Coronary Reactive Hyperemia

Olivecrona, Göran

2007

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This thesis is dedicated to my dear wife Cecilia and our wonderful children Axel, Jacob and Emma.

I also dedicate the thesis in loving memory of my younger brother Lars.
Medicus curat, natura sanat
1 List of Publications

This thesis is based on the following papers, referred to in the text by their Roman numeral:

I. Wang LW, Olivecrona GK, Gotberg M, Olsson ML, Sörhede Winzell M, Erlinge D. ADP acting on P2Y13 receptors is a negative feedback pathway for ATP release from human red blood cells. *Circulation research* 2005; Feb 4;96(2):189-196


## Contents

1 List of publications .......................................................... 5  
2 Contents ........................................................................... 7  
3 Abbreviations .................................................................... 9  
4 Abstract ............................................................................ 11  
5 Introduction ......................................................................... 13  
6 Hypotheses of the thesis .................................................... 21  
7 Materials and methods ...................................................... 23  
8 Reagents, Ethics and Statistics ........................................... 31  
9 Results .............................................................................. 33  
10 Main findings ..................................................................... 45  
11 Discussion ......................................................................... 47  
12 Populärvetenskaplig sammanfattning (Summary in Swedish) .. 57  
13 Acknowledgements ............................................................ 59  
14 Sources of Funding ............................................................. 61  
15 References ......................................................................... 63  
16 Appendix: Original Papers I-V ......................................... 75
3 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAR</td>
<td>Area At Risk</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>AMI</td>
<td>Acute Myocardial Infarction</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine Monophosphate</td>
</tr>
<tr>
<td>APV</td>
<td>Average Peak Velocity (cm/s)</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BE</td>
<td>Base Excess</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>EDHF</td>
<td>Endothelium Derived Hyperpolarizing Factor</td>
</tr>
<tr>
<td>IS</td>
<td>Infarct Size</td>
</tr>
<tr>
<td>LAD</td>
<td>Left Anterior Descending Artery</td>
</tr>
<tr>
<td>LV</td>
<td>Left Ventricle</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial Infarction</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>NO</td>
<td>Nitrous Oxide</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cell</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth Muscle Cell</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single Photon Emission Computed Tomography</td>
</tr>
<tr>
<td>t-PA</td>
<td>Tissue Plaminogen Activator</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine Diphosphate</td>
</tr>
<tr>
<td>UTP</td>
<td>Uridine Triphosphate</td>
</tr>
</tbody>
</table>
4 Abstract

**Introduction:** The mechanism of post ischemic reactive hyperemia is still unknown but now thought to be multifactorial and perhaps involving purinergic signalling. Purines such as ATP and ADP have recently been discovered to play a vital role in the regulation of vascular tone. We postulated that ADP could play a vital role in the mechanism of coronary post ischemic reactive hyperemia and that increased concentrations of plasma ADP could also stimulate a negative feed back loop governing release of ATP from red blood cells as well as an increased release of t-PA. It was also hypothesized that hypothermia may have effects on coronary reactive hyperemia and that rapidly induced hypothermia prior to reperfusion in an infarction model could reduce myocardial infarct size.

**Material and Methods:** All studies were performed in a closed chest porcine model in which coronary reactive hyperemia was studied after a ten minute occlusion of the LAD distal to the first diagonal branch, and blood flow measured in the distal LAD with a Doppler flow wire. Blood samples were collected in a peripheral artery, in the Coronary Sinus and in a central vein. Hypothermia was induced with an endovascular catheter and cold saline solution. In the infarction model in conjunction with hypothermia, the occlusion of the LAD was maintained for 40 minutes before reperfusion. Risk area was analyzed by SPECT and final infarct size by MRI. **Results:** (1) The P2Y_{13} receptor on red blood cells is stimulated by ADP and when activated attenuates ATP release from red blood cells to plasma. (2) Activation of the coronary endothelial P2Y_{1} receptors causes hyperemia in coronary arteries. (3) Inhibition of coronary P2Y_{1} receptors reduces peak blood flow during reactive hyperemia by 46 \%. (4) Coronary t-PA release during ischemia and reperfusion is mediated through P2Y_{1} receptors. (5) Mild systemic hypothermia reduces peak flow in coronary arteries by a 43 \% reduction in peak reactive hyperemia. (6) Rapidly induced hypothermia during anterior myocardial infarction before reperfusion reduces final infarct size compared with rapidly induced hypothermia in conjunction with or after reperfusion. (7) Rapidly induced hypothermia before reperfusion in the setting of anterior myocardial infarction in pigs abolishes microvascular obstruction. **Conclusions:** ADP is important in both regulation of microvascular circulation as well as stimulating a large part of the increased blood flow seen during reactive hyperemia and the subsequent release of t-PA. Additional research on reactive hyperemia, now with the use of hypothermia, led to the conclusion that mild hypothermia also attenuates the blood flow seen in coronary reactive hyperemia. Further research in the infarction model, using hypothermia as adjunctive treatment resulted in a nearly halved final infarct size and an abolishment of microvascular obstruction in the animals treated with hypothermia compared to controls. This may be due to a reduction of the reperfusion injury and our research indicates that a major part of the myocardial reperfusion injury may occur during the short period of reactive hyperemia which is within the same timeframe in which the initiation of myocardial tissue swelling can be seen, an increased release of t-PA, ATP and ADP can be measured, and microvascular obstruction develops as visualized by MRI.
5 Introduction

Heart disease is still the number one cause of death in the western world. This is despite the enormous progress that has been made in treating cardiovascular disease during the last 20 years. In 1987 the first major thrombolysis trial, ISIS 2, was published which heralded a new active strategy of treating patients with acute myocardial infarcts. It is also during this time we have seen an exponential increase in the number of angioplasties performed while the number of coronary artery bypass grafting surgery (CABG) procedures has reached a plateau. Treatment of myocardial infarction has in our generation gone from a passive symptomatic treatment with prolonged bed-rest (several weeks!) to an extremely active treatment, first with thrombolytic drugs in combination with aspirin and later with balloon angioplasty to dislodge the blood clot causing the infarct. The median hospital stay in Lund is now only 4 days following a myocardial infarct. The mortality rate of myocardial infarction today compared to 20 years ago has been reduced by approx 75%.

Yet there are still areas to be explored within cardiovascular research and in particular within coronary artery disease beginning with the very basics of physiology of the coronary circulation. There is also still a need for continuation of research to salvage myocardial tissue lost during a myocardial infarct.

Hyperemia

Hyperemia is the increased blood flow that occurs in response to an organ or a tissues demand of oxygen. It can be thought of as an active hyperemia when caused by an increased demand of oxygen such as in a muscle performing work. Reactive hyperemia (or post-ischemic reactive hyperemia) is the increased blood flow to an organ or a tissue following a temporary blockage of an artery. This increased blood flow seen during reactive hyperemia and active hyperemia is well known to exist but has been difficult to explain.

It is intuitively easy to understand why the blood flow to a leg performing a strenuous exercise would increase during work. The increase in metabolism in the skeletal muscles of the leg causes a sharply increased cellular use of nutrients and oxygen. Obviously cells would then release large quantities of vasodilatory substances which then in turn would relax blood vessel tonus on a capillary level and thus increase blood flow. Active hyperemia in skeletal muscle has been measured to increase blood flow by 20- to 25-fold to that seen at rest.

Reactive hyperemia is similar but may not be exactly the same as active hyperemia. During the ischemic period, when the artery is blocked, local metabolic activities are thought to set in motion a vasodilatory response once the blockage is removed. Cardiac blood flow during reactive hyperemia has been measured to increase over 700% compared to that seen at baseline.

If we make a closed fist and then choke off the blood supply by applying pressure to the two arteries in the wrist before we open the hand it will be white, but will turn red when we release pressure over the arteries. There is as yet no firm clear mechanism for how a local tissue can rapidly increase blood flow in response to a stimulus. Such mechanism must abide by several limiting criteria. First the mechanism must be able to occur very rapidly upon stimulus, as well as to be able to be inhibited quickly. The mechanism must be able to exert its effect on blood flow only in the local tissue where the hyperemia is to occur. In Arthur C Guyton’s latest physiology textbook (2006), it is proposed that the affected tissue can cause hyperemia in two manners. First, through a response with release of vasodilatory substances such as adenosine, carbon dioxide, adenosine phosphate compounds, histamine, potassium ions and hydrogen ions. Secondly hyperemia could be caused by a lack of oxygen which could affect opening of more precapillary sphincters in the affected tissue, thereby increasing blood flow. It has been unclear how many of these pathways actually do play a part in...
hyperemia and if such, to what extent. Each hypothesis has its proponents and opponents in the research community and no consensus has thus been reached on the cause of hyperemia. It would then seem that the mechanism of hyperemia still remains the enigma it has been since the start of vascular physiology.

The role of ATP and red blood cells in arterial vascular control

Recent research by Bengt Saltins group in Copenhagen and by Randy Sprague and Mary Ellsworth in St. Louis has proposed an entirely new theory of the cause of hyperemia\(^6,12\). The theory consists of Adenosine Triphosphate (ATP) and its release from red blood cells in response to increased demand of oxygen from a tissue. ATP has long been thought of as an energy-substrate in cells as well as a component in DNA/RNA. Little thought had been given to ATP as a messenger substance although Björn Folkow had as early as 1949 found out that ATP was a potent vasodilator\(^13\). Folkow also noticed that the content of haemolysed red blood cells could also cause increased blood-flow.\(^13\) Today we know that red blood cells contain massive amounts of ATP (mmol compared to µmol) in comparison to extracellular plasma and also contain the membrane-bound glycolytic enzymes needed for the production of ATP\(^12,14,15\). Actually one can think of the red blood cells as big bags containing mainly oxygen and ATP. We all know why red blood cells contain oxygen, but it has not been clear what function ATP has had in red blood cells.

The class of substances which ATP belongs to is called nucleotides (consisting of purines and pyrimidines). Other members of that class include Adenosine Diphosphate (ADP), Uridine Triphosphate (UTP), and Uridine Diphosphate (UDP). The first proposal for cell surface receptors for nucleotides was made by Drury in 1929\(^16\). However renewed interest in the field of nucleotides as extra-cellular signal substances and their targets on membrane bound receptors did not occur until the publication of Burnstock's classic review article in 1972\(^17\) where he coined the name P2 receptors as the target of extracellular nucleotides. Nucleotides have since slowly come to be accepted as important extracellular signal substances and P2 receptors have become the focus of an increasing amount of research. Initially the theory of nucleotides acting as extracellular signal substances and the existence of P2 receptors met a great deal of scepticism in the research community. It was then not until 1978 that the existence of plasma membrane receptors for extracellular nucleotides was formally recognized\(^18\) even though this was based only on the pharmacological and functional evidence.

There are now 15 known P2 receptors (Table 1) consisting of two subtypes, P2X (ionotropic ligand-gated ion channel receptors) and P2Y (metabotropic G-protein coupled receptors). P2 receptors are found in almost all mammalian cells where they regulate important activities of the cells. It is therefore a new research-field in development with effects of many P2 receptors remaining to be elucidated. Only a limited number of specific stable agonists and antagonists have hitherto been developed to some of the P2-receptors and this has thus far limited research to P2 receptors.

