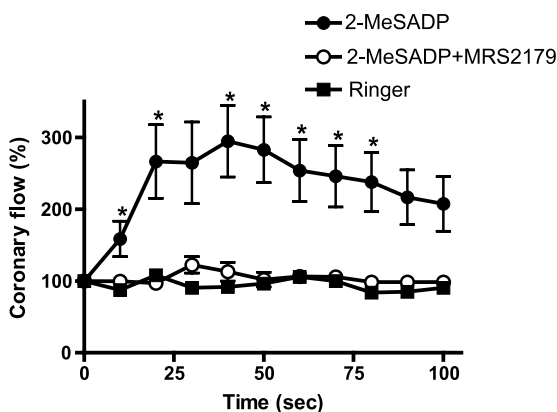


Figure 1. Ringer's acetate solution infused into coronary vessels of pigs, open circles, did not alter coronary flow from baseline. Infusion of 2-MeSADP increased flow by 300%, closed circles. The 2-MeSADP-mediated flow increase was essentially aborted by simultaneous infusion of MRS 2179, closed squares. Data are expressed as percentage of baseline flow (100%) and shown as means ± S.E.M., * $P < 0.05$, ** $P < 0.01$ ($n = 5$).

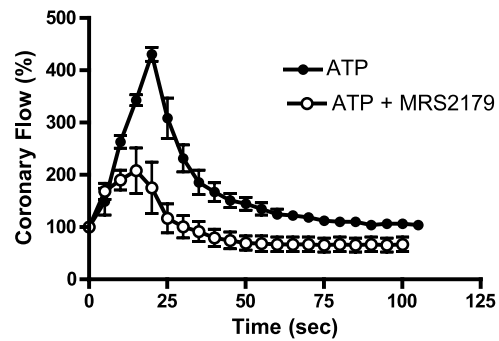


Figure 2. Intracoronary infusion of ATP increased flow in pig coronary arteries by a factor of 5, closed circles. The ATP-mediated flow increase was then reduced by 50% during simultaneous intracoronary administration of MRS 2179, open circles. Data are expressed as percentage of baseline flow (100%) and shown as means ± S.E.M., * $P < 0.05$, ** $P < 0.01$ ($n = 9$).

($n = 5$, $P < 0.05$) (Figure 1). Following a 30-min washout period, the dilations to 2-MeSADP without MRS 2179 could be repeated with similar results as the initial dilatation (data not shown). MRS 2179 alone did not have any effect on basal coronary flow.

ATP delivered selectively in the LAD caused an increase of flow in the LAD by a factor of 5. When ATP was delivered together with MRS 2179 there was significant reduction of flow in the LAD by approximately 50% ($n = 9$), demonstrating that a major portion of the ATP-induced flow is mediated through its degradation-product ADP acting on P2Y₁ receptors (Figure 2).

UTP delivered selectively in the LAD caused an increase of flow in the LAD by a factor of 3.5. When UTP was delivered together with MRS 2179 there was no difference in increased flow in the LAD, demonstrating the selectivity of MRS 2179 ($n = 3$, Figure 3).

Post-ischemic flow in the LAD increased nearly sevenfold in the 10 pigs treated with balloon inflation alone in the LAD ($n = 10$). In contrast, a significant 46% reduction of flow in the early phase (1–2.5 min following balloon deflation corresponding to the period of the fastest flow during post-ischemic hyperemia) was observed in the

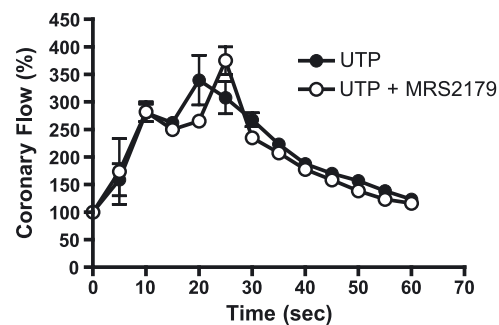


Figure 3. Intracoronary infusion of UTP increased flow, in pig coronary arteries, approximately by a factor of four, closed circles. The UTP-mediated flow increase was not blocked by MRS2179, open circles. Data are expressed as percentage of baseline flow (100%) and shown as means ± S.E.M., $P = NS$ ($n = 3$).

