Myocardial ischemia and reperfusion injury, clinical and experimental studies

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Gee Toto, I don't think we are in Kansas anymore.

From the Wizard of Oz, Frank Baum 1900

Gee Walker, we're in Gothenburg!

2010-05-17

Abstract

Acute myocardial infarction is the consequence of an occluded nutrient coronary artery. Reperfusion reduces infarct size and enhances the rate of survival. But reperfusion may also, in itself, cause reversible injury, stunning and arrhythmias, as well as irreversible lethal reperfusion injury. The aim of this thesis was to gain knowledge about the complex pathophysiology behind myocardial reperfusion injury.

Two different patient populations with AMI, treated with primary percutaneous coronary intervention were investigated. Presumptive underlying causes for reperfusion injury such as reactive oxygen species (ROS) production, neutrophil activation, signs of inflammation and myocardial cellular damage were studied. In a part of the patient population, delayed-enhanced magnetic resonance imaging (DE-MRI) was performed to estimate infarct size. An experimental porcine infarction with reperfusion was investigated, in which myocardial microdialysis samples and biopsies were analysed with proteomics, Western Blot and real-time-polymerase chain reaction. Mouse cardiomyocytes (HL-1 cells) were analysed after exposition to hypoxia. The HL-1 cells were further investigated with aspects of FKBP12 and FKBP12.6 release in hypoxia, energy depletion, acidosis, ROS activation and re-establishing of physiologic conditions, simulating ischemia and reperfusion at varying durations.

Markers for inflammation increased over time, whereas the markers for ROS production and neutrophil activation were at the maximum level at baseline and during the first day. Biomarkers, showing myocardial injury, were useful for infarct size estimation compared with DE-MRI when obtained correctly. FKBP12 and FKBP12.6, increased during ischemia/hypoxia in both the experimental models. Viability of HL-1 cells matched severity of duration and intensity of ischemia. FKBP12 and FKBP12.6 increased during simulated ischemia, while the mRNA expression was depressed suggesting dissociation from receptors regulating intracellular calcium flows. Clinical symptoms and signs of reperfusion injury may partly be explained by release of FKBP12 and FKBP12.6, this causing disturbance of the intracellular cell contraction. These findings may indicate further important mechanisms in ischemia/hypoxia and reperfusion in the heart.

Keywords: myocardium, ischemia, reperfusion injury, FKBP12, FKBP12.6

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List of original papers

This thesis is based on the following papers which will be referred to in the text by their Roman numerals (I to V).

I. K Åström Olsson, J Harnek, AK Öhlin, N Pavlidis, B Thorvinger, H Öhlin. No increase of P-malondialdehyde after primary coronary angioplasty for acute myocardial infarction.
Scand Cardiovasc J 2002; 36(4):237-240.

II. K Åström-Olsson, E Hedström, L Mattsson Hultén, O Wiklund, H Arheden, AK Öhlin, A Gottsäter, H Öhlin. Dissociation of the inflammatory reaction following PCI for acute myocardial infarction.
J Inv Cardiol 2007;19:452-456.

III. E Hedström, K Åström-Olsson, H Öhlin, F Frogner, M Carlsson, T Billgren, S Jovinge, P Cain, G.S Wagner, H Arheden. Peak CKMB and cTnT accurately estimates myocardial infarct size after reperfusion.
Scand Cardiovasc J 2007;41:44-50.

IV. K Åström-Olsson, L Karlsson, L Mattsson Hultén, P Davidsson, V Mantovani, C Månsson, SO Olofsson, O Wiklund, L Grip. Myocardial release of FKBP12 and increased production of FKBP12.6 in ischemia and reperfusion, experimental models. *Biochem & Biophysical Res Comm 2009;390:1299-1304.*

V. K Åström-Olsson, L Li, L Akyürek, J Borén, A Gottsäter, H Öhlin, L Grip. Studies of HL-1 mouse cardiomyocytes regarding viability and release of FKBP12 and FKBP12.6 after hypoxia, energy depletion, acidosis, and ROS activation with or without subsequent reestablishment of physiologic conditions.
Manuscript

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Abbreviations

AMI	= acute myocardial infarction
PCI	= percutaneous coronary intervention
hs-CRP	= high-sensitivity C-reactive protein
CK	= creatine kinase
CK-MB	= creatine kinase monobasic fraction
cTnT	= cardiac troponin T
MDA	= malondialdehyde
Iso-P	= 8-Isoprostane-prostaglandin $F_{2\alpha}$
IL-6	= interleukin 6
IL-8	= interleukin 8
TNFα	= tumour necrosis factor α
NGAL	= neutrophil gelatinase-associated lipocalin
MPO	= myeloperoxidase
MMP-9	= matrix metalloproteinase-9
ROS	= reactive oxygen species
TIMI	= thrombolysis in myocardial infarction
GPIIb/IIIa	= glycoprotein receptor IIb/IIIa
ELISA	= enzyme-linked immunosorbent assay
LAD	= left anterior descending artery
SOD	= superoxide dismutase
ECG	= electrocardiogram
ASA	= acetyl salicylic acid
MPTP	= mitochondrial permeability transition pore
RyR	= ryanodine receptor
FKBP12	= FK binding protein 12
FKBP12.6	= FK binding protein 12.6
SR	= sarcoplasmic reticulum
ER	= endoplasmic reticulum
HL-1 cells	= cardiomyocytes (mice), derived from atrial tumor cells (AT-1)
WB	= Western blot
RT-PCR	= real time-polymerase chain reaction
SERCA	= sarco-endoplasmic reticulum calcium ATPase
IP_3R	= inositol 1,4,5-triphosphate receptor
Da	= dalton
DMTU	= dimethylthiourea
EC	= excitation-contraction
STEMI	= ST-elevation myocardial infarction
RISK	= reperfusion injury salvage kinase
GAPDH	= glyceraldehyde-3-phosphate dehydrogenase

Introduction

Ischemic heart disease

Cardiovascular disease with all its appearances and complications is responsible for approximately 50% of all deaths in the world today [WHO 2008]. Acute myocardial infarction (AMI) is the most common cause of death. Today in Sweden the single cause of death for men is shown to be 16% and for women 11% [Swedeheart 2008]. The history of cardiovascular disease is well-known; already in 1785 William Heberdeen described the symptoms and signs of angina pectoris. From 1800 onwards, this description of chest pain indicated more severe symptoms as well as deaths, so-called heart attacks. The development and establishment of diagnostic tools for verifying ischemic heart disease greatly intensified during the 1900s. A paper concerning the new electrocardiogram (ECG) diagnostic tool was published in 1903 by the 1924 Noble Prize recipient Willem Einthoven. This method, using electrical currents from heart activity, was originally discovered by Augustus D. Waller from London, who showed that the heart's rhythmical electrical stimuli could be monitored directly from a person's skin (published in the Journal of Physiology (London) 1887). As early as 1917, James Herrick reported a case of an AMI diagnosed with ECG and almost ten years later, in 1926, a fully portable ECG instrument was available in the market. The ECG method was then further developed and elaborately refined by several famous names such as Thomas Lewis, Emanuel Goldberger and, later, by Frank Wilson during the 1930s [Fye 1994]. As complimentary tools for diagnosing myocardial infarction, biomarkers indicating myocardial injury in the peripheral blood became available during the 1950s.

The first cardiac catheterisation was performed in 1929 by Werner Forssmann from Eberswalde, later Berlin, Germany, when he inserted a catheter through his antecubital vein into the right atrium of the heart. For this method, Dr Forssmann was awarded the Nobel Prize in 1956. This is considered to be the start of the era of cardiac and subsequently coronary catheterisations. During the 1950s, angiography of the coronary vessels was further evolved at the Cleveland Clinic, Cleveland, Ohio, USA. An important milestone for the usage and development of this method was the arterial puncture

technique, which was introduced in 1953 by Sven-Ivar Seldinger at the Karolinska Hospital, Stockholm, Sweden [Braunwald 1992].

The thrombus as a cause of a thrombotic event at a vulnerable plaque rupture resulting in an occluded vessel was not fully accepted as the rationale for infarction until the 1980s. Suspicion of the occluded vessel was dominant but smooth muscular spasm around the artery was also discussed, as well as an accumulation of catecholamines causing local metabolic disturbances leading to AMI. The argument behind these other mechanisms was that in autopsy material of patients who had died of sudden death, only 10% of these patients had presented with visible coronary occlusion. DeWood and his team observed and followed the time course of the total occlusive thrombus during an AMI with coronary angiography, and published their study in 1980 in New England Journal of Medicine [DeWood 1980]. The year prior to this, Rentrop and his team had demonstrated a rapid recanalisation of an occluded coronary vessel after intracoronary administration of streptokinase directly into the infarct-related artery [Rentrop 1985]. Throughout this time, other sources for infarctions were discussed and finally Erling Falk demonstrated the vulnerable atherosclerotic plaque with a thrombus attached to the intimal part of the vessel, obstructing the lumen and thereby causing an interrupted nutrient blood flow to the distal myocardium. The vessel occlusion with the thrombus was thoroughly illustrated by findings at autopsy cases of patients who had died during AMI [Falk 1983].

Streptokinase was introduced as a thrombolytic therapy as early as 1958, but at that time the therapy was challenged with severe and even fatal bleeding complications, these appearing due to extreme dosing and leading to a reluctance to propagate the treatment. The large randomized trials, GISSI-2 1990 and ASSET 1990, during the 1980s were then introducing lower doses of thrombolytics, and newer agents as recombinant tissue type plasminogen activator (r-TPA) together with and without addition of heparin [GISSI-2 1990, Wilcox 1990]. A breakthrough in acute coronary care emerged however after the ISIS-2 already in 1988 where 17187 patients underwent thrombolytic treatment with streptokinase and/or oral ASA [ISIS-2 1988], and the stunning outcome was that the effect of ASA decreased the mortality of AMI. The old-fashioned treatment of AMI prior to 1980 was to administrate analgesics, sedatives and oxygen. After ISIS-2 was published

in 1988, the role of the antiplatelet regimen was fully established and generally accepted [ISIS-2 1988].