What is known is that the same nucleotide may have very different effects on different cells within the same organ. For instance, ATP acting on P2X\(_1\) receptors on the adventitial cells of an artery causes smooth muscle cells of the artery to constrict\(^15,20\) while ATP acting on P2Y\(_2\) receptors (and ATP’s subsequent degradation product ADP acting on P2Y\(_1\) receptors) on the endothelial cells of an artery causes smooth muscle cells to relax with ensuing vasodilatation as a result\(^21\). Thus the dualistic role of purines was established\(^22,23\).
Using purines and pyrimidines as signal substances has one very clear advantage. They can act very locally because they are quickly degraded or re-uptaken. Extracellular ATP in the circulation is thus quickly degraded to ADP, AMP and adenosine by ectonucleotidases such as ATPase and 5’-nucleotidase or by the cell membrane protein NTDPase 1 (CD39), which degrades both ATP as well as ADP within milliseconds (Figure 1). Perhaps then, ultra short acting ATP could be the answer to the century-old enigma of the mechanism controlling hyperemia.

Table 1
P2-Receptor classification, intracellular signalling (I.c.), ligands and selective agonists and antagonists

<table>
<thead>
<tr>
<th>P2 subtype</th>
<th>I.c. Signalling</th>
<th>Ligand</th>
<th>Selective agonist</th>
<th>Selective antagonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2Y1</td>
<td>IP3</td>
<td>ADP (ATP)</td>
<td>MRS2365</td>
<td>MRS 2179, MRS2500</td>
</tr>
<tr>
<td>P2Y2</td>
<td>IP3</td>
<td>UTP=ATP</td>
<td>MRS2498, UTPgS</td>
<td>---</td>
</tr>
<tr>
<td>P2Y4</td>
<td>IP3</td>
<td>UTP (≠ATP in rodents)</td>
<td>UTPgS</td>
<td>---</td>
</tr>
<tr>
<td>P2Y6</td>
<td>IP3</td>
<td>UDP</td>
<td>MRS2666, MRS2633, UDPbS</td>
<td>MRS2578</td>
</tr>
<tr>
<td>P2Y11</td>
<td>IP3, cAMP</td>
<td>ATP</td>
<td>AR-C67085MX, NF546</td>
<td>NF157</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Clopidogrel, Prasugrel, AZD6140, INS50589, AR-C9931 (cangrelor)</td>
</tr>
<tr>
<td>P2Y12</td>
<td>cAMP</td>
<td>ADP</td>
<td></td>
<td>MRS2211</td>
</tr>
<tr>
<td>P2X1</td>
<td>Positive ion channel</td>
<td>ATP</td>
<td>α-β-mATP</td>
<td>NF023, NF449</td>
</tr>
<tr>
<td>P2X2</td>
<td>Positive ion channel</td>
<td>ATP</td>
<td>---</td>
<td>NF770</td>
</tr>
<tr>
<td>P2X3</td>
<td>Positive ion channel</td>
<td>ATP</td>
<td>α-β-mATP</td>
<td>A317491, NF110</td>
</tr>
<tr>
<td>P2X4</td>
<td>Positive ion channel</td>
<td>ATP</td>
<td>Ivermectin potentiates</td>
<td>---</td>
</tr>
<tr>
<td>P2X5</td>
<td>Positive ion channel</td>
<td>ATP</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>P2X6</td>
<td>Positive ion channel</td>
<td>ATP</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>P2X7</td>
<td>Positive ion channel</td>
<td>ATP</td>
<td>---</td>
<td>KN62, KN04, MRS2427</td>
</tr>
</tbody>
</table>

Perhaps then, ultra short acting ATP could be the answer to the century-old enigma of the mechanism controlling hyperemia.
Randy Sprague’s group published two reports in 1995 and 1996 that showed that the only component present in whole blood that was required for vasodilatation in humans was the red blood cell (RBC). Further reports followed which documented that RBC release ATP in response to physiological stimuli such as reduced oxygen tension and/or mechanical deformation of the RBC. The RBC could thus be thought of as an oxygen demand sensor in the blood stream which could release ultrashort-acting ATP in response to decreased tissue levels of oxygen which would then stimulate vasodilatation. In vivo studies in man has demonstrated that ATP is released in active hyperemia (working skeletal muscle circulation) depending on the number of unoccupied O₂ binding sites and ATP derived from RBC’s is involved in the control of blood-flow distribution.

**Can the red blood cell regulate ATP release depending on concentrations of ATP in the blood?**

**(Paper I)**

In the circulation, the ATP released from RBC’s in response to low oxygen tension then binds to P2Y₁ receptors on the endothelium and stimulates vasodilatation by the release of nitric oxide (NO), prostaglandins, and endothelium-derived hyperpolarizing factor (EDHF). Thus, the RBC functions as an O₂ sensor, contributing to the regulation of blood flow and O₂ delivery by releasing ATP depending on the oxygenation state of haemoglobin.

In mammalian physiology, important signalling systems are usually regulated by negative feedback systems; for example, noradrenalin and ATP released from sympathetic nerves is inhibited by presynaptic α2 and P1 (A1 subtype) receptors.
We hypothesized that ATP release from RBC’s could perhaps be regulated by a P2 receptor-mediated negative feedback pathway which is the topic of the first manuscript (I) of this dissertation.

**What is the role for the selective ADP/ATP-dependent P2Y_{1} receptors in hyperemia (Paper II)**

Although active hyperemia and reactive hyperemia share many similarities it was not clear if reactive hyperemia is also dependent of ATP or its degradation-product, ADP, for the increase in blood flow. 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 \(^{37}\). 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 justifly the flow increase \(^{38}\). 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) \(^{33,35,38,39}\). Adenosine stimulates SMC relaxation directly by acting on specific receptors (mainly A_{2A}) on the SMC\(^{35}\). \(K_{ATP}\) 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 vaso-relaxation. Indeed, the \(K_{ATP}\) channel inhibitor glibenclamide has been shown to inhibit a part of the reactive hyperemia \(^{40,41}\). 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 \(^{41-45}\).

Rongen et al. have proposed a role for P2 receptors in reactive hyperemia, but no experimental evidence has yet been published probably because of the previous lack of specific antagonists \(^{46}\). In previous studies, it has been demonstrated that adenosine and even more potently ATP administered through intracoronary injection and infusion can cause a pharmacologic hyperemia nearly as prominent as reactive hyperemia \(^{46-48}\). 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 \(^{33,49}\), and concentrations of ATP and ADP have also been shown to increase during coronary reactive hyperemia in the venous outflow in the Coronary Sinus \(^{30}\). P2Y\(_{1}\) receptors are found in abundance on the endothelial cells of the coronary vessel wall and can promote hyperemia in response to selective P2Y\(_{1}\) agonists \(^{51}\). Research has shown that P2Y\(_{1}\) receptors promote smooth muscle relaxation through both NO and EDHF \(^{33-35,39}\). Recently, a selective P2Y\(_{1}\) receptor inhibitor, MRS2179, has become available \(^{35,39,52}\), facilitating further exploration of these effects. We therefore hypothesized that the selective P2Y\(_{1}\) antagonist MRS2179 could reduced postischemic reactive hyperemia in the coronary circulation. The results are covered in the second manuscript (II) of this dissertation.

**t-PA-release during ischemia (Paper III)**

Tissue-type plasminogen activator (t-PA) is an enzyme of great importance in maintaining the endothelial wall of blood vessels free of thrombi formation. t-PA itself is synthesized and stored both in endothelial cells as well as vascular neurons\(^{33,54}\). Release of t-PA is caused by
several substances (β2 receptor activators, Platelet activating factor, Isoproterenol, Metacholine, Bradykinin, and extracellular nucleotides (such as ATP, ADP and UTP) as well as by cardiac sympathetic nerve stimulation and local ischemia. The actual mechanism by which the above mentioned factors cause the release of t-PA is still unclear but it has been postulated that the release may be an EDHF mediated phenomenon. ADP activates P2Y1 receptors on the endothelium and on platelets which causes smooth muscle cell relaxation and activation of platelets in arteries. Endothelial P2Y1-receptors, when activated by ADP, mediate SMC relaxation through both NO and EDHF. We therefore postulated that by blocking the P2Y1 receptor, and thus EDHF, we would find a decreased release of t-PA during and immediately following cardiac ischemia, if t-PA release is indeed mediated through EDHF and thus dependent upon the P2Y1 receptor.

Myocardial infarction
The acute coronary syndrome and its pathophysiology
The vast majority of acute coronary syndromes involve the rupture of a vulnerable plaque in a coronary artery and the subsequent formation of a thrombus at the site which may partially or fully occlude the artery. If the rupture is small it may heal by itself with few or no symptoms. A larger rupture and / or a more aggressive formation of thrombus may however cause intermittent symptoms giving rise to a condition known as unstable angina. In such scenario it is believed that the thrombus-formation is in a dynamic state and that several factors including interactions between the endothelium, platelets, coagulation factors and the fibrinolytic system may increase or decrease the size of the thrombus thus causing intermittent symptom in the patient. Symptoms may also be caused by rheological and hemodynamic factors such as seen in patients with anaemia or those performing exercises. The condition of unstable angina may progress to a very small injury of the heart which is then commonly called Non-ST Elevation Myocardial Infarct (NSTEMI). Unstable angina may also progress to the complete occlusion of the coronary artery and then give rise to an acute myocardial infarction which is also called ST Elevation Myocardial Infarct (STEMI). A STEMI may also be the first symptom of a ruptured plaque in a coronary artery without any precursor symptoms.

Acute myocardial infarction and evolution of treatment
In acute myocardial infarction when there is a complete occlusion of the coronary artery (STEMI) supplying a region of the heart muscle, irreversible cellular damage starts to occur after approximately 20 minutes through a number of well known mechanisms. It was not until after the results of the pivotal study ISIS 2 that reperfusion became a therapeutic option in the treatment of acute myocardial infarction, albeit by pharmacological thrombolysis in combination with aspirin. Mortality was reduced by 40% and reperfusion by thrombolysis became the treatment choice for acute myocardial infarction within 12 hour of symptoms.

During the 1990’s interventional cardiologists became proponents of treating patients with acute myocardial infarction by means of mechanical reperfusion with coronary angioplasty balloons (Primary PCI). Several trials were conducted comparing thrombolysis vs. PCI in the setting of acute myocardial infarction which indicated an advantage in using Primary PCI, at least in patients with more than 2 hours of symptoms. Although meta-analysis of data from 23 randomized trials (n=7739) indicated a significant 22% reduction in short term mortality among PCI-treated patients, the one-year mortality data from 26 205 patients in the Swedish RIKS-HIA registry indicated a 50% reduction in mortality for patients treated with PCI compared with patients treated with thrombolysis.
Reperfusion injury

The idea of salvaging myocardial tissue in myocardial infarction is a fairly recent concept as stated by Eugene Braunwald in his 1974 editorial in The New England Journal of Medicine—“Reduction of myocardial infarct size”81. Reperfusion injury is hypothesized to be the additional myocardial injury sustained by the heart during the process of reperfusion of an infarct-related artery. Whether such an entity exists or not is still a matter of debate82-86. What is certain is however that in patients with ongoing STEMI, the earlier reperfusion takes place the more myocardial tissue is saved87 and mechanical reperfusion seems to save more myocardial tissue than reperfusion through thrombolysis88.