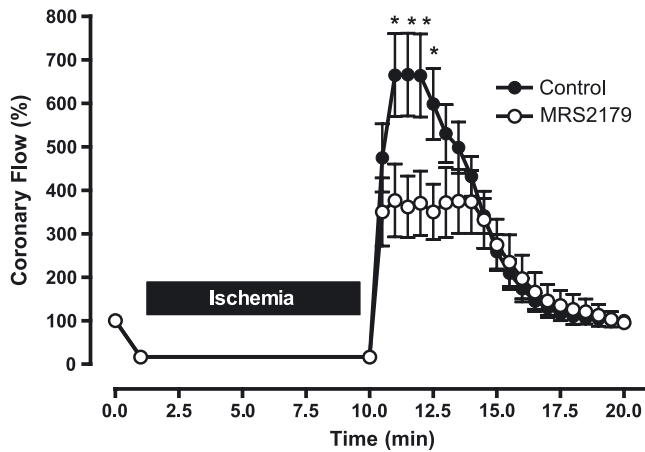


Figure 4. The ensuing reactive hyperemia following a 10-min coronary occlusion was measured as a nearly sevenfold increase of flow (closed circles). Infusion of MRS 2179 reduced the early post-ischemic flow increase by 46%. Data are expressed as percentage of baseline (100%) and shown as means \pm S.E.M., * $P < 0.05$ ($n = 8-10$ pigs).

eight pigs receiving bolus doses of MRS 2179 ($P < 0.05$, Figure 4). During infusions of NaCl, 2-MeSADP and MRS 2179, there were no significant differences in blood pressure or pulse rate between the groups at the analyzed time intervals (Figure 5). There was no difference in basal coronary flow rates between the control and MRS 2179 groups (10.2 ± 4.9 and 11.3 ± 3.7 cm/s, mean \pm S.D., $P = \text{NS}$). The flow rates returned to initial values at the end of the experiments.

The analyzed blood-gas samples of the 18 pigs in the occlusion/reperfusion group showed no statistical difference between the pigs receiving MRS2179 and the group treated as controls (Table 1).

Discussion

The main finding in these experiments is that the selective $P2Y_1$ blocker MRS 2179 significantly reduces the early peak flow by 46% in coronary reactive hyperemia in pigs. This is supported by the flow increase caused by the selective $P2Y_1$ agonist 2-MeSADP, which could be completely blocked by MRS 2179.

The mechanism of reactive hyperemia is still not completely understood but appears to be multifactorial in origin. Investigations of the effects of adenosine [9, 21, 22], prostaglandins [23–26], and K_{ATP}^+ channels [7, 8, 11, 27–29] and the role of NO [11, 12, 30, 29–33], acting alone or in combinations with each other, have been performed. Earlier research on post-ischemic reactive hyperemia in the heart has been performed both *in vivo* in large animals, as well as in Langendorff models in rodents. However, using rodents in a Langendorff model has its inherent drawbacks due to the lack of ATP release from red blood cells in response to ischemia. In large animals, *in vivo* models have shown that adenosine by itself contributes to about 1/3 of the reactive hyperemia with NO contributing slightly less

[9, 10]. Saito et al. [9] showed that adenosine did not contribute to peak reactive hyperemia but instead reduced payment of flow debt following peak flow. Morrison et al. [34] have shown in recent work that the contribution of adenosine to reactive hyperemia occurred 3 min after reperfusion and thus well after peak flow in an A_{2A} receptor knockout mouse model. Similar findings were seen using a selective A_{2A} antagonist [11]. Thus, adenosine plays an important role in post-ischemic reactive hyperemia in the heart but seems to contribute to flow only well after peak flow has occurred.

K_{ATP}^+ channels in smooth muscle cells have also been implemented as a major contributor to reactive hyperemia in the heart. The experiments with K_{ATP}^+ channel blockers (glibenclamide) have mainly been assessed in Langendorff models in rodent hearts and to some extent in large *in vivo* models. In these experiments, K_{ATP}^+ channel blockers significantly reduced reactive hyperemia [7, 11]. Interestingly, K_{ATP}^+ channel blockers did not seem to affect reactive hyperemia in the forearm [35]. Prostaglandins have been found to contribute only marginally to reactive hyperemia, both in the heart and in the forearm [7, 26].