Over the last few decades interest has been focused directly on coagulation cascades for diminishing the risk of thrombus as a source of myocardial events. Interest has focused on agents working as inhibitors of factor II (direct thrombin inhibitors such as bivalent hirudins and bivalirudin, or univalent such as melagatran, argatroban or dabigatran), and agents working as inhibitors of factor Xa (such as the low molecular heparins, or fondaparinux) by preventing clot formation [Moser 2009]. Another platelet-aggregating inhibiting agent was introduced during the 1990s: the thienopyridines. The working mechanism is to block the P2Y12 units of the adenosine diphosphate (ADP)-receptors on the platelet's surface and thereby make it impossible for the platelets to aggregate and form thrombi. The first generation of the thienopyridines showed effectiveness but was associated with severe side effects such as agranulocytosis and also related with liver disorders. The second generation of thienopyridines (clopidogrel) was introduced in the mid-1990s and released worldwide in 1997 for commercial use. At that time the severe side effects were overcome, and this new treatment has established a role in the acute coronary syndrome together with ASA [Quinn 1999, CURE study investigators 2000]. A third generation of these agents are entering the cardiology world at present.

There are also other ways of blocking clot formations, by agents directly blocking the surface glyco-protein receptors IIb/IIIa (GPIIb/IIIa) on activated platelets making it impossible for these platelets to aggregate by binding fibrinogen between the activated GP ligands and thereby inhibiting formation of thrombi [EPIC 1994, Tcheng 2003].

A different strategy for the treatment of AMI has been direct angioplasty with stenting of the coronary arteries: primary percutaneous coronary intervention (pPCI). Clinical trials with acute angioplasty as a treatment for AMI were performed during the 1990s and 2000s, with the beneficial outcome of hitherto the least extension of the myocardial infarct. This method has thereby become a tool that has diminished the expansion of heart failure due to ischemic injury. In the beginning (the mid-1980s), stents were only used when bail-out procedures were needed, during situations when the risk of reocclusion of the coronary artery vessel after deflating the angioplasty balloons was immediately threatening. During the end of the 1990s, metallic stents were structurally improved and

simultaneously enhanced anti-thrombotic (antiplatelet) and anti-coagulant drugs were used. Due to this progress the treatment of infarctions has become safer, and is performed in a way that should guarantee the restoration of coronary artery blood flow and thereby assure the nutrition to the myocardium and the transportation of toxic degradation products away from the injured and infarcted area. Several studies during the 1990s showed that the mechanic resolution of the thrombus and dilation of underlying plaques with pPCI challenged the medical reperfusion therapies and confirmed a better outcome and lowered mortality for AMI [Stone 1998, Widimský 2000, Andersen 2003].

Myocardial reperfusion injury

If almost complete reperfusion is attained, one would assume that by re-establishing the coronary blood flow, and thus saving the myocardium at risk, no further injury would occur. Notwithstanding, a number of patients experience an acute clinical deterioration with severe symptoms such as acute myocardial failure occurring as pulmonary edema, cardiogenic shock, and frequently occurring ventricular arrhythmias, even fatal arrhythmias. These observed facts have been referred to as reperfusion injury, and this concept was called "reperfusion, the double–edged sword" by Eugene Braunwald [Braunwald 1985]. The reperfusion injury is often divided into two different categories: the reversible and the irreversible injury.

Reversible injury includes stunning which is defined as reversible dysfunction of the myocardium, systolic or diastolic, after an episode of ischemia and reperfusion, and a range of arrhythmias. Irreversible reperfusion injury is defined as a reperfusion-induced cell death of myocytes that were still viable at the time of blood flow restoration [Yellon 2007]. The two types (reversible and irreversible) of reperfusion injury are described below.

Reversible reperfusion injury

The most common and very obvious clinical symptom of reversible reperfusion injury is acute heart failure, elicited by reperfusion. This is a situation which is important clinically but not as crucial as irreversible injury, as stunned myocardium will eventually recover to almost normal function compared to that of non-ischemic myocardium from

within a few hours to a few weeks, depending on the duration of ischemia and species affected [Bolli 1998, Kloner 2001]. The historic definition of stunned myocardium has been well established since 1975 and is composed of four items [Heyndrickx 1975]. Stunning or so-called post-ischemic left ventricular dysfunction is shown with the following objective findings:

1. Normal myocardial perfusion
2. Preserved contractile reserve
3. Enzyme release
4. Delayed but full recovery of function

These findings are uncontroversial except for the enzyme release, which has been considered to be a sign of cell death. Heyndrickx suggests that a rise in plasma creatine kinase (CK) as well as creatine kinase monobasic fraction (CKMB) indicates that severe ischemia reversibly alters the cell membrane permeability sufficiently to lose all cell components with a molecular weight up to 80 000 Dalton (Da). Thus, enzyme release is not only a sign of cell necrosis [Heyndrickx in Kloner 1993].

Bolli describes stunned myocardium in both experimental and clinical settings, and his definition of stunned myocardium is:

"A stunned myocardium is a mechanical dysfunction that persists after reperfusion despite the absence of irreversible damage and despite restoration of normal or near-normal coronary flow". The two essential points of this definition are [Bolli 1998]:

1. A post-ischemic dysfunction, no matter how severe or prolonged, is a fully reversible abnormality.
2. The dysfunction is not caused by a primary deficit of perfusion.

The classic model of myocardial stunning is the canine experiment with a coronary occlusion lasting less than 20 minutes in an open-chest model [Heyndrickx 1975]. The short ischemic time did not result in any myocardial necrosis but showed a delayed functional recovery of the reperfused myocardium. Similar results have been obtained with closed-chest canine models and from experiments in other species [Bolli 1998]. Myocardial stunning has also been shown in canine ischemic hearts that were exposed to more than 20 minutes and less than three hours of coronary occlusion, resulting in

infarcted areas with surrounding viable myocardial tissue with a delayed recovery of function.

The historical evidence for the concept of stunned myocardium post-thrombolytic treatment was established by Satler [Satler 1986]. Patients receiving streptokinase as thrombolytic treatment were evaluated for their response to inotropic stimulation (a brief infusion of isoprotenerol) after successful reperfusion. The patients that were considered to be successfully reperfused had an improvement in the regional wall motion and ejection fraction measured with radionuclide angiography, multi gated acquisition scan (MUGA), compared with those patients in whom thrombolysis was unsuccessful [Satler 1986]. Several clinical studies of patients receiving thrombolytic treatment have demonstrated that the initial decreased motility of the left ventricular wall may partly recover within a few weeks [Kloner 1993, Ito 1993]. The time course of stunning in patients with reperfused AMI has been shown with contrast echocardiography in a study by Ito [Ito 1993]. This study showed that the recovery time for the restoration of the ventricular function was about fourteen days. Ito studied patients with anterior transmural infarctions treated with thrombolytic therapy, and evaluated them with coronary angiography at day 1 and at day 28 with repeated echocardiographical examinations [Ito 1993].

Acute systolic myocardial dysfunction-symptoms and signs

Stunning presents itself clinically as acute heart failure. The scenario is a patient with hypotension of various degrees, often with severe dyspnoea, sometimes even progressing to a complete pulmonary edema. Heart failure occasionally progresses to the serious situation of a cardiogenic shock, with signs of peripheral hypoperfusion, multiple organ failure and sometimes cerebral confusion. Even in these severe cases, patients may survive with the assistance of mechanical and medical treatment and the adjacent parts of infarcted myocardium will eventually recover.

Diastolic dysfunction-symptoms and signs

Stunning may manifest itself as a diastolic dysfunction. Impaired myocardial relaxation is observed in the affected myocardial area soon after the supporting coronary artery is

occluded. Myocardial relaxation is the first to be affected by the ischemia as the diastolic dysfunction is the most energy-demanding process of the cardiac cycle [Bolli 1998]. Impaired relaxation and increased filling pressure may lead to heart failure even in situations when the systolic function is normal.

Hibernation- symptoms and signs

It is a matter of controversy whether hibernation represents a form of reperfusion injury. Hibernation is defined as a chronic reduction of the cardiac muscle function due to decreased blood flow. This decreased blood flow is usually caused by a tight coronary artery stenosis. This situation is not attributable to an acute occlusion caused by a thrombus obliterating the nutritious vessel. The hibernating myocardium has the potential to recover when the nutrient blood supply is restored. Symptoms and signs of hibernation are those of heart failure [Braunwald 1992].

Arrhythmias, symptoms and signs

The appearance of arrhythmias is another part of the reversible symptoms, although even sustained arrhythmias might lead to a fatal outcome. The most common arrhythmias in the reperfusion period are idioventricular tachycardia occasionally, even rarely, leading to a fatal ventricular fibrillation, or the appearance of a nodal tachycardia, or a bradycardia that might sometimes lead to an asystolia. If these arrhythmias are discovered and treated quickly, they will not interfere with the long-time outcome of the myocardial infarction [Bolli 1998].

Reversible reperfusion injury - other presentation

The no-reflow phenomenon -symptoms and signs

The no-reflow phenomenon is also depicted as a manifestation of reperfusion injury. This phenomenon has been characterized as forms of both irreversible and reversible reperfusion injury. Despite verified opening of the occluded epicardial coronary vessel, the distal flow to the myocardium is severely impaired, resulting in a total loss of microcirculation in some areas. The coronary artery blood flow goes to zero (thrombolysis in myocardial infarction-0, TIMI-0) from full normal flow within a few

seconds. This frequently occurs during large AMIs. The no-reflow phenomenon is associated with reduced left ventricular ejection fraction, left ventricular remodeling and poor clinical outcomes with a poor clinical prognosis [Ito 2006].