Hears and Bolli characterized four mechanism of reperfusion injury: arrhythmias, stunning, lethal reperfusion injury (cell death) and accelerated necrosis89. Other proposed mechanisms of reperfusion injury include calcium overload, complement activation, mitochondrial injury, effects of oxygen-derived free radical generation, increased osmotic stress, endothelial dysfunction with reduced flow and neutrophil activation85, 89-92. Of importance may also be the swelling of myocytes and tissue oedema which may occur as early as after 30 sec following reperfusion93. Histological studies have shown reperfusion injury to be associated with tissue oedema, microvascular / endothelial injury, myocytes damage and cell necrosis4. Research on reducing reperfusion injury has focused on preconditioning (repeated short periods of occlusions prior to the myocardial infarct)95-98, postconditioning (repeated short cycles of reclosure following the opening of an infarct-related vessel)99-102, administration of pharmacological agents103 and on hypothermia104-115. Although animal research have indicated a positive effect of all above mentioned treatment strategies, none of the strategies tested in humans (pharmacological treatment, postconditioning or hypothermia) have proved to clearly reduce infarct size in a large randomized trial.

Hypothermia during myocardial ischemia

(Paper IV and V)

Therapeutic mild hypothermia has experimentally in large animals been shown to limit myocardial infarct size if applied prior to reperfusion104-107, 113, but not following reperfusion115. It has also been shown that the earlier hypothermia is applied to an ischemic myocardium, the more tissue stands to be salvaged113. Animal research also indicate that attaining a temperature less than 35°C prior to reperfusion is needed to see a positive effect of hypothermia107. With the invention of endovascular systems to rapidly cool a large body mass without need for topical cooling Dae et al. successfully performed the first safety trial in a pig model using the SetPoint System (Radiant Medical, Inc)116. Dae et al. then developed the first large animal infarct model employing endovascular cooling prior to reperfusion in which an absolute reduction of 36% in final infarct size was seen104. The importance of the latter experiment by Dae et al. is that it was performed animals with a weight similar to humans and hypothermia was initiated well after onset of ischemia simulating a real life situation. In light of the positive results from prior animal experiments, several human trials employing hypothermia through endovascular cooling as adjunct treatment to primary PCI in the setting of myocardial infarction were performed. Unfortunately the results of these trials have not been able to substantiate an effect of hypothermic treatment. However, post hoc analysis of the patients in the two only large randomized trials (COOL-MI and ICE-IT) who reached a temperature of <35°C before reperfusion showed a reduction in infarct size suggesting a benefit of induction of hypothermia before reperfusion. It is also important to note that only a minority of the patients randomized to hypothermia in COOL-MI and ICE-IT actually reached the target temperature of < 35°C prior to reperfusion of the infarct-related artery. It is unclear why so few patients randomized to cooling did not actually reach target temperature.
prior to reperfusion, but it is our belief that the angiography in these patients was generally completed far before the patients had reached their target temperature due to the slow cooling process, and that a delay to the start of the PCI and reperfusion in order to reach target temperature was deemed unethical. Due to the large body mass of humans in the clinical setting it is difficult to achieve adequate hypothermia without delaying reperfusion therapy. With external cooling or endovascular cooling alone it takes 30 min to 1 h for the patients to reach target temperature104, 106, 111, 112. The mechanism(s) by which mild hypothermia exerts its effect is still unknown, although it is a common belief that the decrease in metabolic demand in the hypothermic myocardium is one explanation for the reduced infarction size. Thus, there is a need to better understand the protective mechanisms of hypothermia, in order to design future human trials in a better way.

Hypothermia in reactive hyperemia
Since reactive hyperemia is an occurrence which immediately follows reperfusion, and thus potentially could be involved in the development of a reperfusion injury, we wanted to examine if reactive hyperemia could be attenuated by mild hypothermia. Myocardial tissue oedema has been documented to occur as rapidly as after 30 s following reperfusion in a 15 minute ischemic model93 and is thus in the same time frame when reactive hyperemia reaches its peak flow within 5 minutes of reperfusion97. There is also the appearance of an excess of O2-derived free radicals during the first minute of reperfusion with a peak at between 4 and 7 minute post reperfusion117, and during this time frame a generalized mitochondrial and cell swelling can also be visualized on electronic microscopy93.

We hypothesized that the large blood flow seen during coronary reactive hyperemia could be attenuated by mild systemic hypothermia and that this may be a mechanism by which hypothermia could reduce reperfusion injury.

Hypothermia during acute myocardial infarct
Although a number of animal experiments and several human trials have been performed in which hypothermia has been used to attenuate myocardial infarct size, there is still no evidence on when a crucial cell saving effect of therapeutic hypothermia occurs, or for how long hypothermic treatment needs to be maintained. Animal studies indicate that a positive effect of hypothermia is only seen if cooling is initiated before reperfusion104, 107. The effect of this may be due to two things. First of all initiation of hypothermia may inhibit further infarct evolution in itself. Secondly, induction of hypothermia prior to reperfusion may reduce the postulated reperfusion injury thought to occur in conjunction with the opening of an infarct related vessel which may also be the reason why neither of the two large randomized human cooling trials showed a positive effect of hypothermia in the setting of myocardial infarction. We therefore wanted to test if there was a difference between rapidly induced hypothermia shortly before reperfusion and rapidly induced hypothermia in conjunction with reperfusion. Data from such an animal experiment could help establish if treatment with hypothermia has its main benefit in the short period of reactive hyperemia during reperfusion, and could also give insights to why the trials COOL-MI and ICE-IT failed to meet their endpoints.
6 Hypotheses of the thesis

I. The P2Y_{13} receptor on the red blood cell functions as a negative feedback-loop for ATP release from the red blood cell.

II. The endothelial P2Y_{1} receptor mediates coronary reactive hyperemia.

III. t-PA release can be mediated by P2Y_{1} receptors.

IV. Hypothermia can reduce coronary reactive hyperemia.

V. Rapidly induced hypothermia shortly before reperfusion reduces myocardial infarct size compared to rapidly induced hypothermia shortly after reperfusion.
7 Materials and methods

The closed chest pig model

In our research we needed to develop an animal model of sufficient size to accommodate catheters and angioplasty balloons for the catheterization of the heart, and also to allow for frequent blood samples. In the last manuscript we also needed to perform Magnetic Resonance Imaging (MRI) and gated single photon emission computed tomography (SPECT). We also wanted to use a “closed chest model” in order to physiologically simulate “real” conditions. Cardiovascular research involving coronary arteries in large animal models frequently use an “open chest model”. An open chest model means that a sternotomy or a thoracotomy is performed in order to have access to the heart from the outside. One can then use the heart for various experiments such as permanently or temporarily ligating coronary arteries. The advantage of using an open chest approach is that most materials used are reusable and the basic surgical technique is fairly straightforward. The closed chest model offers most of the same possibilities when research is focused on coronary arteries but in this model access to the arteries is gained through catheters commonly engaging the left coronary artery. This requires use of a great deal of expensive single use (disposable) materials which increases costs as well as the need to have access to a catheterization laboratory and skills in interventional cardiology. When using large animals in research involving coronary arteries, the pig or the dog is commonly selected. We choose the pig model for several reasons, first of all the anatomy and the distribution of the coronary vascular system in pigs is much more similar to humans than to dogs. The pig also has a, to humans, comparatively corresponding heart-to-body ratio of about 0.005 for both. Compared to humans, the pig heart has one major difference. This difference is in regards to the venous system. In humans the great cardiac vein (the continuation of the anterior ventricular vein) with returning blood from the heart-muscle confluences with the middle and small cardiac veins to form the Coronary Sinus which returns the venous blood to the right atrium with the ostium located at the base of the right atrium. The pigs have a completely different anatomy in that the left Azygos Vein enters the right atrium at the base and the Coronary Sinus confluences with the Azygos Vein a few centimetres before the ostium to the right atrium. This makes catheterization of the Coronary Sinus in pigs much more challenging than in humans.

The pig was also chosen as model because it is readily available in large quantities and is deemed ethically to be more suitable for research than primates or dogs.

Animal preparations
(papers I-V)

Anaesthesia

Healthy, domestic male and female pigs were fasted overnight with free access to water and were premedicated with azaperone (Stresnil Vet., Leo; Helsingborg, Sweden), 2 mg/kg intramuscularly 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 AB, 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. Min 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.
Catheterization of the Coronary Sinus
(Papers I and III)
A 14 F or 12F introducer sheath (Boston Scientific Scimed, Maple Grove, MN, USA) was inserted into the surgically exposed right jugular vein through which a short 10 F Coronary Sinus catheter of our own design was introduced into the ostium of the Azygos vein in the right atrium. Then a 6F coronary MPA catheter (Onset, Cordis Co. Miami, FL, USA) was passed through the 10 F catheter and the Azygos Vein and into the Coronary Sinus. An angiogram was obtained using 5 -10 ml of the contrast medium Omnipaque™ 300 mg I/ml (Nycomed, Oslo, Norway) to ensure correct positioning of the catheter.

General Catheterizations
(Paper I – III)
A 6 F introducer sheath (Boston Scientific Scimed, Maple Grove, MN, 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 GMBH. Boeblingen, Germany).
A 6 F introducer sheath (Boston Scientific Scimed, Maple Grove, MN, USA) was inserted into the surgically exposed left carotid artery and a 6F JL 3.5 or 4.0 Wiseguide™ (Boston Scientific Scimed, Maple Grove, MN, 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), (not in paper I) and a 0.014-inch PT choice™ guidewire (Boston Scientific Scimed, Maple Grove, MN, USA) into the distal portion of the LAD. A 3.0 x 20 mm over the wire Maverick™ angioplasty balloon (Boston Scientific Scimed, Maple Grove, MN, 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 (Figure 2). Continuous coronary velocity flow profiles were displayed and recorded using the Doppler flow wire connected to a FloMap monitor (Cardiometrics, Mountain View, CA). Flow was measured in units of average peak velocity (APV) in centimeters per second.

Figure 2
Schematic illustration of the model used in the closed chest pig model. The left coronary artery is engaged with a coronary catheter, through which an over-the-wire angioplasty balloon is placed in the LAD, distal to the first diagonal branch. A Doppler flow wire runs adjacent to the angioplasty balloon with the tip placed in the distal LAD.
General Catheterizations
(paper IV)

A 6 F introducer sheath (Boston Scientific Scimed, Maple Grove, MN, USA) was inserted into the surgically exposed left femoral artery as described previously.

A 12 F introducer sheath (Boston Scientific Scimed, Maple Grove, MN, USA) was inserted into the surgically exposed left femoral vein. A 10.7 F cooling (temperature control) catheter (Celsius Control™, Innercool Therapeutic, San Diego, CA, USA) was inserted through the sheath and positioned in the Inferior Vena Cava (Figure 3). Body temperature was measured with a temperature probe (TYCO Healthcare Norden AB, Solna, Sweden) placed in the distal part of the esophagus. The catheter and the temperature probe were then connected to the console and the system was set to maintain a temperature of 37.0° C. The normal temperature of a pig is located somewhere between 37.0° and 38.5° C according to our laboratory veterinarian and the temperature of the pigs was thus set at the lower end of normal.