Recent research has demonstrated that red blood cells release ATP in response to ischemia [36, 37]. ATP is then

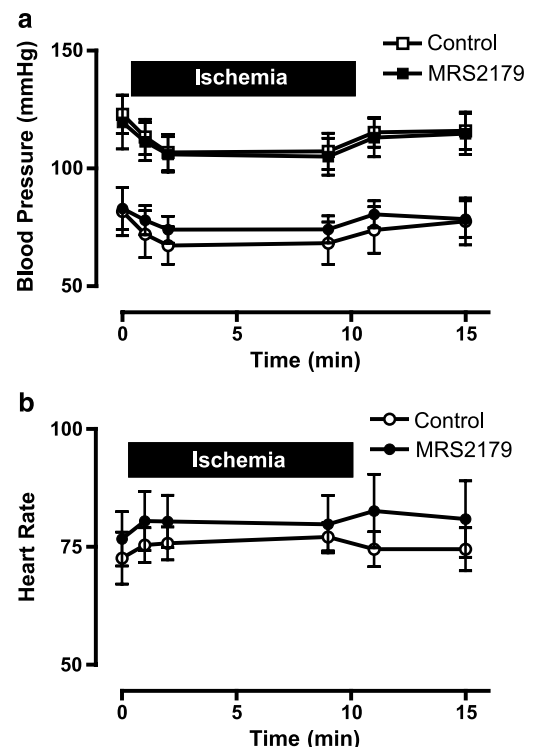


Figure 5. a) In the pigs subjected to coronary occlusion blood pressure was measured at baseline, during ischemia and during reperfusion. There was no statistical difference in the either diastolic or systolic blood pressure between controls ($n = 10$, open symbols) or pigs treated with MRS 2179 ($n = 8$, closed symbols). Mean diastolic and systolic blood pressure are expressed as \pm S.E.M., $P = \text{NS}$. b) In pigs subjected to coronary occlusion the heart rate was measured at baseline, during ischemia and during reperfusion. There was no statistical difference in heart rate between controls, open circles, or pigs treated with MRS 2179, closed circles. Data are mean heart rate expressed as \pm S.E.M., $P = \text{NS}$.

Table 1. Arterial blood-gas analysis of pigs with occlusion and reperfusion of a coronary vessel treated with MRS 2179 ($n=8$) or used as controls ($n=10$).

	Baseline		1 min reperfusion		5 min reperfusion	
	Control	MRS2179	Control	MRS2179	Control	MRS2179
pH	7.47 ± 0.05	7.49 ± 0.03	7.45 ± 0.07	7.48 ± 0.03	7.46 ± 0.06	7.47 ± 0.05
pCO ₂	5.4 ± 0.8	5.3 ± 0.5	5.6 ± 1.0	5.3 ± 0.4	5.3 ± 0.7	5.3 ± 0.6
pO ₂	46.8 ± 19.0	43.7 ± 16.3	40.6 ± 14.6	46.1 ± 20.2	35.4 ± 3.9	36.3 ± 4.4
Na ⁺	132 ± 2	135 ± 1	133 ± 2	134 ± 1	132 ± 2	135 ± 1
K ⁺	3.5 ± 0.2	3.6 ± 0.3	3.4 ± 0.2	3.7 ± 0.3	3.4 ± 0.3	3.6 ± 0.1
Hb	89 ± 8	92 ± 3	91 ± 8	93 ± 4	88 ± 5	92 ± 3
O ₂	97.1 ± 0.2	97.2 ± 0.2	97.9 ± 1.5	97.2 ± 0.2	97.1 ± 0.1	97.2 ± 0.2
HCO ₃	29.0 ± 1.9	30.0 ± 0.3	28.6 ± 1.6	29.4 ± 0.3	28.2 ± 1.3	29.2 ± 0.7
Base excess	5.6 ± 0.8	6.1 ± 0.2	4.9 ± 0.8	5.6 ± 0.3	5.1 ± 0.8	5.3 ± 0.4

Samples were collected at baseline and at 1 and 5 min following reperfusion. There was no statistical differences between pigs treated with MRS 2179 or pigs used as controls. Data are expressed as mean ± S.E.M., $P = NS$.