Stunning mechanisms

The physiological aspects of reversible reperfusion injury

Acute systolic myocardial dysfunction mechanism

Acute systolic dysfunction refers to an acutely impaired ventricular contraction. The loss of cardiac inotropy (decreased contractility) causes a downward shift in the Frank-Starling curve. An effect of this shift results in a reduced stroke volume and a compensatory rise in preload. Preload is often measured as ventricular end-diastolic pressure or pulmonary capillary wedge pressure [Braunwald 1992].

Diastolic dysfunction mechanisms

The mechanism behind the diastolic dysfunction is explained as a dysfunctional myocyte relaxation [Jennings 1990]. The relaxation of the myocyte consists of this following mechanism: Cytosolic calcium ions (Ca^{2+}) is pumped into the sarcoplasmic reticulum (SR) or out of the cell, this results in an increase of the extracellular level of $Ca^{2+}$and can thus be measured in peripheral blood. This active pump mechanism (SERCA) uses adenosine triphosphate (ATP) for its energy demands. If a coronary occlusion occurs and by this the nutrition supply to the distal myocardium is barred, this energy source is supported via the anaerobic glycolysis within seconds. This energy source is unfortunately short-lived and exhaustion occurs already after 30 seconds. This leads to the ATP production from creatine phosphate decreasing and ATP consumption increasing, which provides a dysfunctional myocyte relaxation. Decreased compliance of the left ventricle results in increased filling pressures and pulmonary capillary pressure. In the acute ischemic setting, a pulmonary edema may develop as a result of diastolic dysfunction even in patients with normal systolic function [Jennings 1990].

Arrhythmia mechanisms

Mechanisms for arrhythmias appearing in the reperfusion period have not yet been fully clarified. One proposed hypothesis is based on the inequality of the Ca^{2+} ions inside the cardiomyocyte [Wehrens 2005]. An overload of Ca^{2+} inside the cytosol would bring the cardiomyocyte to a hypercontracture state, which may be a reasonable explanation for the stunned myocardium as well as the origin of arrhythmias due to the ion imbalance over the cell membranes, thus causing electrical disturbances and thereby creating re-entry foci responsible for new electrical circuits in the injured myocardium. The calcium homeostasis theory offers a reasonable mechanical explanation for the appearance of arrhythmias in the acute reperfusion phase. Further speculations and theories are presented below in the section concerning biochemical aspects.

Hibernation mechanisms

One hypothesis regarding the pathogenesis of hibernation is that the myocardium is chronically adapted to a low-resting blood flow. However, in experimental animal models of hibernation, low-resting blood flow is not an obligatory prerequisite for the development of hibernation. Consequently, an alternative explanation of hibernation has been developed suggesting that it may be caused by repetitive stunning and if this hypothesis is valid, hibernation must be regarded as a reperfusion injury.

No-reflow phenomenon mechanisms

The progressive microvascular function may be caused by destruction or obstruction of capillaries, or by introduction of microemboli to the arterioles. Microemboli may arise from cholesterol-rich plaques which may induce arteriole spasm, leading to congestion, thrombosis and sluggish flow. This microemboli-no-reflow situation is more common during AMI, at ad hoc PCI performed in unstable angina situations and PCI in diseased saphenous vein grafts in coronary artery bypass graft (CABG) patients. This form of no-reflow is usually transient and may correspond to the reversible form. The irreversible form is caused by a more permanent capillary obstruction by the plugging of neutrophil leukocytes causing inflammation processes, myocyte swelling and interstitial edematous

surroundings [Ito 2006]. Neutrophils are shown infiltrating and migrating into the interstitial area of the myocardium already during the ischemic event and early in the reperfusion period [Go 1988]. These components are considered to be one of the important factors of irreversible reperfusion injury and they do not necessarily participate in reversible myocardial injury [Vinten-Johansen 2004]. Another possible mechanism may be microvascular damage as a direct consequence of ischemic injury. Sources of this symptom and speculations about this occurrence are presented below in the section covering the biochemical aspects of reversible reperfusion injury.

The biochemical aspects of reversible reperfusion injury

Reactive oxygen species (ROS)

A reactive oxygen species (ROS) is any oxygen derived molecule that contains an unpaired electron. Their half-life is between 10^{-6}-10^{-9} s and thus ROS are difficult to detect and quantify in vivo [Jeroudi 1994]. It is well known that molecular oxygen (O_2) has sixteen electrons, of which two are unpaired. These unpaired electrons are highly prone to react, and toxic substances are formed during the metabolism where oxygen is used as a substrate. Knowledge of the reactive forms of oxygen has shown that the majority of the super-radicals formed in vivo are removed by endogenous superoxiddismutase (SOD). This enzyme is present in all organisms that use oxygen as a source of energy. SOD removes the radical during the production of hydrogen peroxide H_2O_2 and oxygen in a so-called dismutation reaction:

$2\ O_2^- + 2\ H^+ \Rightarrow H_2O_2 + O_2$

There are both organic and inorganic molecules that can act as ROS. Superoxide O_2^- can be produced enzymatically or as a result of a leakage of electrons to O_2 from the cell electron transport chain (ETC). Due to the short half-lives of ROS and extremely low measurable levels in peripheral fluids, ROS detection methods have been confined to measuring indirect end products in other chain reactions such as lipid peroxidation, where aldehydes such as malondialdehyde (MDA) have been used as a marker for ROS production [Öhlin 1988].

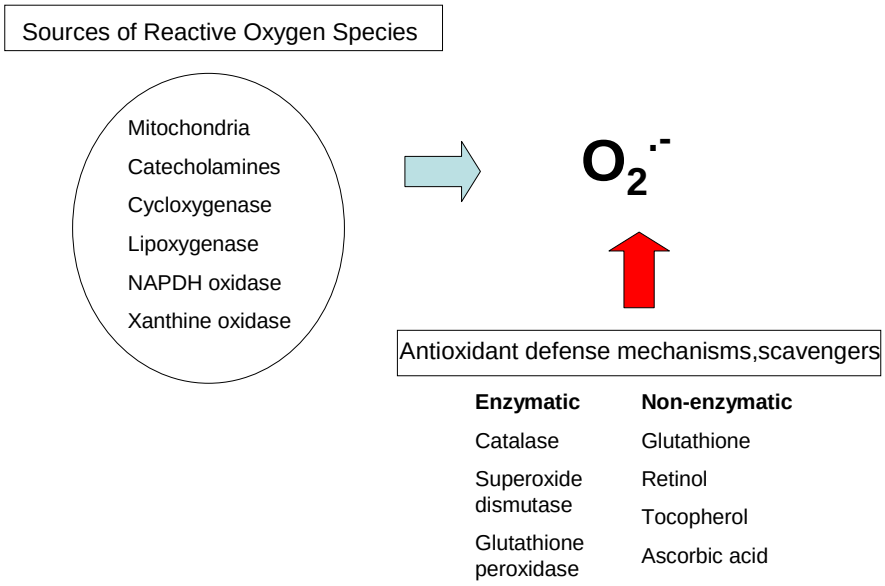

Figure 1. Different sources of reactive oxygen species.

ROS are created and found in the cytosol of cardiomyocytes, in endothelial cells, in leukocytes and mitochondria [Zweier 2006]. The vascular endothelial cells contain the enzyme xanthine oxidase that catalyses the oxidation of hypoxanthine together with H_2O and oxygen to xanthine and H_2O_2. The xanthine oxidase also catalyses the next step, which is the oxidation of xanthine together with H_2O and oxygen to uric acid and H_2O_2. Xanthine oxidase activity is inhibited by the scavenger allopurinol, and thereby removes the radicals that arise during the production of H_2O_2.

Lipid peroxidation

This term actually means rancidness of lipids (elements present in all cell membranes). A peroxidation sequence is initiated by the removal of a hydrogen atom (H) from a methylene group of a poly unsaturated fatty acid (PUFAH) by a free radical (e.g. the hydroxyl radical OH^-). This reaction leaves behind an unpaired electron on the carbon remains. Free unpaired and highly reactive electrons react with O_2, to form a peroxy

radical. This peroxy radical is capable of abstracting a hydrogen atom from another fatty acid, and by this starting a chain reaction of peroxidation. The first step, where the fatty acid (an unsaturated lipid) reacts with the highly reactive hydroxyl ion (OH^-), is known as initiation, forming a lipid radical which after contact with oxygen propagates and finally ends up in lipid peroxide. This is a chain reaction which results in cell membrane injuries and eventually the production of end products such as aldehydes (one is MDA), which may then be measured in peripheral blood as an indirect marker of ROS production [Öhlin 1988, Öhlin 1995].