Figure 3
Illustration of the Celsius Control™ cooling catheter used

A 6 F introducer sheath (Boston Scientific Scimed, Maple Grove, MN, USA) was inserted into the surgically exposed left carotid artery and the left coronary artery was catheterized as described previously, and an angiogram obtained.

The catheter was used to place a Doppler flow velocity transducer as earlier described into the distal portion of the LAD together with a PT choice™ guidewire into the distal portion of the LAD. A 3.0 or 3.5 x 20 mm over the wire Maverick™ angioplasty balloon was then positioned in the mid portion of the LAD, proximal to the flow velocity transducer but distal to the first diagonal branch, followed by the withdrawal of the PT choice guidewire. Continuous coronary velocity flow profiles were displayed and recorded as described earlier.

General Catheterizations
(paper V)

The endovascular cooling catheter was inserted, as earlier explained above, into the Vena Cava. Body temperature was measured in the distal part of the esophagus as previously described. The catheter and the temperature probe were then connected to the Celsius Control
and the system was set to maintain a normal pig body temperature of 38.0°C. A 6 F introducer sheath (Boston Scientific Scimed, Maple Grove, MN, USA) was then inserted into the surgically exposed left carotid artery upon which a 6 F JL4 Wiseguide™ was inserted into the left main coronary artery. The catheter was used to place a 0.014-inch PT Choice™ guide wire into the distal portion of the LAD and a 3.0-3.5 x 12 mm Maverick monorail™ angioplasty balloon was then positioned in the mid portion of the LAD, immediately distal to the first diagonal branch.

**Protocol**

**Infusion of saline solution in the LAD**

The lumen of the angioplasty balloon was connected to an infusion pump, Asena CC (Alavis Medical, Bristol, England). The infusion pump was initially used to infuse Ringer’s acetate solution and NaCl (0.9%) 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 vivo pig model with microdialysis for determining ATP levels in blood (Paper I)**

Through a catheter, the microdialysis probe (CMA70) was inserted and the microdialysis tip was placed in the Coronary Sinus. A 6F introducer sheath was inserted into the surgically exposed left carotid artery. Through the left carotid artery, a catheter was placed in the left anterior descending artery (LAD). Finally, 2 ml of 2-MeSADP (10^-5 mol/L) was infused in the LAD for 1 minute. Samples were collected by microdialysis (5 µL/min) from the Coronary Sinus every 5 minutes, and ATP measured. ATP was measured by ATP Bioluminescent assay kit (Sigma) according to the supplier’s instructions.

**Infusion of 2-MeSADP and MRS2179 in the LAD (Paper II)**

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 MRS2179 (10^-3 M) at a rate of 1 ml/min. To test the effect of MRS2179 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 MRS2179 on P2Y_1 receptors, 5 ml of UTP (10^-4 M) was infused into the LAD (n = 3). Following the 30-min washout period, 5 ml of UTP (10^-4 M) together with 5 ml of MRS2179 (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.

**Inhibition of P2Y_1 receptors with MRS2179 during ischemia and reperfusion of the LAD (Paper II)**

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 MRS2179 (10^-3 M) at a rate of 1 ml/min. An occlusion of the LAD, distal to the first diagonal branch, was achieved with inflation of the angioplasty balloon for a period of ten min. During the first and tenth min of
coronary ischemia, 2.5 ml of MRS2179 (10^{-3}M) was delivered distal to the occlusion in the LAD in 8 pigs. 10 pigs were used as controls, in which the same volumes of NaCl 0.9% were infused. Reactive hyperemia measured in Average Peak Velocity (APV) in cm/s, was performed only once in each pig. Blood gas analysis was performed at baseline and at one and ten min following reperfusion in the 18 pigs treated with balloon-inflation.

Measurement of t-PA during simultaneous inhibition of P2Y_{1} receptors with MRS2179 during ischemia and reperfusion of the LAD

(Paper III)

Infusions of MRS2179 and 2-MeSADP were performed in an identical fashion as earlier described in measurement of reactive hyperemia, see above.

To test the effect of MRS2179 on t-PA release following reactive hyperemia, an occlusion of the LAD, distal to the first diagonal branch, was achieved with inflation of the angioplasty balloon for a period of ten min. During the first and tenth min of coronary ischemia, 2.5 ml of MRS2179 (10^{-3}M) was delivered distal to the occlusion in the LAD in 8 pigs. 10 pigs were used as controls, in which the same volumes of NaCl 0.9% were infused. t-PA samples were collected in the Coronary Sinus and in a peripheral artery at baseline, during ischemia and at 1 and 5 minutes following reperfusion (balloon deflation). Reactive hyperemia t-PA measurements were only measured once in each pig.

t-PA measurements

Plasma concentrations of t-PA were determined by commercial ELISA kits (TintElize® t-PA, Biopool AB, Umeå, Sweden and COALIZA® PAI, Chromogenix, Haemochrom Diagnostica AB, Mölndal, Sweden). All samples from one experiment were assayed in duplicate on the same microtest plate. Intra-assay variation coefficients were 2.7 and 3.1% for respective assay. Plasma glucose, cholesterol, and triglycerides were analyzed by standard methods at the Department of Clinical Chemistry at Sahlgrenska University Hospital.

Measurement of coronary reactive hyperemia during mild hypothermia

(Paper IV)

In all 16 pigs baseline registrations were performed. Regardless of initial temperature, all pigs were cooled or warmed (as needed) to a baseline temperature of 37°C, which was then maintained for 30 minutes. The pigs were then randomized to the hypothermia group or to the control group using a simple randomization by drawing folded notes out of a box which read “cool” or “warm”. The pigs randomized to hypothermia were then cooled with the endovascular cooling catheter to a temperature of 34.0°C, prior to balloon inflation, and temperature was then maintained until sacrifice. The pigs randomized to the control group were actively maintained at 37°C using the endovascular cooling catheter until sacrifice.

In all pigs, the LAD was then occluded distal to the first diagonal branch by inflation of the angioplasty balloon for a period of ten min. The coronary blood flow in the LAD was measured before, during and after occlusion of the LAD. During the reperfusion phase, coronary blood flow was measured at every 10 sec. Flow was measured in APV, in cm/sec. Occlusion of the LAD was performed only once in each pig.

Blood gases were collected through the MPA catheter in the Coronary Sinus and the Femoral Artery sheath during baseline, early ischemia (one minute after balloon inflation in the LAD), early reperfusion (one minute following balloon deflation in the LAD) and late reperfusion (ten minutes following balloon deflation in the LAD).
At baseline, measurements of blood pressure, pulse and APV were performed. Blood pressure and pulse were measured continuously.

**Rapid induction of hypothermia in pigs with myocardial infarction**

*(Paper V)*

**Ischemia protocol**

After a stable core body temperature of 38.0°C was achieved, ischemia was induced by inflation of the angioplasty balloon for 40 min. An angiogram was performed after inflation of the balloon and before deflation of the balloon in order to verify total occlusion of the coronary vessel and correct balloon positioning. After deflation of the balloon a subsequent angiogram was performed to verify restoration of blood flow in the previously occluded artery.

**Hypothermia protocol**

The pigs were randomized to rapid hypothermia before reperfusion, (pre-reperfusion hypothermia, n=8) or immediately after reperfusion (post-reperfusion hypothermia, n=8). A normothermic group (n=5) was also studied in order to provide comparison between different hypothermia protocols and normothermia. Hypothermia was induced by a rapid intravenous infusion of 1000 ml of 4°C cold saline into a central vein together with the Celsius Control™ endovascular cooling system after 25 min of ischemia or immediately after reperfusion when coronary blood flow was restored (Figure 4). Target temperature was 33°C and successful cooling was defined as a temperature of ≤ 35°C. Hypothermia was then actively maintained for 30 min followed by passive rewarming with blankets.

**Figure 4**

Protocol for induction of hypothermia. In the pre-reperfusion group, hypothermia was started after 25 min of ischemia (15 min before reperfusion) and in the post-reperfusion group, hypothermia was started immediately after reperfusion. The normothermic group was maintained at 38.0°C.
In vivo assessment of area at risk by SPECT

SPECT was used to assess the area at risk (AAR) as percent of left ventricular myocardium. Five hundred MBq of $^{99m}$Tc-tetrofosmin was administered intravenously ten minutes before deflation of the angioplasty balloon. The anesthetized pigs were then imaged in a supine position with a dual head camera (ADAC Vertex, Milpitas, CA, USA) at 32 projections (40 s per projection) with a 64 X 64 matrix yielding a digital resolution of 5 X 5 X 5 mm. Iterative reconstruction using maximum likelihood-expectation maximization (MLEM) was performed with a low-resolution Butterworth filter with a cut-off frequency set to 0.6 of Nyquist and order 5.0. No attenuation or scatter correction was applied. Finally short and long-axis images were reconstructed. Quantification of the size of AAR was performed automatically as the extent of the perfusion defect as determined by commercially available software (Auto QUANT™ 4.3.1 and a standard database; ADAC, Milpitas, CA, USA)\textsuperscript{124}.

Infarct size and microvascular obstruction assessed by ex vivo MRI

Ex vivo imaging of the heart was undertaken using a 1.5 T Philips Intera CV MR scanner (Philips, Best, the Netherlands) according to a previous described protocol\textsuperscript{125}. In brief, a commercially available gadolinium-based contrast agent (Magnevist, *gadopentetate dimeglumine*, Gd-DTPA, Schering Nordisker AB, Järfälla, Sweden) was administered intravenously (0.2 mmol/kg) both 60 and 15 minutes prior to removal of the heart. The heart was removed 4h 22 min ± 47 min after initiation of reperfusion. After removal, the heart was immediately rinsed in cold saline and the ventricles were filled with balloons containing deuterated water. T1-weighted images (TR = 20ms, TE = 3.2ms, flip angle = 70° and 2 averages) with an isometric resolution of 0.5 mm covering the entire heart were then acquired using a head coil. The MR images were analyzed using freely available software (Segment 1.457, http://segment.heiberg.se)\textsuperscript{126}. The endocardial and epicardial borders of the left ventricular myocardium were manually delineated in short-axis ex vivo images. This defined the volume of the left ventricular myocardium. The infarct size (IS) was first determined as the volume of infarcted myocardium (cm$^3$). The infarct volume was calculated as the product of the slice thickness (cm) and the area of hyperenhanced pixels (cm$^2$) with a signal intensity above the infarction threshold defined as > 8 SD above the mean intensity of non-affected remote myocardium. Microvascular obstruction was defined as hypointense regions in the core of the infarction which had signal intensity less than the threshold for infarction. These regions were manually included in the infarct volume. The volume of microvascular obstruction (cm$^3$) was calculated as the difference between the infarct volume before and after manual inclusion of regions of microvascular obstruction. Furthermore, the size of microvascular obstruction was expressed as percent of the total infarct volume. Penultimately, the infarct size was expressed as percent of left ventricular myocardium. One animal in the normothermic group suffered from severe bradycardia which required manual open chest cardiac compression in order to secure the adequate circulation of the second injection of contrast media. The remote myocardium was somewhat increased in signal intensity and the threshold for infarction in this animal was therefore defined as pixels which were 3SD above the remote myocardium as determined by visual assessment. Finally, infarct size was expressed as a percentage of the area at risk (IS/AAR) in order to adjust for any difference in area at risk between the groups.