rapidly degraded to ADP, which in turn binds to the vascular endothelium at the site of the P2Y₁ receptor. Selective blockers of the P2Y₁ receptors have recently become available [20], allowing us to test the role of P2Y₁ receptors during post-ischemic reactive hyperemia. The porcine *in vivo* model in our experiment was chosen because the presence of whole blood and a live model was essential. The use of angioplasty ‘over-the-wire’ balloons allowed for precision in attaining both accurate and localised induction of ischemia, and delivery of infusions. The physiological alterations induced by open chest experiments could thus be avoided. The infusion of Ringer’s acetate solution at the same rate as later infusions of 2-MeSADP and MRS 2179 did not alter measurements of flow from baseline. The selective P2Y₁ receptor agonist 2-MeSADP induced a predicted increase in flow, which could be completely abolished by co-infusion of 2-MeSADP and the selective P2Y₁ receptor blocker MRS 2179. In contrast, UTP, which activates P2Y_{2/4} receptors, stimulated a flow increase that was unaffected by MRS 2179, demonstrating the selectivity for P2Y₁ receptors to post-ischemic-hyperemia, the LAD was occluded, and MRS 2179 was infused into the ischemic portion of the heart supplied by the LAD. The 46% reduction of peak flow achieved during reactive hyperemia indicates that P2Y₁ receptors are of major importance as an activator of endothelium-derived smooth muscle cell relaxing factors such as NO and EDHF. NO and EDHF has been shown to mediate a major part of early reactive hyperemia [8, 10–12], and both are released by ADP acting on P2Y₁ receptors [3–6]. The K_{ATP}⁺ channel inhibitor glibenclamide blocks the remaining early reactive hyperemia [11]. Interestingly, glibenclamide is also an inhibitor of P2Y₁-mediated vasodilatation [6]. It is therefore possible that a part of the hyperemia blocked by glibenclamide is stimulated by ADP via endothelial P2Y₁ receptors and that glibenclamide in part acts downstream of the P2Y₁ activation and not only after intracellular metabolic regulation of K_{ATP}⁺ channels.

The time profile of the mediators of reactive hyperemia is highly interesting. Previous studies using adenosine

deaminase, theophylline [9], selective A_{2A} antagonists [11], or A_{2A} knockout mice [34], have shown that adenosine mediates the late phase of reactive hyperemia. This adenosine is probably derived from degradation by ecto-nucleotidases of the ADP that we now demonstrate mediates a major part of the early peak phase. We would like to propose that ATP is released during ischemia from red blood cells; cardiomyocytes, endothelial cells and platelets, and that ATP could mediate an even earlier part of the hyperemia (Figure 6). This has not been tested yet due to the lack of selective antagonists. (The presence of ATP- and UTP-responsive endothelial P2Y_{2/4} receptors has been demonstrated before [3] and was confirmed here by the hyperemic effect of UTP.) ATP is then degraded to ADP, which mediates peak hyperemia via endothelial P2Y₁ receptors, followed by degradation of ADP to adenosine resulting in late-phase hyperemia mediated via A_{2A} receptors on SMC (Figure 6).

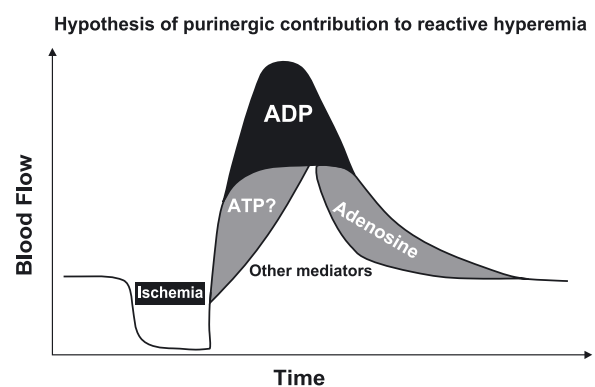


Figure 6. This figure illustrates a hypothesis of smooth muscle cells relaxation in response to accumulation of purinergic substrates in coronary vessels following post-ischemic reperfusion. Red blood cells, heart myocytes, endothelial cells, platelets and sympathetic nerves release ATP during hypoxia. ATP may contribute to the very early reactive hyperemia, although this has not been proven due to lack of specific antagonists. ATP is quickly degraded to ADP which stimulates P2Y₁ receptors on the endothelium, thus initiating the peak flow during the early phase of reactive hyperemia, as demonstrated in the present study. ADP is then degraded to adenosine that stimulates A_{2A}-receptors and thus maintains reactive hyperemia during the mid- to late phase [7–10].

In conclusion, our experiments suggest that ADP stimulating P2Y₁ receptors mediates a major part of peak reactive hyperemia in the heart. Inhibition of the P2Y₁ receptor could potentially attenuate the reperfusion injury incurred during primary angioplasty in the setting of acute myocardial infarction [38].

Acknowledgements

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