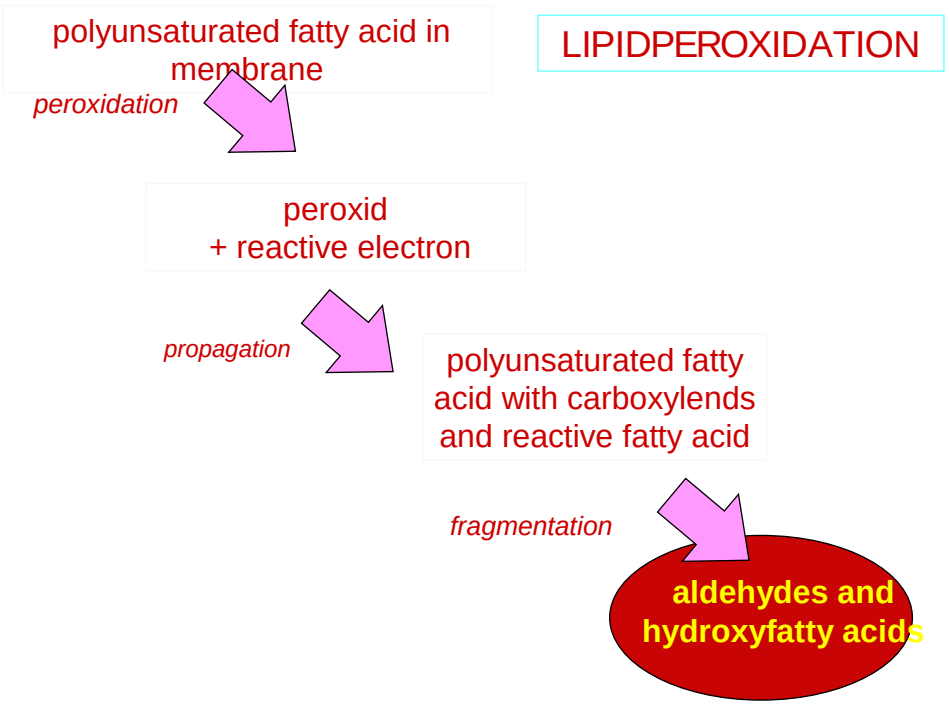

Figure 2. Modified by the author after Öhlin 1988. The lipid peroxidation cascade chain.

The hypothesis concerning ROS as a source of reversible reperfusion injury is supported in several animal studies. According to the hypothesis, ROS are produced during ischemia and reperfusion and cause disturbances in cellular function by direct toxicity or initiating cell membrane damages [Bolli 1998]. Bolli describes mongrel dogs undergoing an open-chest model of induced myocardial ischemia, where the left anterior descending coronary artery (LAD) was ligated for 15 minutes and then reperfused for four hours with the administration of an effective and highly permeable free radical scavenger,

dimethylthiourea (DMTU), in one group, and another group without DMTU serving as a control. The DMTU group showed a significant and sustained improvement in recovery in contractile function compared with the control group. The authors concluded that the myocardial dysfunction occurring after a brief episode of regional ischemia is mediated in part by the hydroxyl radical (acting as a ROS).

The production of ROS during reperfusion has been confirmed with spin trap alpha-phenyl *N-tert*-butyl nitrone (PBN) and electron paramagnetic resonance (EPR) spectroscopy. This was used by Bolli and his group in another open-chest ischemic model on dogs with a 15 minute occlusion of a coronary artery. They showed a linear positive relation between the extent of ROS production and the magnitude of ischemic flow reduction [Bolli 1987, 1991]. The conclusion of this study was that the greater the degree of hypoperfusion, the greater the succeeding production of free radicals and, by assumption, the severity of reperfusion injury.

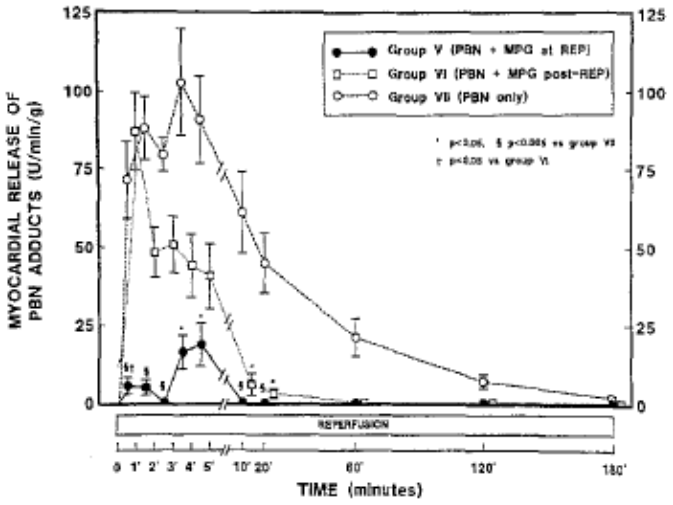

Figure 3. The burst of free radicals and scavengers detected by spin trap EPR in a canine experiment from Bolli 1998. Reprinted from Progr in Cardiovasc Dis 1998;40:477-517. Bolli R. Basic and clinical aspects of myocardial stunning with permission from Elsevier.

To verify the ROS hypothesis, further studies in dogs were performed with different ROS scavengers such as superoxide dismutase (SOD), desferrioxamine (an iron-chelator), mercaptopropionyl glycine (MPG) and catalase. These scavengers showed an enhanced

recovery of function after reperfusion by suppressing ROS production. Studies were also performed on pigs and rabbits with similar positive results regarding attenuation of post-ischemic stunning [Bolli 1998]. Other studies in dogs, with other scavengers such as oxypurinol (a xanthine oxidase inhibitor) and *N*-acetylcysteine, showed attenuation of myocardial stunning after 90 minutes of occlusion and 24 hours of reperfusion. Similar results were shown in pigs with 45 minutes of coronary occlusion and 72 hours of reperfusion [Bolli 1998]. Näslund et al showed in a porcine closed-chest experimental model that infarct size could be limited by administration of SOD as an adjunct to reperfusion, but this effect was limited due to a narrow time window [Näslund 1990].

Every malfunction described of the stunned myocardium may possibly be caused by ROS. Cellular components such as proteins and lipids are presumably targets of free-radical initiated reactions leading to protein denaturation and enzyme inactivation, or peroxidation of polyunsaturated fatty acids contained in cellular membranes (lipid peroxidation). This responds well to the picture of disturbed cell integrity and dysfunction such as impaired ion homeostasis due to leakages and depletion of essential energy sources.

Calcium ions and reversible reperfusion injury (Ca^{2+} overload theory)

For contraction to occur, cardiac muscle cells require both extracellular calcium and sodium ions. Like skeletal muscle, the initiation and upshot of the action potential in cardiac muscle cells is derived from the entry of sodium ions (Na^{+}) across the sarcolemma in a positive feedback loop. However, an inward flux of extracellular Ca^{2+} ions through L-type calcium channels (LTCC) (also known as dihydropyridine receptors, DHP) sustains the depolarization of cardiac muscle cells for a longer duration. Calcium-induced calcium release from the sarcoplasmic reticulum (SR) occurs under normal excitation-contraction (EC) coupling. Once the intracellular concentration of Ca^{2+} increases, Ca^{2+} ions bind to the protein troponin, which initiates contraction by allowing the contractile proteins, myosin and actin, to associate through cross-bridge formation. Ca^{2+} ions are stored inside the SR. Their outflow is regulated via the cardiac ryanodine receptor type 2, (RyR2) and this function is dependent on the cytosolic-located stabilizing

proteins FKBP12 and FKBP12.6 and their connection to RyR2. This complex allows the ion channels to function correctly [Olson 2004, Wehrens 2005].

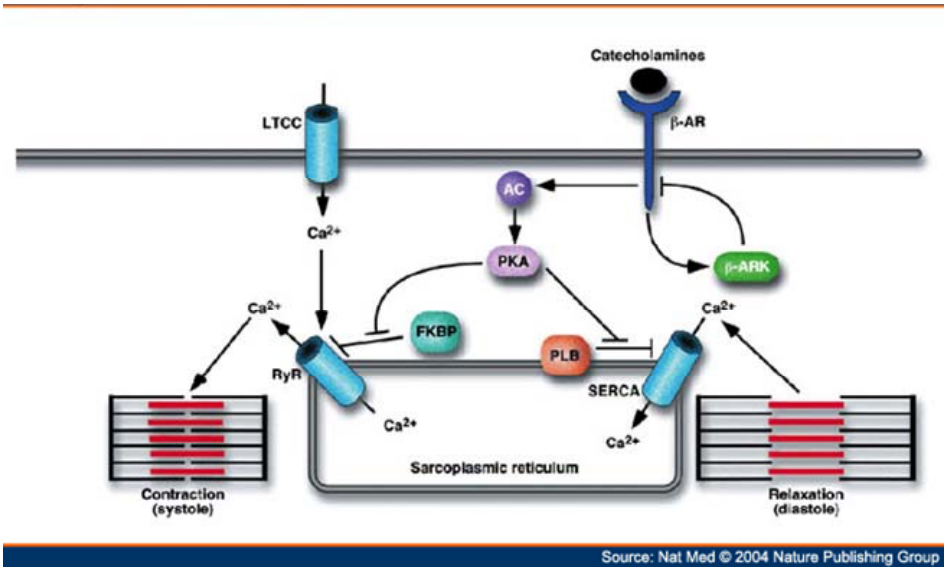

Figure 4. The intracellular calcium regulation in connection to muscular contraction. Reprinted with permission of Nature Medicine from Olson E. A decade of discoveries in cardiac biology. Nature Med 2004;10:467-474.

FK binding protein12 and 12.6 and cardiomyocyte calcium regulation

FKBP12 and its homologue FKBP12.6 are cis-trans peptidyl-prolyl isomerases, with a molecular weight of approximately 12 kDa. These proteins are known to be associated with RyR2. One RyR2 complex consists of four large subunits, each with a molecular weight of 565 kDa, and each subunit of RyR2 binds one FKBP12 alternatively FKBP12.6 unit among several other proteins. The function of FKBP12 and FKBP12.6 has been established to be a stabilizing agent of the interaction within the subunits of the RyR2, and its main assignment is to regulate the calcium gating function [Yano 2005]. FKBP12 and FKBP12.6 similarly interacts with another receptor regulating intracellular ion exchange, the inositol 1,4,5-trisphophate receptor (IP_3R), located on the cytosolic side of the endoplasmic reticulum (ER). By binding and release from the RyR and IP_3R, respectively, these protein receptor complexes govern the release of Ca^{2+} from the SR and ER [Bultynck 2001, Carmody 2001]. A dissociation of FKBP12 and FKBP12.6 from

the cardiac forms of the receptors, the RyR2 and IP_3R2, respectively, may lead to leakage of Ca^{2+} ions from the reservoir into the cytosol, thereby contributing to a hypercontractile state and electrical instability in the cardiomyocyte [Bultynck 2001, Carmody 2001, Yano 2005].