Patchiness index

Infarct homogeneity was assessed by a patchiness index based on infarct surface area. The high resolution ex vivo MR images allowed quantification of the surface area of the infarct. Infarct surface area (cm$^2$) was automatically determined as the product of the slice thickness (cm) and
the distance along the pixel border between infarcted and non-infarcted pixels (cm) in each slice. For equally homogeneous infarcts, a larger infarct volume will yield a larger surface area. A patchiness index (cm$^{-1}$) was therefore calculated as the infarct surface area (cm$^2$) divided by the infarct volume (cm$^3$). Thus, the patchiness index provided a method for estimating the homogeneity of the myocardial infarction adjusted for infarct size.
8 Reagents, Ethics and Statistics

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

Ethics
The Ethics Committee of Lund University approved the project.

Statistics
Paper I
Data are expressed as mean ± SEM. n indicates the number of subjects tested. The Student \( t \) test was used. Differences were considered significant at \(*P<0.05\), \(**P<0.01\), and \(***P<0.001\) (two-tailed test), compared with control if not indicated.

Paper II
Calculations and statistics were performed using the GraphPad Prism 3.02 software. Values are presented as mean ± SEM. 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.

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

Paper IV
Calculations and statistics were performed using the GraphPad Prism, version 4.0 software. Values are presented as mean ± SEM. Statistical significance was accepted when \( P < 0.05 \) (two-tailed test). Two-way analysis of variance (ANOVA) test followed by Bonferroni post test was used.

Paper V
Calculations and statistics were performed using the GraphPad Prism 4.0 software. Values are presented as mean ± SEM. Statistical significance was accepted when \( P < 0.05 \) (Mann-Whitney’s test).
9 Results
The P2Y1 receptor mediates early coronary reactive hyperemia

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 5, 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 5). 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 MRS2179 (10^{-3} M) at a rate of 1 ml/min, (n=5, P< 0.05) (Figure 5). Following a 30-min washout-period, the dilatations to 2-MeSADP without MRS2179 could be repeated with similar results as the initial dilatation (data not shown). MRS2179 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 MRS2179 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 6).

Figure 5
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 6
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)
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 MRS2179 there was no difference in increased flow in the LAD, demonstrating the selectivity of MRS2179 (n = 3, Figure 7).

![Graph showing flow increase](image1)

**Figure 7**
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).

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 eight pigs receiving bolus doses of MRS 2179 (P < 0.05, Figure 8).

![Graph showing flow change](image2)

**Figure 8**
The ensuing reactive hyperemia following a ten-minute coronary occlusion was measured as a nearly seven-fold increase of flow (closed circles). Infusion of MRS2179 reduced the early post-ischemic flow increase by 46%. Data are expressed as percentage of baseline (100%) and shown as means ± s.e.m, * p < 0.05, (n=8-10 pigs).
During infusions of NaCl, 2-MeSADP and MRS2179, there were no significant differences in blood pressure or pulse rate between the groups at the analyzed time intervals as listed in paper II. There was no difference in basal coronary flow rates between the control and MRS2179 groups (10.2 ± 4.9 and 11.3 ± 3.7 cm/s, mean ± S.D., P =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 2).

### Table 2
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). Samples were collected at baseline and at one and five minutes following reperfusion. There were no statistical differences between pigs treated with MRS 2179 or pigs used as controls. Data are expressed as mean ± s.e.m, p =NS.

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The P2Y13 receptor on erythrocytes regulates ATP release

*Regulation of Plasma ATP Concentrations in an In Vivo Pig Model*

After intracoronary injection of 2-MeSADP, samples were obtained from the venous effluent of the heart in the Coronary Sinus with a microdialysis probe. Analysis showed decreased concentrations of plasma ATP after intracoronary injection of 2-MeSADP (Figure 9).

![Figure 9](image-url) Effects of 2-MeSADP on plasma ATP concentrations in an in vivo pig model. Bar graphs show plasma ATP concentrations sampled from the venous outflow of the heart with a microdialysis probe in the coronary sinus 5-10 min after intracoronary injection of 2-MeSADP (10 μmol/L, 2 ml). n=8.
t-PA release is mediated through P2Y₁ receptors

**Infusion of ADP analogue and P2Y₁ antagonist in the non-ischemic heart**

Intracoronary infusion of the stable ADP agonist 2-MeSADP (10⁻⁵M) at a rate of 1 ml/min caused a significant increase of the t-PA level in the Coronary Sinus compared to infusion of Ringer’s acetate (data not shown). The effects of 2-MeSADP (10⁻⁵M, n=9) on t-PA release was significantly inhibited when infused together with the P2Y₁ receptor antagonist MRS2179 (10⁻³M, n=6) at a rate of 1 ml/min. t-PA increased to a maximum (at 1 min) of 1.81±0.32 ng/ml compared to 0.78±0.28 ng/ml in the presence of MRS2179, *p<0.05, (Figure 10A). t-PA was sampled selectively in coronary sinus. Following a 30 min washout period, the effects of 2-MeSADP on t-PA release could be repeated with similar results as during the initial infusion of 2-MeSADP 10⁻⁵M (data not shown). MRS2179 alone did not have any effect on t-PA release (data not shown).

![Figure 10](image)

**Figure 10**

(A) Illustrates that an ADP analogue release t-PA in the pig coronary circulation and that the effect is mediated by the P2Y₁ receptor: Release of t-PA due to continuous intracoronary infusion of 2-MeSADP (10⁻⁵M) (1ml/min) alone (filled circles, n=9), or 2-MeSADP (10⁻⁵M) jointly infused with the P2Y₁ antagonist MRS2179 (10⁻³M) at a rate of 1 ml/min (open circles, n=6). t-PA was sampled selectively in the Coronary Sinus. * = p<0.05 compared to baseline. § = p<0.05 compared to 2-MeSADP alone.

(B) Illustrates an estimate of differences in total t-PA release: Continuous infusion of 2-MeSADP (10⁻⁵M) at 1 ml/min increased flow in the LAD significantly as measured with the FloMap Doppler wire (data not shown). 2-MeSADP (10⁻³M) jointly infused with MRS2179 (10⁻³M) at a rate of 1 ml/min did not increase flow. When release of t-PA was measured in the coronary sinus and factored with the measured flow in the LAD, there was a significant increase of t-PA release due to infusion of 2-MeSADP. The estimate of differences in total t-PA release was calculated by multiplying with the relative increase in flow compared to baseline.

The effect was even more prominent when an estimation of total t-PA release was performed by factoring the measured t-PA level in the coronary sinus with measured blood flow in the LAD with a FloMap Doppler wire, (Figure 10B). Continuous infusion of 2-MeSADP (10⁻⁵M) at 1 ml/min increased flow in the LAD significantly as measured with the FloMap Doppler wire, as previously shown. When release of t-PA was measured in the Coronary Sinus and factored with the measured flow in the LAD, there was a significant increase of t-PA release due to infusion of 2-MeSADP. The estimate of differences in total t-PA release was calculated by multiplying with the relative increase in flow compared to baseline. Unfortunately this closed chest model does not allow determination of coronary blood flow in ml, only in cm/s, APV. However, our control experiments with angiographic measurements of vessel diameter during
flow increase, demonstrated a minor increase in LAD vessel diameter. Thus, any error due to lack of correction for vessel diameter (i.e. quantification in ml), would underestimate a flow increase and would thus if anything underestimate the findings of the experiment.

**Cardiac ischemia experiments**

There was no observed difference in t-PA levels in peripheral arterial samples from the Femoral Artery of t-PA during the baseline, ischemic or reperfusion periods, (Figure 11). t-PA was measured in a peripheral artery (Femoral Artery) before, during and after coronary ischemia. Levels of t-PA in the peripheral arterial circulation were at these points unaffected by coronary infusion of MRS2179 during the first and tenth minute of coronary ischemia, 2.5 ml of MRS2179 (10⁻³M).

**Figure 11**  
No systemic changes in t-PA concentrations during ischemia were observed: t-PA was measured in a peripheral artery (Femoral artery) before, during and after coronary ischemia. Levels of t-PA in the peripheral arterial circulation were at these points unaffected by coronary infusion of 2.5ml of MRS2179 (10⁻³M) during the first and tenth minute of coronary ischemia.

Net t-PA release over the coronary bed was inhibited by the ADP P2Y₁ receptor blocker MRS2179, (Figure 13). t-PA was simultaneously measured in the Coronary Sinus (venous) and the Femoral Artery (arterial) before, during and after 10 minutes of coronary ischemia. The difference of measured levels of arterial and venous t-PA yielded a significantly decreased release of t-PA during the greater part of the ischemic period and the early phase of ischemia reperfusion by coronary infusion of MRS2179 during the first and tenth minute of coronary ischemia, 2.5 ml of MRS2179 (10⁻³M). * = p<0.05.
reperfusion by coronary infusion of MRS2179 during the first and tenth minute of coronary ischemia, 2.5 ml of MRS2179 (10^{-3} M).

Figure 13
Net t-PA release over the coronary bed was inhibited by the ADP P2Y1 receptor blocker MRS2179: t-PA was simultaneously measured in the Coronary Sinus (Venous) and the Femoral Artery (Arterial) before, during and after 10 minutes of coronary ischemia. The difference of measured levels of arterial and venous t-PA yielded a significantly decreased release of t-PA during the greater part of the ischemic period and the early phase of reperfusion by coronary infusion of MRS2179 during the first and tenth minute of coronary ischemia. (2.5 ml of MRS2179, 10^{-3} M). n=7-8, *p<0.05.

Figure 14
An estimate of relative changes in total t-PA release: The greatest effect of MRS2179 to decrease measured levels of t-PA during the ischemia-reperfusion phases occurred at one minute after reperfusion. The same was observed regarding coronary flow in the LAD as previously reported. This figure illustrates the arterial venous difference factored with the measured flow in the LAD at one minute and 5 minutes following reperfusion. A significant decrease of total t-PA release corrected for flow changes was measured at one minute, but not at 5 minutes following reperfusion. Coronary LAD blood flow was abolished during ischemia, making it impossible to estimate total t-PA release during this period. n=7-8, *p<0.05.

The greatest effect of MRS2179 (to decrease measured levels of t-PA) during the ischemia-reperfusion phases occurred at one minute after reperfusion. The same was observed regarding peak coronary blood flow in the LAD Figure 8. A significant decrease of estimated relative total t-PA release corrected for flow changes was observed at one minute, but not at 5 minutes following reperfusion. Coronary LAD blood flow was abolished during ischemia due to the inflated balloon, making it impossible to estimate total t-PA release during this period. During infusions of NaCl, 2-MeSADP and MRS2179 there were no significant differences in blood pressure or pulse rate between the groups at the analysed time intervals, (see paper III). There was no difference in basal t-PA levels or coronary flow rates between the group treated with MRS2179 and controls. The t-PA levels and the flow rates returned to initial values at the end of the experiments in all animals. The blood-gas samples analysed of the 18 pigs in the occlusion/reperfusion group showed no statistical difference between the pigs receiving MRS2179 and the group treated as controls.
Hypothermia can reduce coronary reactive hyperemia

Coronary blood flow in the LAD increased dramatically during the early reperfusion phase. Peak flow was observed in both groups within 3 minutes following start of reperfusion (deflation of the balloon). The peak flow observed during post ischemic reactive hyperemia was significantly reduced by 43% in the 8 pigs randomized to hypothermia compared to the 8 pigs in the control group (p<0.01, Figure 15). There was no observed difference in coronary flow between the groups during baseline or after 7 minutes from reperfusion.