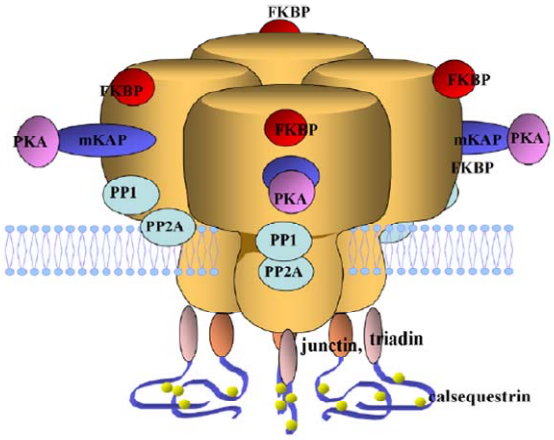

Figure 5. The ryanodine receptor. Reprinted from Pharm&Ther 2005;107:377-391. Yano et al, Abnormal ryanodine receptor function in heart failure, with permission from Elsevier.

FKBP12 is a predominantly cytosolic protein. The two known isoforms, FKBP12 and FKBP12.6, consist of 85% of the same peptide sequences. The ratio of FKBP12 to FKBP12.6 in mammalian ventricular cardiomyocytes is in the order of 10:1 in the cytosol. This higher concentration of FKBP12 in the cytosol and higher affinity for FKBP12.6 to the RyR2 may indicate that the two isoforms exert different intracellular actions, which has also been shown to be species dependent [Jeyakumar 2001, Seidler 2007].

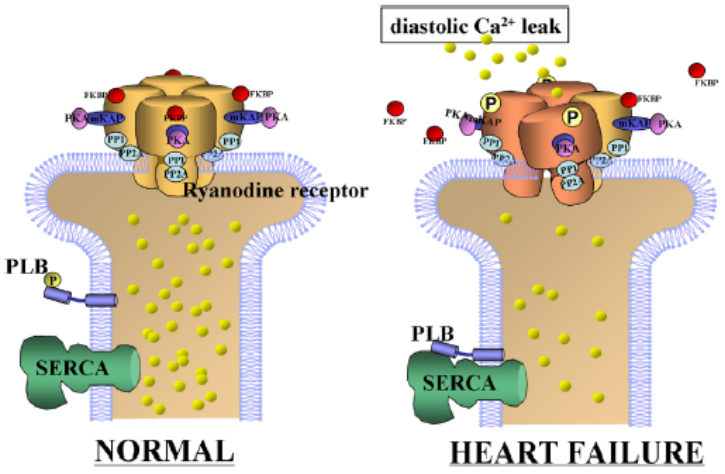

Figure 6. The malfunction of the ryanodine receptor at heart failure resulting in leakage of calcium ions out of the SR to the cytosol. Reprinted from Pharm&Ther 2005;107:377-391. Yano et al, Abnormal ryanodine receptor function in heart failure, with permission from Elsevier.

The majority of studies concerning FKBP12 and FKBP12.6 and their association with RyR2 have been performed on dogs, where the isoform FKBP12.6 is dominant and FKBP12 is hardly detectable [Timerman 1996, Lehnart 2004, 2006, 2007, Wehrens 2005, Zalk 2007]. However, in dogs the affinity of FKBP12 for RyR2 is 500 times lower than for FKBP12.6, hence binding is negligible under physiological conditions. In other species (including rabbit and human), the affinity of FKBP12 for RyR2 is only seven times lower than for FKBP12.6 [Carmody 2001, Jeyakumar 2001].

The protein-receptor complex (FKBP12 alt. FKBP12.6 and RyR2) regulation of Ca^{2+} is described briefly below, among other regulating receptor complexes. The Ca^{2+} influx triggers a release from the intracellular reservoir (the SR) through RyR2 controlled and stabilized by FKBP12 and FKBP12.6 in the normal state. This intracellular release from SR is then normally regulated by an uptake from the cytosol of Ca^{2+} by sarco-endoplasmic reticulum Ca^{2+} pumps (SERCA). Simultaneously, a discharge out of the cytosol through the Na^{+}/Ca^{2+}exchanger (NCX) is initiated. SERCA has three known isoforms, of which SERCA2a is considered to be the isoform present mainly in cardiomyocytes [Lucats 2007]. The re-uptake pump SERCA is regulated by

phospholamban (PLB, in the literature also abbreviated as PLN), which in an unphosphorylated state inhibits SERCA activity and consequently results in an overload of intracellular Ca^{2+} [Olson 2004, Yano 2005, Lehnart 2009]. Thus, disturbances in the function of these receptors fit well with the clinical presentation of cardiac ischemia-reperfusion injury where muscular stunning and arrhythmias appear as dominant features [Kloner 1993].

In chronic heart failure, this regulation of the Ca^{2+} flow malfunctions and FKBP12 has been shown to dissociate from the RyR2. This allows the outflow of Ca^{2+} ions to increase out from the SR into the cytosol. This pathogenic mechanism has been shown in an experimental model with heart failure with an explanted human heart muscle [Yano 2005]. Stunned myocardium might be explained by cytosolic overload of Ca^{2+} during ischemia/reperfusion with interference with the EC uncoupling. Increased cytosolic calcium can activate protein kinases, phospholipases and other degradative enzymes [Bolli 1998]. When the cytosol is overloaded with Ca^{2+} the contractile proteins will be affected resulting in a hypercontracture position, a tetani, constituting one possible mechanism for the development of stunning.

Animal experiments demonstrated that calcium-channel blockers (e.g. nisoldipine) given to conscious pigs as a cardioprotective treatment attenuated myocardial stunning. The mechanism behind the protective effect of the calcium-channel blockers was considered to consist of a decreased influx of calcium during ischemia, resulting in decreased ATP consumption, attenuation of ischemic injury and to provide a secondary effect of reduced reperfusion injury [Bolli 1998]. The impact of differences regarding the experimental animal models was further tested during the 2000s. The ultra-short-acting calcium antagonist Clevidipine was tested in both open- and closed-chest models, as well as different anaesthetic methods. The results from this study however failed to reveal any protective and infarct size-reducing effects of this ultra-short-acting calcium antagonist [Odenstedt 2004]. Calcium-channel blockers have not yet been proven to ameliorate stunning in human clinical trials [Boden 2000].

FKBP12 is known to interact with several other biological situations and a few of these are therefore briefly presented below. In many cases FKBP12 and FKBP12.6 act in association with calcineurin, an important regulator of many intracellular processes [Bultynck 2001, Carmody 2001]. FKBP12 also acts as a ligand for the transforming growth factor-β (TGF-β) family [Wang 1996]. Among other effects in different cell systems, TGF-β_1 has been demonstrated to have cardioprotective effects after myocardial ischemia-reperfusion [Lefer 1993]. Additionally, FKBP12 may interact with the nuclear factor of activated T cells (NFAT) that promote interleukin 2 production and, as a transcription factor, may promote left ventricular hypertrophy, sympathetic nerve sprouting and have an impact on left ventricular survival or resistance to ischemic injury [Molkentin 1998, Obansanjo-Blackshire 2006, Rana 2009]. Thus, FKBP12 exerts many vital biological activities that may be essential for the myocardial response to ischemia or reperfusion.

A link between the calcium and ROS theories

Both of these hypotheses regarding the possible sources or mechanisms of reversible reperfusion injuries do not exclude each other. They may, however, represent different aspects of the same pathophysiology. ROS production in reperfusion can cause dysfunction of the SR and this may alter the Ca^{2+} flow across the sarcolemma. Together this will result in EC uncoupling and intracellular Ca^{2+} overload. ROS may damage the contractile proteins and impair their responsiveness to calcium [Bolli 1998, Yellon 2007].

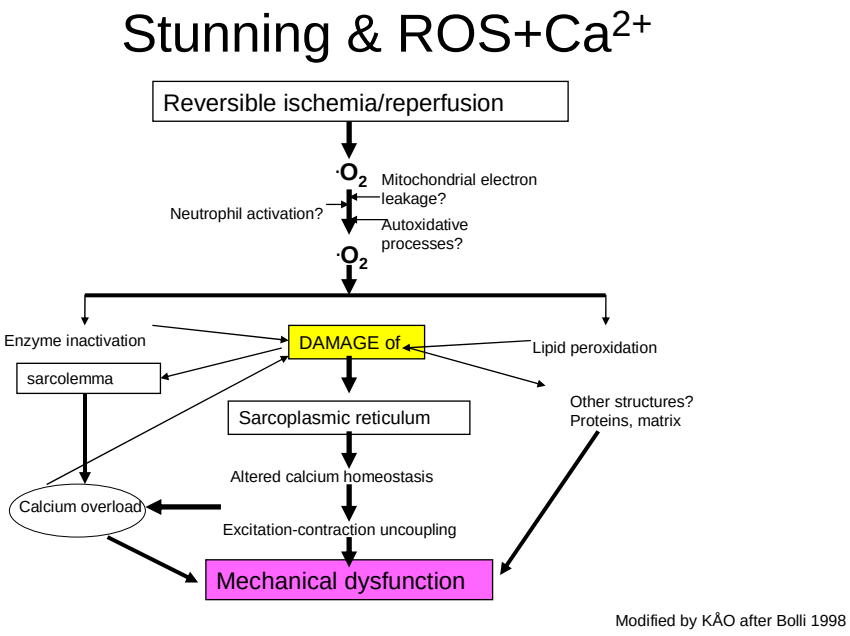

Figure 7. A schematic flow-sheet describing the links between stunning and ROS activation with the role of calcium ions. Reprinted and slightly modified by the author after Bolli 1998, from Progr in Cardiovasc Dis 1998;40:477-517. Bolli R. Basic and clinical aspects of myocardial stunning with permission from Elsevier.

Inflammation and stunning

Neutrophil leukocytes infiltration and accumulation have been shown to accelerate in reperfusion in a canine model of myocardial ischemia and reperfusion, with ligation of LAD and reperfusion after three hours [Engler 1986]. Neutrophils are important factors in the defence system of cells, by identifying and destroying foreign invaders with the assistance of intracellular signals and thereby starting the immunological response: inflammation. In myocardial ischemia and reperfusion inflammation, signals are generated by endothelial cells and cardiomyocytes and the neutrophil responses are directed against the signalling tissues. Because of this, an injury against viable myocardial cells is started, and the reperfusion injury is initiated. Activated neutrophils are capable of releasing substances injurious to the myocardium as proteolytic enzymes (i.e. elastase), ROS production and cytokines [Vinten-Johansen 2004].

The role of neutrophils in reversible myocardial reperfusion injury has been widely debated [Vinten-Johansen 2004]. The infiltration of these blood components is well described in a canine open-chest model with short and long ischemia and reperfusion. This study showed that the amount of neutrophil leukocytes in the myocardium was decreased after short-term ischemia (12 minutes) and reperfusion, and the authors concluded that neutrophils would not cause reperfusion injury after reversible ischemic injury. They also demonstrated that more neutrophils accumulated rapidly in the reperfused myocardium after the long-term ischemia (40-90 minutes), making it more likely that neutrophils may be responsible for irreversible reperfusion injury [Go 1988]. Therapies against neutrophil accumulation have so far been disappointing and have not shown any usable approaches in humans [Yellon 2007].

Irreversible reperfusion injury

Irreversible reperfusion myocardial injury is defined as reperfusion-induced cell death of the cardiomyocytes that were still viable at the time of blood flow restoration [Yellon 2007]. Thus, irreversible reperfusion injury extends the initial infarction caused by ischemia with further clinical deterioration in the shape of heart failure and arrhythmias.

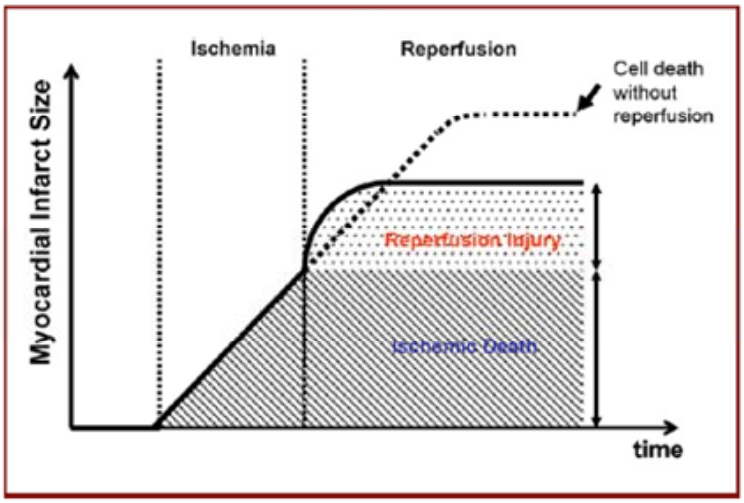

Figure 8. The presumed extension of the reperfusion injury above the ischemic cell death at an AMI. Reprinted from Arch of Cardiovasc Dis 2008; 101:491-500, Monassier JP, Reperfusion injury in acute myocardial infarction. From bench to cath lab. Part 1: basic considerations, with permission from Elsevier.

The most convincing support for the existence of irreversible reperfusion injury is that the extent of a myocardial infarct might be reduced by an intervention used at the beginning of myocardial reperfusion.

Background

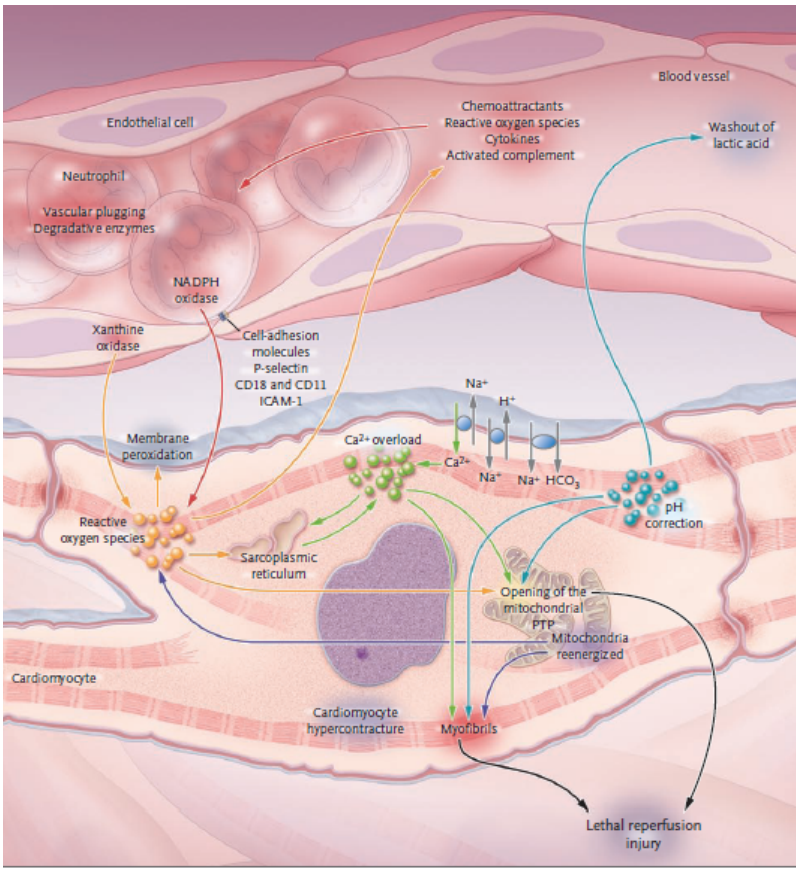

Figure 9. Assumed causes to myocardial reperfusion injury. Reprinted with permission from NEJM 2007;357:1125 Yellon & Hausenloy Myocardial reperfusion injury. Copyright © Massachusetts Medical Society.

Different biochemical mechanisms responsible for the development of lethal irreversible reperfusion injury have been proposed. ROS, as one of the responsible mechanisms, may arrive from xanthine oxidase principally derived from vascular epithelial cells, or by neutrophil leukocytes, accumulating in the ischemic area. Other sources of late ROS production are nicotine adenine dinucleotide phosphate (NADPH) oxidases inside the neutrophils and the release of free electrons from the ETC in the mitochondria, due to dysfunction of mitochondrial permeability transition pores (MPTPs), and activation of

the complement system. Reperfusion induces an increase of intracellular Ca^{2+}, which is due to sarcolemmal membrane damage and ROS-induced dysfunction of the SR. This increase results in Ca^{2+}-overload. This excess induces cardiomyocyte death by causing hypercontracture and early MPTP opening. Another mechanism is calcium overload of the cytosol due to dysfunction of regulatory receptors located at the SR and/or ion exchangers at the cell membrane, at least partially caused by ROS. ROS may also act as directly damaging agents initiating lipid peroxidation causing membrane rupture leading to cell death [Yellon 2007].

Correction of pH

The myocardium is exposed to acidic conditions during ischemia due to a switch from the aerobic to anaerobic metabolism of glucose, where the Krebs cycle is not engaged and therefore production of lactic acid, protons and CO_2 is started, which in itself inhibits glycolysis. Reperfusion will restore the pH to physiologic levels, with a wash-out of accumulated lactic acid and further activation of Na^+ /H^+ ion-exchange (NHE) systems. However, this return to the physiologic stage has been shown to contribute to further extension of reperfusion injury [Monassier 2008, Yellon 2007]. The MPTPs, are described as key determinants of cardiomyocyte death. Early and irreversible opening of the pores following an episode of ischemia-reperfusion causes mitochondrial dysfunction. The inner mitochondrial membrane will lose its potential and a depletion of the mitochondrial NADH pool (the energy supply) will occur, with this the oxidative phosphorylation will be uncoupled, initiating further cascade reactions inside the mitochondria. These cascades include internal generation of adenosine, bradykinin, and opioids, that activate protective mediators such as protein C (PKC), serin-threonin kinase (Akt) and extracellular signal-regulated kinase (Erk1/2). All of these are incorporated in the reperfusion injury salvage kinase (RISK) pathway. Early opening of the MPTPs also

leads to an activation of the mitochondrial apoptosis cascade through caspase, and the cardiomyocytes will follow their involuntary cell death caused by reperfusion [Hausenloy 2009].

Another possible explanation for lethal irreversible reperfusion injury is the induction and activation of apoptosis. Apoptosis is the genetic programme of cell death. This is required for normal embryonic development and for maintaining essential tissue homeostasis.

Therapies against irreversible reperfusion injury

ROS

The action of ROS has been considered as more aimed at reversible reperfusion injury that at irreversible reperfusion condition. For ROS mechanisms please see chapter reversible reperfusion injury.

ROS as a cause of irreversible reperfusion injury has been tested in clinical trials by using substances considered as ROS scavengers, mechanisms which possibly would attenuate reperfusion injury. Scavengers such as superoxiddismutase (SOD), trimetazidine (TMZ), edaravone and allopurinol have been evaluated.

SOD was tested already in 1994 by Flaherty et al in a human study with negative results concerning the effect on recovery of the left chamber function compared with controls [Flaherty 1994].