As expected, there was a reduction in heart rate observed among the pigs randomized to the hypothermia group during the entire period of hypothermia (Figure 16a). The difference in heart rate was maintained at the same level during baseline, ischemia and reperfusion, and unaffected by the increased coronary flow measured during reactive hyperemia. Systolic blood pressure was non-significantly reduced in the hypothermia group, but the mean arterial blood pressure was similar or even slightly increased in the reperfusion phase compared to the control group (Figure 16b).
Heart rate (HR) was measured during baseline, ischemia, and reperfusion in both the control group (n=8) and the hypothermic group (n=8). During the entire period of hypothermia HR was lower in the hypothermic group (A). Mean arterial pressure, expressed as mm/Hg, was similar and reduced in both groups during ischemia and reperfusion compared to baseline (B).

Blood gas analysis showed a significant decrease in peripheral arterial base excess (BE) at one and ten minutes following reperfusion in the control group compared to the hypothermic group, See paper IV. Samples collected in the Coronary Sinus showed a prominent reduction in BE in both groups at one minute after reperfusion, but BE did not differ significantly between the groups, See Paper IV. Other measurements of blood gas analysis did not show any significant differences in regard to O2 saturation or pH between the groups (not shown).

In separate pigs (n=4) the diameter of the coronary vessels was measured at the baseline temperature of 37°C (n=4) and at the hypothermic target temperature of 34°C (n=2). The vessel diameter was again measured during contrast injection in the LAD in both hypothermic and control pigs during early and late reperfusion. The diameter of the LAD never varied more than 10% from baseline, for each pig, and in our experiments compensation for diameter changes did not markedly influence the results.

**Rapidly induced hypothermia before reperfusion reduces myocardial infarct size.**

One pig in the pre-reperfusion hypothermia group died of intractable ventricular fibrillation shortly after initiation of ischemia. One pig in the post-reperfusion hypothermia group died of pulse-less electrical activity 30 min after initiation of ischemia. One pig in the normothermic group died of pulse-less electrical activity 15 min after initiation of ischemia. Thus, seven pigs in the pre-reperfusion hypothermia group, seven pigs in the post-reperfusion hypothermia group, and five pigs in the normothermia group were available for analysis.

**Temperature measurements**

Measurements of central temperature during the experiment are shown in figure 17. At the time of balloon inflation there was no difference in temperature between the groups. In less than 10 minutes after initiation of hypothermia, the temperature had been lowered to below 35.0°C in all animals. There was a significant difference in temperature at the time of reperfusion between the hypothermia groups (pre-reperfusion hypothermia: 34.2 ± 0.4°C; post-reperfusion hypothermia: 37.8 ± 0.2°C; p <0.001).
Central body temperature

Figure 17
Core body temperature (oesophageal) measurements in the different groups. The combination of infusion of cold saline with endovascular cooling caused a rapid reduction in core body temperature. Data are expressed as mean ± SEM.

Assessment of area at risk and infarct size
As shown in figure 18 there was no difference in AAR between the groups (pre-reperfusion hypothermia: 38 ± 2 %, post-reperfusion hypothermia: 38 ± 3 %, normothermia: 40 ± 4 %, n.s. for all comparisons).

Pre-reperfusion hypothermia caused a 40 % relative reduction in relative infarct size (IS/AAR) compared to post-reperfusion hypothermia (44 ± 5 % vs. 74 ± 3 %; p<0.001, Figure 19) and 44 % compared to normothermia (79 ± 4, p=0.003). There was no significant difference in IS/AAR between post-reperfusion hypothermia and normothermia (p=0.15).
The infarct volume as percent of left ventricular volume (uncorrected for AAR) also differed markedly: pre-reperfusion hypothermia: 17 ± 2 % (% of left ventricle) compared to post-reperfusion hypothermia 28 ± 3 % (p<0.001), and normothermia 31 ± 4% (p=0.002), (Figure 20).

Furthermore, 6 out of 7 pigs in the post-reperfusion hypothermia group and all 5 pigs in the normothermia group had hypointense zones in the infarction, typical for microvascular obstruction. None of the 7 pigs in the pre-reperfusion hypothermia group displayed microvascular obstruction (Figure 21). The difference in size of the regions of microvascular obstruction was also significant: Pre-reperfusion hypothermia, 0 % compared to post-reperfusion hypothermia (10 ± 5 %; p<0.001), pre-reperfusion hypothermia compared to normothermia: (30 ± 5 %; p=0.003), but also between post-reperfusion hypothermia and normothermia (p=0.02).
**Microvascular obstruction**

![Bar chart showing microvascular obstruction](image)

**Figure 21**

Microvascular obstruction measured by *ex vivo* MRI, expressed as a percentage of the left ventricular mass. Pre-reperfusion hypothermia totally abolished microvascular obstruction. Post-reperfusion hypothermia significantly decreased the extent of microvascular obstruction compared to normothermia. (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).

Data are expressed as mean ± SEM.

**Patchiness**

In the pre-reperfusion hypothermia group a patchy appearance of the myocardial infarctions was observed (Figure 22). In contrast, in the post-reperfusion hypothermia and normothermic groups the infarctions were more homogeneous in appearance. There was a significant difference in the previously described patchiness index between the groups pre-reperfusion hypothermia (15.7 ± 2.7 cm⁻¹) compared to post-reperfusion hypothermia (7.4 ± 0.3 cm⁻¹; p=0.02), and compared to normothermia (5.8 ± 0.5 cm⁻¹; p=0.005). There was also a significant difference in patchiness index between the post-reperfusion hypothermia and normothermia groups (p=0.048).
Figure 22
This figure illustrates the visual comparison between typical examples from the respective groups. Area at risk (AAR) measured by SPECT is shown in a bird's-eye plot of the left ventricle, left at risk (LR) from ex vivo MRI. White lines denote the slice position of the two ex vivo MRI slices in relation to each other.
Note the patchy pattern of the myocardial infarction in the MRI images from the pre-reperfusion hypothermia group. In the MRI images from the post-reperfusion hypothermia and normothermia hypothermia areas of microvascular obstruction are seen within the area of infarction.
10 Main findings

1. The P2Y₁₃ receptor on the red blood cell is stimulated by ADP, and when activated attenuates ATP release from red blood cells to the extracellular plasma.
2. Activation of the coronary endothelial P2Y₁ receptors causes hyperemia in coronary arteries.
3. Inhibition of coronary P2Y₁ receptors reduces peak coronary blood flow during reactive hyperemia by 46%.
4. Coronary t-PA release during ischemia and reperfusion is mediated through P2Y₁ receptors.
5. Mild systemic hypothermia reduces peak coronary blood flow by 43% during reactive hyperemia.
6. Rapidly induced hypothermia during anterior myocardial infarction before reperfusion significantly reduces final infarct size compared with rapidly induced hypothermia in conjunction with or after reperfusion.
7. Rapidly induced hypothermia before reperfusion in the setting of anterior myocardial infarction in pigs abolishes microvascular obstruction.
11 Discussion

The pig model
For the experiments in regard to the P2 receptors (Papers I-III) it was essential to work with a live animal model instead of using a Langendorf model because the experiments depended on the circulation of RBC's that could release ATP. The pig model was selected also because large animals could more easily facilitate the closed chest approach with insertion of large lumen catheters. The closed chest pig model allowed us to study coronary ischemia, infarction and reperfusion in a physiologically appropriate setting while still retaining control of the coronary circulation. The commonly used method of ligation of a coronary artery in an open chest model creates unnecessary trauma to the animal. Due to the similar coronary anatomy in pigs, compared to man, it is a common large animal model to use in cardiovascular research. However, earlier cardiovascular research in the 1950’s through the 1980’s commonly used the dog as the animal model. In regards to coronary research this may have serious disadvantages. The coronary anatomy in man and pigs is more similar to each other than either is to the dog and differs also to the presence of an extensive collateral network seen in dogs which is not seen in pigs and in healthy humans. A recent trial by Hedstrom et al. showed that patients with acute myocardial infarcts shows a similar infarct expansion over time compared to that of dogs in contrast to that seen in pigs. How is this possible?

Of considerable importance is that the experimentally induced infarcts in pigs and in dogs is generally performed in young animals. In contrast, patients treated for acute myocardial infarctions are generally well above middle age and are in various stages of an atherosclerotic coronary disease. Many of these patients have already developed visible collaterals at the time of catheterization and in those with no visible collaterals there may still be collaterals, but to small to be recognized. A patient may also have had several episodes of ischemia prior to the occluding infarct which may have been silent episodes or actual episodes of chest-pains which could have resulted in preconditioning.

The P2Y₁₃ receptor acts as a negative feedback loop on the surface of red blood cell’s to attenuate ATP release
Our finding of a negative feedback system for ADP release fits well with previous evidence of ATP as an extracellular transmitter, as first proposed by Burnstock, and it is possible that this negative feedback system plays an important role in the control of peripheral and coronary circulation. In underperfused organs, hypoxia stimulates an increase in intracellular cAMP, which leads to the release of ATP to the blood plasma. This increase of cAMP could be enhanced by adrenaline, that is in itself elevated during hypoxia. Extracellular ATP stimulates endothelial cells to release dilatory factors such as NO, mediating vasodilatation and improved oxygen delivery to the organ. To shut down the ATP release on the venous side (where the blood is still deoxygenated), and to avoid unnecessary ATP release, the degradation product ADP acting on P2Y₁₃ receptors could inhibit cAMP and decrease ATP-release from RBC’s. ATP and ADP are then degraded by ectonucleotidases to adenosine, which is quickly taken up in the RBC’s where it can be recycled to ATP by the glycolytic pathway.

The described negative feedback pathway may be important to avoid high extracellular plasma concentrations of ATP. At levels above 100 µmol/L, ATP concentrations may exceed the catalytic capacity of ectonucleotidases and, could in fact, stimulate ATP release by increasing permeability of the RBC’s. At high concentrations of ATP, a self sustaining process may thus be instigated, which may contribute to the irreversible stage of circulatory shock that can develop rapidly in severely ill patients. It is possible that in extreme conditions
such as circulatory or septic shock with acidosis and hypoxia, high ATP levels could be deleterious, leading to a drop in blood pressure. However, in most situations increased ATP levels ought to have positive effects by increasing tissue perfusion.