TMZ acts by inhibiting the fatty acid metabolism and shifting the metabolism at the ischemic myocardium from fatty acid oxidation to carbohydrate (glucose) utilization. In a clinical study, no reduction in mortality was demonstrated [Marzilli 2003]. The antioxidant mechanism of TMZ is attenuation of intracellular acidosis during ischemia and acceleration of the restoration of phosphorylation during reperfusion [Marzilli 2001].

Edaravone acts as an inhibitor of the lipoxygenase metabolism of arachidonic acid by trapping OH^- radicals. This scavenger showed a reduction in infarct size measured with biochemical markers and the appearance of Q-waves on the ECG, and less oxidative stress as measured by thioredoxin levels. Clinical signs were reduction of frequency of reperfusion arrhythmias [Tsujita 2006].

Allopurinol is an inhibitor of the enzyme xanthine oxidase. This enzyme and its function for cardioprotection in cardiovascular surgery was discussed as a therapeutic entity by Xia and Zweier [Xia 1995]. Very low levels of xanthine oxidase are found in human heart tissue, whereas the levels are higher in vascular beds. Guan et al tested oral allopurinol administered 4.5 hours prior to pPCI for AMI, and an improvement in the LVEF and a decrease of isoprostanes as markers for ROS production were shown [Guan 2003].

The neutrophil theory

The role of neutrophils in irreversible myocardial reperfusion injury is widely debated and this research field has been well described [Vinten-Johansen 2004]. Damage of the myocardial tissue by activated neutrophils is believed to be caused by proteolytic enzymes and ROS production.

Animal experiments regarding the neutrophil theory and reperfusion injury

Reduction in infarct size has been studied and shown to be significant for different species such as dogs, cats and pigs using leukocyte depletion achieved by using neutrophil-specific filters, administrating anti-serum containing antibodies against neutrophils as well as chemical methods such as chemotherapy. These studies have been the subjects of criticism due to the fact that the cited therapies induced neutropenia prior to vessel occlusion, mimicking AMI, which may influence and change the natural development of the infarction [Vinten-Johansen 2004].

Human studies and the neutrophil theory

Several human studies based on the neutrophil theory have been performed during the last decade, and none of them have shown any significant effect on reducing myocardial reperfusion injury [Vinten-Johansen 2004, Yellon 2007]. Different antibodies, such as antiCD18 and antiCD11 directed against the surface of the neutrophils, and inhibiting activation and accumulation have failed to show any effect with regard to reducing reperfusion injury in clinical human trials such as LIMIT-AMI 2001 (thrombolysis with

r-TPA and antiCD18/ placebo) [Baran 2001] and HALT-MI 2002 (pPCI and CD11/CD18 versus placebo) [Faxon 2002].

P-selectin plays an essential role in the initial recruitment of leukocytes to the site of injury during the early inflammatory reaction caused by ischemia. P-selectin moves from an internal cell location to the endothelial cell surface when the endothelial cells are activated by molecules such as histamine or thrombin. P-selectin is found both in endothelial cells and in activated platelets. An antagonist to P-selectin was used for studying reperfusion injury in the PSALM study but no effects regarding infarct size reduction could be shown [Mertens 2006].

Pexelizumab, a C5 complement inhibitor, did not reduce infarct size when measured with the biomarker CKMB area under the curve (AUC) in the COMMA trial, but long-term mortality (days and weeks post-infarction) was significantly reduced. This effect was suggested to depend on the reduction of delayed myocardial damage by an anti-inflammatory effect and improved healing and remodeling through cell death mechanisms. C5 inhibition prevents apoptosis which occurs during the first two weeks in humans [Granger 2003]. Later studies with pexelizumab combined with pPCI during AMI failed to show any significant effect on 30- or 90-day mortality or infarct size reduction [Armstrong 2007].

FIRE [Atar 2009] was using FX06 as a cardioprotective drug. The mechanism of FX06, a peptide, is anti-inflammatoric by binding to the vascular endothelial cadherin. FX06 was administered at pPCI for AMI, and showed a significant reduction of the necrotic core zone of the infarct measured five days post-infarction with delayed enhanced magnetic resonance imaging (DE-MRI) with 58% reduction, this finding was, however, a secondary endpoint.

One important difference between animal and experimental models is that humans presenting with AMI usually show different co-morbidity, as previous manifestations of atherosclerosis, cardiovascular disease, diabetes mellitus and/or inflammatory disorders with or without a concomitant need of other pharmacological treatment. All these differences make it difficult to translate findings into a clinical perspective. Another variation is that the timing of the administration of presumptive cardio-protective drugs is

specific in an experimental setting with animal models, but cannot be exact in a clinical situation as patients present with varying durations of their AMI. Animal models differ also with regards to coronary collateral flow, especially dogs [Bolli 2004, Yellon 2007], though dogs are considered as a useful work model [Hedström 2009]. Regards have also to be taken to the fact that animals in experimental models are unconscious and it is well-known that anaesthetics, such as isoflurane for example, present a cardioprotective effect [Tanaka 2004].

As Vinten-Johansen also conclude, a focus on neutrophils participating in the apoptosis reaction, and the involvement of the neutrophils in the longer term reperfusion beyond the acute phase of 4-6 hours may also be considered.

Calcium overload

During the 1980-1990s porcine and canine experimental results with calcium-channel blockers such as nifedipine, verapamil, diltiazem or a NHE inhibitor, showed positive results regarding reduction of infarct size [Klein 1984, Carry 1989, Gumina 1999]. However, the ultra-short-acting calcium-channel blocker Clevidipine failed to reduce infarct size in a closed-chest porcine model [Odenstedt 2004]. The positive results obtained earlier with the other calcium-channel blockers (nifedipine, verapamil and diltiazem) have not shown any successful translation to the human clinical setting. INTERCEPT, a multinational clinical trial, in which diltiazem was tested as an adjunctive to thrombolytic treatment of AMI, failed to show any reduction of the primary endpoints: mortality, revascularization, and refractory angina [Boden 2000]. A newer agent, MCC-135 (Caldaret), a NHE inhibitor which also promotes the uptake of Ca^{2+} into the SR has been tested, but the study failed to show any reduction of infarct size [Bär 2006, Jang 2008].

All other hitherto published human studies have been of mixed clinical settings with regards to infarct size duration, varied timing for administration of the active drug, and simultaneous co-administration of different thrombolytic drugs and mechanical treatment with PCI as adjunct or bail-out procedure, when the medical therapy has failed. The endpoints for these studies have been inhomogeneous, and in some studies results have

been measurements of recovery of LV-function, short-term mortality, or indirect evidence of ROS production.

Infarct size has been measured with different methods such as routine biochemical markers and their AUC from CKMB, TnT or troponin I, with nuclear investigations such as single photon emission computed tomography (SPECT) and positron emission tomography (PET), or with ECG methods such as resolution of the ST-elevation or development of Q-waves. Recently, clinical studies have begun using DE-MRI for quantitative measurements of infarct size, and as sub-analysis in the DE-MRI-investigation of the necrotic core zone of the infarcted area. This has been estimated as a dimension of infarct size. So far, due to all these varieties, the performed clinical studies have been difficult to interpret and to transfer to the routine medical situation.

Correction of pH

Animal experiments (performed on rat cardiomyocytes) with buffering solutions aimed at reducing reperfusion injury showed promising results [Bond 1991]. Nevertheless, the approach to address the pH restoration process has not been successfully translated to the human setting, and trials with ion-exchange inhibitors, such as eniporide, failed to show any cardioprotection [Zeymer 2001].

Mitochondrial permeability transition pore (MPTP), ischemic preconditioning (IPC), ischemic postconditioning (IPost) and ischemic perconditioning (IPer)

The opening of the MPTPs causes the disruption of the inner mitochondrial membrane. The membrane will lose its potential, a depletion of the mitochondrial NADH pool will occur, and the oxidative phosphorylation will be uncoupled leading to additional cascade reactions inside the mitochondria. This process constitutes a source of free electrons which are fundamental for ROS production, and can lead to further cascade reactions with fatal cell injuries as a consequence. The early opening of the MPTPs also activates the mitochondrial apoptosis cascade which is yet another mechanism for accelerated cell death following ischemia and reperfusion.

Therapies against the opening of MPTPs will be briefly described below.

Ischemic preconditioning (IPC)

A procedure performed prior to reperfusion is the IPC [Zhao 2003, 2006, Yellon 2007, Hausenloy 2009]. This is described as salvage of ischemic myocardium caused by episodes of transient myocardial ischemia and reperfusion administered to the myocardium before the sustained ischemic period. The mechanisms proposed behind IPC are that it stimulates internal secretion of adenosine, bradykinin, and opioids, which will then recruit a complex system of intracellular signalling pathways resulting in ROS production and further activation of further pathways such as protein kinase C (PKC), serin-threonine kinase (Akt) and extracellular signal-regulated kinase (Erk1/2) as cardioprotective signals in the RISK pathway. Akt, also known as protein kinase B (PKB), is involved in cellular survival pathways by inhibiting apoptotic processes. Akt is known to stimulate nitric oxide synthase (NOS) to produce nitric oxide (NO) [Bolli 2007, Downey 2007, Hausenloy 2009].