The P2Y1 receptor mediates coronary reactive hyperemia

The selective P2Y1 blocker MRS2179 was found to significantly reduce the early peak flow in coronary reactive hyperemia in pigs. This is supported by the flow increase caused by the selective P2Y1 agonist 2-MeSADP, which could be completely blocked by MRS2179. The mechanism of reactive hyperemia is still not completely understood but appears to be multifactorial in origin. Investigations of the effects of adenosine43, 132, 133, prostaglandins 134-137, K+ATP channels40, 41, 45, 138-140 and the role of NO42, 43, 139, 141-144, acting alone or in combinations with each other, have been performed. Earlier research on reactive hyperemia in the heart has been performed both in vivo in large animals, as well as in Langendorf models in rodents. However, using rodents in a Langendorf model has its inherent drawbacks due to the lack of ATP release from RBC’s in response to ischemia. In large animals, in vivo models have shown that both ATP and ADP are released during reactive hyperemia and adenosine by itself contributes to about 1/3 of the reactive hyperemia with NO contributing slightly less43, 44 and is mainly seen in the later phase of reactive hyperemia45, 147. During reactive hyperemia increased levels of ATP and ADP have been measured43. ATP is then 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 available46, 146, allowing us to test the role of P2Y1 receptors during 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 MRS2179 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 MRS2179. In contrast, UTP, which activates P2Y2/4 receptors, stimulated a flow increase that was unaffected by MRS2179, demonstrating the selectivity for P2Y1 receptors of MRS2179. To test the contribution of P2Y1 receptors to reactive hyperemia, the LAD was occluded, and MRS2179 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 have been shown to mediate a major part of early reactive hyperemia45, 146, and both are released by ADP acting on P2Y1 receptors33, 35, 39. The K+ ATP channel inhibitor glibenclamide blocks the remaining early reactive hyperemia45. Interestingly, glibenclamide is also an inhibitor of P2Y1-mediated vasodilatation24. 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, theophylline43, selective A2A antagonists45, 148, or A2A knockout mice145, 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.

In conclusion, our experiments suggest that ADP stimulating P2Y1 receptors mediates a major part of peak reactive hyperemia in the heart, and inhibition of the endothelial coronary P2Y1.
receptors could potentially attenuate the reperfusion injury incurred during primary angioplasty in the setting of acute myocardial infarction\textsuperscript{94}.

**The purinergic hypothesis of coronary reactive hyperemia**

We would like to propose that ATP is released during coronary ischemia from red blood cells; cardiomyocytes, endothelial cells and platelets, and that ATP could mediate an even earlier part of the reactive hyperemia (Figure 23). This has not been tested yet due to the lack of selective antagonists. (The presence of ATP- and UTP-responsive endothelial P2Y\textsubscript{2/4} receptors has been demonstrated before\textsuperscript{33} and was confirmed here by the hyperaemic effect of UTP.) ATP is then degraded to ADP, which mediates peak hyperemia via endothelial P2Y\textsubscript{1} receptors, followed by degradation of ADP to adenosine resulting in late-phase hyperemia mediated via A\textsubscript{2A} receptors on SMC (Figure 23).

![Hypothesis of purinergic contribution to reactive hyperemia](image)

**Figure 23**

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 P2Y\textsubscript{1} receptors on the endothelium, thus initiating the peak flow during the early phase of reactive hyperaemia, as demonstrated in the present study. ADP is then degraded to adenosine that stimulates A\textsubscript{2A} receptors and thus maintains reactive hyperemia during the mid to late phase.

In the early phase of reactive hyperemia the high rate of oxygen depletion causes an increased release of ATP. ATP is then degraded rapidly to ADP and local plasma concentrations of ADP increase. At a critical plasma level of ADP and when myocardial oxygen demand decreases in the later phase of reactive hyperemia, ADP interaction with the membrane bound P2Y\textsubscript{13} receptor on RBC’s will attenuate further ATP release through the negative feedback mechanism until equilibrium is attained (Fig 24).
Figure 24
Negative feedback system (shown in yellow) regulating plasma ATP levels via the P2Y13 receptor on the membrane of the RBC which is important in the control of plasma ATP levels and tissue circulation.

t-PA release and function
We have for the first time shown that intra-coronary administration of the ADP analogue 2-MeSADP increases release of t-PA in the coronary venous outflow via activation of endothelial coronary P2Y1 receptors. We have also for the first time shown that cardiac release of t-PA in response to cardiac ischemia can in turn be inhibited by selectively blocking the ADP receptor, P2Y1, with MRS2179.
The mechanism of t-PA release during reactive hyperemia is still not completely understood but appears to be multifactorial in origin. Earlier research on ischemic and post-ischemic release of t-PA in the heart has been performed both in vivo in large animals, as well as in ex-vivo cardiac models in rodents. In large animals, in vivo models have shown that ischemia causes the release of t-PA and that this may be caused by a number of factors but seems to be independent of blood flow. During severe coronary ischemia, as seen during and shortly following a coronary occlusion, ADP is released (or derived from ATP) from cardiac myocytes, endothelial cells, red blood cells, platelets and sympathetic nerves. The ADP released into circulation in response to ischemia can thus stimulate release of t-PA, perhaps in part through the P2Y1 receptors. Endothelial P2Y1 receptors mediate SMC relaxation through EDHF. NO and EDHF have been shown to mediate a large part of early reactive hyperemia, and both are released by ADP acting on P2Y1 receptors. Furthermore, ATP infused in the human forearm (which results in formation of ADP as well), stimulates increased blood flow via EDHF and results in a substantial release of t-PA. Because the ADP receptor P2Y1 mediates increases in blood flow during reactive hyperemia, we wanted to examine the role of P2Y1 receptors during ischemic and post-
ischemic release of t-PA. We found that the selective P2Y1 receptor agonist, the stable ADP analogue 2-MeSADP, induced a significant increased release of t-PA and as expected, a significant increase in coronary flow. However, both these findings could be completely abolished by simultaneous infusion of the selective P2Y1 receptor blocker MRS2179. To test the contribution of P2Y1 receptors to the release of t-PA during ischemia and during reactive hyperemia, the LAD was occluded, and MRS2179 was infused into the ischemic portion of the heart supplied by the LAD. This resulted in a significant reduction in t-PA release. This indicates that levels of ADP in the coronary circulation play an important role in the regulation of local t-PA release in the heart by acting on P2Y1 receptors. Several earlier reports have suggested an important role for both extracellular nucleotides and EDHF in the release mechanism for t-PA. Thus, ADP release could cause pro-fibrinolytic effects. This is however in stark contrast to the effect ADP has on platelets. ADP, in fact, activates platelets by stimulation of the P2Y1 (and P2Y12) receptors. Thus our findings indicate a dualistic action of ADP by means of a pro-fibrinolytic (by means t-PA release) and a counteracting prothrombotic (by means of platelet activation) role for ADP. The effect of MRS2179 on ischemia induced t-PA release is only partial. Other mechanisms than the P2Y1-receptor that could explain the remaining effect include other P2-receptors, such as P2Y2, P2Y6 or P2X4 which are expressed in the endothelium. Furthermore, novel mechanisms of ADP mediating relaxation in pig coronary arteries via adenosine release have recently been described and could be involved.

In conclusion, our experiments suggest that ADP stimulating P2Y1 receptors mediate a major part of the cardiac release of t-PA seen during coronary ischemia and during the subsequent reactive hyperemia in the heart. This may counter-balance the well-known platelet stimulating effects of ADP during acute myocardial ischemia. It is possible that the inhibitor of ADP induced platelet aggregation, clopidogrel, shifts this balance in favour of the fibrinolytic ADP effects described here.

Hypothermia in coronary ischemia and reperfusion

**Reactive hyperemia during hypothermia**

In general, hypothermia is thought to reduce the metabolic needs of cells and specifically perhaps by reducing the oxygen demand in the hypothermic tissues. However, little is known about the actual mechanisms by which hypothermia can reduce cell death in ischemic tissues and the reduced oxygen demand does not fully explain the positive effects of hypothermia.

Several animal studies have shown that hypothermia reduces infarct size in the setting of myocardial infarct (usually caused by the ligation of LAD). Duncker et al. found a correlation between infarct size and temperature with the smallest infarct size found at the lowest temperature. Other reports have indicated a positive temperature-related effect of hypothermia in relation to infarct size. On the contrary Maeng et al. found no benefit of hypothermia induced in conjunction with, or after reperfusion. Others have also shown in small animal models that the earlier hypothermia is applied to ischemic myocardial tissues the lesser the infarct size will be, and that this effect can be further enhanced by preconditioning. To date, hypothermia has been tested in five human trials in the setting of myocardial infarction with planned PCI as reperfusion method (COOL MI, ICE-IT, NICAMI, LOWTEMP and a study by Dixon et al.). The NICAMI and the LOWTEMP studies as well as the study by Dixon were only feasibility trials, whereas the randomized trials COOL MI and ICE-IT were designed to test if a reduction of final infarct size at 30-days by SPECT analysis could be attained through treatment with mild
hypothermia. Unfortunately, both COOL MI and ICE-IT were negative trials. At first glance this should have put an end to further cooling trials in the setting of acute MI. However, in both trials, only a minority of patients randomized to cooling reached the target temperature prior to reperfusion, and the few patients that did reach target temperature prior to reperfusion exhibited a significant or a strong reduction in myocardial infarct size at 30 days compared to control-patients, especially among patients with anterior infarcts. The result of the two trials is thus in accordance with the results found in preceding animal studies which exemplifies the importance of reaching target temperature prior to reperfusion of completely occluded vessels.

Recently a sixth human Trial, COOL MI II\textsuperscript{156}, has been started in lieu of these findings. In this trial, only patients with anterior acute myocardial infarcts will be randomized to intravascular cooling or control, and cooling will be initiated earlier than in COOL-MI I. The previous animal and human trials have all looked at the effect of using hypothermia as a method to reduce myocardial infarct size. However, the mechanism(s) by which hypothermia exerts its myocardial protective effect is still unclear but it is hypothesized that it can be explained mainly by a reduction in oxygen consumption and reduced metabolic demands\textsuperscript{111, 112, 150, 151, 157}.

Because hypothermia has been shown to limit infarct size if instituted during ischemia and prior to reperfusion\textsuperscript{104, 107, 113}, we postulated that the protective effect of hypothermia is in part mediated by a reduced reperfusion injury. It is still controversial if a reperfusion injury exists as an entity, but it is also the focus of a great deal of research\textsuperscript{83, 158}. If there is such a thing as reperfusion injury, one of several events taking place during reperfusion which could potentially cause reperfusion injury is the large increase in coronary flow that occurs during reperfusion (reactive hyperemia). The increased coronary flow may in itself be the cause of the early myocardial oedema seen during reperfusion\textsuperscript{91} or responsible for the generation of large quantities of O\textsubscript{2}-derived free radicals seen during the increased coronary flow phase of reactive hyperemia\textsuperscript{157}. We therefore wanted to examine if the phenomenon of post-ischemic reactive hyperemia could be affected by hypothermia and thus explain one possible mechanism, or effect, by which hypothermia may exert its protective effect during reperfusion. We decided to use a 10 minute occlusion time which is an established time period for studying reactive hyperemia in both animals and man.

Our results indicated a significant 43% reduction in peak coronary blood flow during reactive hyperemia in the hypothermic group compared to controls. The reason why hypothermia reduces coronary reactive hyperemia is not fully elucidated. It is probably mediated by a reduction in the release of dilatory mediators, but it could be caused by a reduced responsiveness in the endothelium and vascular smooth muscle cells. Hypothermia also seems to better maintain BE as measured in the peripheral artery (internal Iliac Artery or distal abdominal Aorta) with no significant differences seen in the coronary venous outflow in the coronary sinus. One could speculate if the difference in BE could have influenced the results of coronary flow during reactive hyperemia. However, it is well established that the vascular bed of the coronary circulation tends to dilate in response to ischemia, and it has been suggested that a decrease in extracellular pH could be involved in coronary flow regulation during hypoxia, ischemia, and increased metabolic demand\textsuperscript{159, 160}. Thus the significantly lower arterial BE observed in the control group could potentially translate into a higher coronary flow compared to the hypothermic group. However, there was no observed difference in pH, thus making it unlikely that the difference in BE levels would significantly contribute to a substantial difference in coronary flow.

The heart rate of the pigs in the hypothermic group was clearly lower compared to pigs in the normothermic group during the entire phase of hypothermia. During mild hypothermia in pigs
(and humans) a temperature dependent decline in heart rate is accompanied by an increase in stroke volume and may actually increase cardiac output according to Weisser et al. and others \cite{104,161}, while on the other hand coronary flow remains unchanged during hypothermia \cite{152}. However, although heart rate declines during hypothermia, this is an independent mechanism unrelated to the cardio-protective effects (i.e. infarct size reduction) of hypothermia \cite{162,163}. In regard to blood pressure, the hypothermic group of pigs showed no difference in mean arterial pressure even though a general decline in mean arterial pressure was observed in both groups mainly during ischemia.

In conclusion we found that hypothermia blunts the increase in coronary flow seen during reactive hyperemia in the LAD, in a closed chest porcine model. This finding may explain one of the effects by which hypothermia can reduce infarct size if attained prior to reperfusion. Further research into the infarct-reducing mechanisms of hypothermia is warranted if we are to use hypothermia in the clinical setting of myocardial infarction.

**Postconditioning in acute myocardial infarction**

Postconditioning as a means to reduce infarct size has recently been successfully performed in several animal experiments and one trial in man \cite{99-102}. There have also been some negative or ambiguous reports concerning the effects of postconditioning \cite{164,165}. Interestingly, the reduced flow often employed during the first couple of minutes (cycles of occlusion and reperfusion) during reperfusion in postconditioning experiments may cause a similar effect to that of the blunted reperfusion flow observed in the very early phase of hypothermia in our experiment.

**Rapidly induced hypothermia prior to reperfusion in acute myocardial infarction**

This study demonstrates that rapid induction of hypothermia before reperfusion reduces myocardial infarct size in pigs by 40% compared to rapidly induced hypothermia immediately after reperfusion, and by 44% compared to normothermia. Furthermore, pre-reperfusion hypothermia, and to some extent also post-reperfusion hypothermia, protects the heart from microvascular obstruction compared to normothermia.

The importance of rapid cooling before reperfusion

In the clinical setting today it is not feasible to delay reperfusion therapy in order to wait for induction of hypothermia. The combination of an infusion of cold saline solution and endovascular cooling achieved a reduction in temperature to $<35^\circ C$ in less than 10 min (Fig 17). This protocol could be clinically applicable since it would allow induction of hypothermia before or during angiography and have the patient cooled to $<35^\circ C$ without delaying reperfusion therapy. Post-reperfusion hypothermia was chosen as a control group with active treatment. A normothermic group was added in order to compare the different hypothermia protocols to normothermia.

In order to avoid a spontaneous variation in temperature during the experiment, a normal body (core) temperature in pigs ($38^\circ C$) was established before induction of ischemia. In subgroup analysis of clinical trials, a pre-reperfusion temperature of $<35^\circ C$ was sufficient to reduce infarction size by up to 40% \cite{106,115}. Based on the results, $33^\circ C$ was chosen as target temperature, and the limit for successful hypothermia was $35^\circ C$. An infusion of 1000 ml of cold saline solution caused a reduction of central body temperature to $<35^\circ C$ in all pigs in less than 10 min. The reduction in body temperature was then maintained by the cooling catheter (Figure 17). The difference in temperature between the hypothermia groups was $3.5^\circ C$.\[53\]
C (37.8° C vs. 34.2° C) at the time of reperfusion. Thus, a reduction of central body temperature of 3.6° C before reperfusion was enough to reduce infarct size by 40%. These results are in agreement with previous studies with more long-term hypothermia during ischemia in which reductions in temperature of 3-5° C had prominent effects104, 107, 109, 110, 114, 163, 166.

Infarction size evaluation

Previous studies have used histology with TTC or imaging with SPECT to visualise the extent of myocardial infarction. Ex vivo MRI has been shown to correlate closely to TTC-staining in the setting of acute myocardial infarction with reperfusion for either six hours or one day125, 167. Ex vivo MRI allowed us to achieve high resolution images of the myocardial infarction and was therefore chosen as the method for evaluation of infarct size in our study. SPECT was used to determine area at risk during transmural anterior myocardial ischemia. As shown in figure 3, the placement of the balloon in the LAD after the first diagonal branch induced ischemia in, on average 38 % of the left ventricle, with no difference in AAR between the groups.

Ex vivo MRI demonstrated a more inhomogeneous and patchy appearance of the myocardial infarctions after pre-reperfusion hypothermia. Dae et al described similar findings with scattered islands of reduced Sestamibi uptake in pig hearts when assessing infarct size with SPECT in pigs subject to endovascular hypothermia104. It is possible that preservation of small zones of viable myocardium could be beneficial in the long term preventing aneurysm formation and that cellular hypertrophy in the zones could improve contractility in the area.

Microvascular injury

It has been demonstrated (contrast echocardiography, SPECT, blush angiographic score and MRI) that up to 30 % of patients with STEMI undergoing Primary PCI as reperfusion therapy lack efficient myocardial perfusion following reperfusion168-171 and this may be attributed to microvascular obstruction seen in up 90% of patients treated with primary PCI172. The degree of microvascular injury is associated to the duration of myocardial ischemia and the extent of myocardial infarction but may possibly also be caused by reperfusion injury. Importantly, the presence of microvascular obstruction is associated with a worse clinical outcome168, 169, 171, 173. Hale et al demonstrated that hypothermia during ischemia reduced the extent of microvascular obstruction174. In our study, induction of hypothermia shortly before reperfusion abolished microvascular obstruction while it was prevalent in both the post-reperfusion and the normothermia groups (Fig 3). Interestingly, there was a significant difference in the size of microvascular obstruction between the post-reperfusion hypothermia and normothermia groups, possibly due to the rapid cooling by cold saline. The post-reperfusion cooling was so rapid that it actually cools during a part of the reperfusion period. The duration of this period is difficult to define, but the reactive hyperemia lasts for approximately ten minutes, and we reached target temperature already after five minutes. Thus, mild systemic hypothermia before reperfusion may protect the heart from microvascular obstruction. This suggests that the development of microvascular obstruction is an event that occurs very early in the reperfusion phase and possibly in the period of coronary reactive hyperemia which lends credibility to the hypothesis that the reperfusion injury may be an event caused by microvascular obstruction which may in turn be caused, in part by, the enormous inflow of blood seen during reactive hyperemia.

In conclusion, a rapid induction of hypothermia can be achieved by a combination of cold saline and endovascular cooling. Such hypothermia induced before reperfusion is effective in
reducing the myocardial infarct size and protecting the heart from microvascular obstruction. This could have significant beneficial effects on clinical outcome after myocardial infarction. This protocol could easily be applied to the clinical setting where hypothermia could be induced without delaying PCI mediated reperfusion. A rapid infusion of large volumes of saline solution in patients with myocardial infarction could have serious side-effects, such as left ventricular overload and pulmonary oedema. A currently ongoing human safety study on patients with acute myocardial infarction will determine whether cold saline as an adjunctive therapy to endovascular cooling can be safely administered (www.clinicaltrials.gov).

**Conclusions**

This thesis bridges research between preclinical and clinical sciences and has led to the conduction of a human trial. We have shown that a negative feedback loop on red blood cells terminates ATP-release which regulates microcirculation. ATP and its degradation product ADP activate endothelial P2 receptors, and from our research we now know that ADP plays a major role in attenuating the increase in blood flow seen during coronary reactive hyperemia. Additional research on reactive hyperemia, now with the use of hypothermia, led to the conclusion that mild hypothermia also attenuates the increased blood flow seen in coronary reactive hyperemia. Further research in a closed chest, porcine, infarction model, using hypothermia as adjunctive treatment resulted in a nearly halved final infarct size and an abolishment of microvascular obstruction in the animals treated with hypothermia compared to controls. This may be due to a reduction of the reperfusion injury and our research indicates that a major part of the myocardial reperfusion injury may occur during the short period of reactive hyperemia which is within the same timeframe in which the initiation of tissue swelling can be seen, an increased release of t-PA, ATP and ADP can be measured, and microvascular obstruction develops as visualized by MRI.


I vårt första arbete (I) prövades en hypotes om att det måste finnas ett återkopplingsystem på den röda blodkroppen som sörjer ytterligare frisättning av ATP i vissa situationer. I försöken på en grismodell kunde vi påvisa att ADP stimulerar en P2Y13 receptor på röda blodkroppar som minskar frisättning av ATP till plasma så länge koncentrationen av ATP:s nedbrytningsprodukt ADP är hög.

I vårt andra försök (II) kunde vi visa, också i en grismodell, att den reaktive hyperemin i hjärtats kranskärl till stor del beror på ATP:s nedbrytningsprodukt ADP och dess effekt på P2Y1 receptorer på kranskärlens endotel som stimulerar till ett ökat blodflöde. Blockering av P2Y1-receptorer nästan halverade den reaktiva hyperemin i hjärtats kranskärl i vårt försök.

I det tredje arbetet (III) kunde vi i vår grismodell visa att P2Y1-receptorer reglerar frisättningen av t-PA (en substans av stor betydelse för att förhindra att blodet levrar sig inne i blodkärlen) och att den blockad av P2Y1-receptorer kraftigt minskade frisättningen av t-PA under reaktiv hyperemi i kranskärlen.

Det är ett faktum att kylbehandling (hypotermi) kunde påverka reaktiv hyperemi i kranskärlen. Vid försöken i vår grismodell kunde vi påvisa att den grupp grisar med aktiv hypotermi-behandling (34°C) fick ett nästan halverat blodflöde i hjärtats kranskärl under reaktiv hyperemi jämfört med kontrollgruppens grisar (37°C).

I vårt sista försök (V) undersökte vi om snabbt inducerad hypotermi precis innan reperfusion i en hjärtnafarkt modell på gris kunde minska infarktstorleken jämfört med en grupp grisar som
fick snabbt inducerad hypotermi precis efter reperfusionen. I detta försök kunde vi se att infarktstorleken minskade med 40 % i de djur som behandlades med hypotermi kort innan reperfusion jämfört med kontrollgruppen.

Sammanfattningsvis har vi i vår forskning undersökt PY1-receptorers effekter på reaktiv hyperemi i kranskärl samt hur de kan reglera frisättning av t-PA och hur P2Y13 receptorer på röda blodkroppar reglerar frisättning av ATP från röda blodkroppar via ett reglersystem. Vidare har vi visat att hypotermi minskar reaktiv hyperemi i kranskärlen och att det kan vara en orsak till att kylbehandling minskar hjärtinfarktstorleken om kylbehandlingen påbörjas innan reperfusionen sker i en hjärtinfarktsmodell.
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15 REFERENCES