The IPC as a cardioprotective mechanism is regrettably not considered to be an option in the clinical setting with an AMI because the infarction event cannot be foreseen and thereby prevented. In a review from 2007, Bolli describes two phases of preconditioning: an early and a late phase. He suggests that early preconditioning would be effective in limiting irreversible lethal reperfusion injury by rapid post-translational modification of pre-existing proteins. The late phase would however be effective against reversible post-ischemic contractile dysfunction, stunning, and synthesis by new cardio-protective proteins, where the inducible isoform of nitric oxide synthetase (iNOS) and cyclooxygenase- 2 (COX-2) are important actors induced by activating several signalling parallel pathways. Bolli mentions the activation of protein kinase C-epsilon proto-oncogenic tyrosine kinases/leukocyte-specific protein tyrosine kinase nuclear factor-jB (PKCε-SRC/Lck-NF-jB), the Janus kinases 1 and 2 and signal transducers and activators of transcription 1 and 3 (JAK1/JAK2-STAT1/STAT3) pathways. The combined and synergistic effects of nitric oxide and cytoprotective prostanoids result in myocardial protection [Bolli 2007].

Ischemic postconditioning (IPost)

Postconditioning has been described as a cardioprotective procedure [Zhao 2003, Hausenloy 2009]. When the blood flow in the reopened coronary artery is repeatedly interrupted a significant reduction of the myocardial infarct size, up to 30-50% in experimental settings, has been demonstrated [Hausenloy 2009]. The mechanism behind the postconditioning is not fully understood, but is supposed to target several of the mediators of lethal reperfusion injury, such as ROS, Ca^{2+}-overload, endothelial dysfunction, opening of the MPTP, apoptosis, neutrophil accumulation, and the edema and pH changes. Like IPC, IPost also initiates activation of cell-surface receptors such as adenosine, bradykinin and opioids, recruiting different signalling pathways such as, for example, PI3K-Akt and MEK1/2-Erk1/2.

Thibault et al studied 38 patients with repeated inflations and deflations of the angioplasty balloon at pPCI at AMI. Infarct size measured with cardiac biomarkers (CKMB and troponin I) was reduced and a functional recovery was improved at six months and one year post-infarction [Thibault 2008].

Ischemic perconditioning (IPer)

Two human studies on IPer were presented at ESC Barcelona 2009. The term refers to the use of simultaneous remote ischemic periods in an extremity (e.g. the upper arm) concomitant with the opening occluded infarct-related coronary artery.

Terkelsen reported a study with patients presenting with ST-elevation myocardial infarction (STEMI), randomized to IPer, as an adjunct to pPCI, versus pPCI alone. IPer consists of episodes of non-lethal ischemia performed simultaneously in a distant organ while the heart suffers from lethal ischemia. Remote IPer did not significantly reduce infarct size measured by the degree of ST-resolution [Terkelsen 2009].

Masotti et al studied STEMI patients undergoing an IPost/IPer protocol consisting of balloon inflations versus routine pPCI. DE-MRI was performed to estimate infarct size and salvage area. ST-resolution was used to estimate successful reperfusion. The results from this study showed no reduction or limitation of infarct size by using IPer and might even jeopardize the salvage of myocardium attained by pPCI [Masotti 2009].

A recent published study concerning IPer at first time STEMI patients and myocardial salvage measured with SPECT showed increased myocardial salvage [Bøtker 2010]. The mechanisms for these protective procedures are assumed to overlap the myocardial adaptive responses to ischemia using the RISK pathway [Hausenloy 2007].

MPTP suppression and cyclosporine

MPTPs have therefore been the subject of the development of cardioprotective drugs and procedures. One of the identified inhibitors is Cyclosporin A, a well-known immunosuppressant drug. This drug and its relation to MPTP was demonstrated as early as 1988 by Crompton et al in experimental settings in animals (rats). The actual work with the pharmacological inhibition of the MPTPs during reperfusion was made by Hausenloy´s group in 2002 [Hausenloy 2009].

A promising attempt to translate these findings to clinical settings has been made by Piot in a study where patients with AMI treated with pPCI received a bolus injection with Cyclosporin A [Piot 2008]. The results were encouraging and the authors reported a reduced infarct size of 30-40% measured with cardiac biomarkers such as CKMB and TnT, and in a subgroup of patients with DE-MRI.

Lately, interest has been focused on strategies that may influence several of the hypothetical mechanisms for lethal irreversible reperfusion injury. Further elucidation of the mechanisms causing reperfusion injury may lead to the development of protection devices or new pharmacological management methods.

Aims of the Work

The general aim of this work was to gain knowledge about the pathophysiology of myocardial reperfusion injury.

The specific aim for each paper was to:

1. Evaluate indirect markers for reactive oxygen species (ROS), MDA and isoprostanes, and patterns of inflammation during acute myocardial infarction (AMI) treated with primary percutaneous coronary intervention (pPCI) as reperfusion therapy.
2. Compare infarct size assessed by serial and clinical routine sampling of biochemical markers CKMB and troponin T with infarct size by delayed contrast-enhanced magnetic resonance imaging (DE-MRI).
3. Search for release of proteins into the myocardium with patterns that may shed light on mechanisms of myocardial reperfusion injury in an experimental porcine model and in cell cultures.
4. To develop a cardiac cell culture model for studying release and production of the intracellular calcium flow regulating proteins FKBP12 and FKBP12.6 during simulated ischemia with or without addition of ROS and with and without simulated reperfusion.

General methods

Laboratory methods for detecting proteins

Proteomics

Proteomics is the study of proteins in a group, particularly their structures and functions. Proteins are essential parts of living organisms as they are the main components of the physiological metabolic pathways of cells. With proteomics, the relative amounts of proteins may be measured, modifications of proteins analysed, and in which cell type specific proteins may be found.

SELDI-TOF: surface-enhanced laser desorption/ionization time-of-flight, an ionization method used together with mass spectrometry for protein analysis.

Western blot technique

The Western blot is a standard method where protein samples are separated electrophoretically, and then transferred from a sodium dodecyl sulfate (SDS)-polyacrylamide gel to a membrane. The membrane is then probed with factors which are specific for amino acids for the identification and verification of proteins. Western blot may also be used for the semi-quantification of proteins, where the protein bands localized on the gel may be densitometrically analysed.

ELISA

Enzyme-linked immunosorbent assay (ELISA) is a standard technique for the quantification of proteins based on the detection of antigen-antibody reactions with light emission.

Real Time RT-PCR

Real-time polymerase chain reaction (RT-PCR), a technique used for gene expression at RNA level by constructing DNA from RNA.

General microdialysis

This method enables a direct sampling from the affected organ, with the result that obtained substances are not released into the usual sampled peripheral blood from other organs. This is considered to be an advantage. It is also possible to sample substances from different parts of the same organ and thereby make it possible to select and obtain samples from different affected parts. The dialysis membrane has pores of a known size and this determines the sizes of the molecular weights that can enter through the membrane. This enables the selection of subjects that may be studied. The concentration of a substance in the microdialysate depends on the recovery rate of the probe. Major determinants include the surface area of the membrane, the membrane pore diameter, the flow rate, the chemical properties of the membranes, and the temperature and tissue pressure. The microdialysis technique has existed since 1960 and been used as a diagnostic tool in central nervous system studies since the 1970s. The myocardial approach in both humans and animal experiments has been thoroughly described by my co-author Dr Mantovani in his thesis published in 2006 by Göteborg University [Mantovani 2006].

Cardiac MRI in general

MRI of the heart can provide useful information about morphology of the heart, myocardial function and myocardial viability. MRI does not involve ionizing radiation. The use of MRI as a tool for evaluating myocardial function and viability has been utilized since the mid-1980s and is now becoming the gold standard when assessing infarct sizes and viable muscle mass [Weinsaft 2007].

Materials and methods, Papers I-V

Ethics

The three first human studies were approved by the local ethics committee at Lund University Hospital, Sweden, where these human studies were performed. Ethics No: LU 180-97 for Paper I, and Ethics No: LU 99-519 for Papers II and III. The investigations were conducted in accordance with the Helsinki Declaration.

The fourth experimental animal study was approved by the local ethics committee for animal research, No 310-2003 Göteborg University, Sweden. The fourth experimental animal investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

The fifth study is an experimental study based on cell cultures.

Materials and methods Paper I

The first human study group consisted of non-consecutive patients admitted directly to the catheterization laboratory for pPCI. Inclusion criteria were AMI with ECG signs of ST-elevation and ongoing chest pain with duration of less than six hours. A totally occluded infarct-related artery verified by coronary angiogram was mandatory.

Blood samples were obtained following a protocol at occluded vessel (baseline), one minute after established maximal TIMI grade flow and then after 5, 10, 30 and 90 minutes of verified reperfusion. Stents were implanted at a rate of 74%. The platelet GPIIb/IIIa inhibitor abciximab (Reopro® Lilly, Indianapolis, IN, USA) was administered in 78% of the patients.

Biochemical methods

Plasma MDA concentrations were measured with a high-performance liquid chromatography (HPLC) method and as thiobarbituric acid-related substances (TBARS) using a fluorimetric method. Routine biomarkers for myocardial damage CKMB and TnT were analysed.

Materials and methods Papers II and III

The second and third human studies consisted of a different group of non-consecutive patients presenting with symptoms of their first-time AMI due for treatment with pPCI. Criteria for inclusion were STEMI in progress with ongoing or recurring chest pain with duration of a maximum of 12 hours. A totally occluded infarct-related artery verified by the coronary angiogram was demanded. All included patients received at least one stent and were given the GPIIb/IIIa inhibitor abciximab (Reopro® Lilly, Indianapolis, IN, USA).

Blood samples for analysis of biomarkers in Papers II and III were obtained prior to opening of the occluded infarct-related vessel (baseline), and then at 1.5, 3 and 24 hours post reperfusion. Another sample was obtained at 48 hours post reperfusion for the markers in Paper III.

Biochemical methods

Circulating levels of markers of neutrophil activation, such as human plasma myeloperoxidase (MPO) and serum neutrophil gelatinase-associated lipocalin (NGAL), and a marker of matrix remodelling, serum matrix metalloproteinase (MMP)-9, were determined by a solid-phase sandwich ELISA. Plasma MDA was analysed with an in–house HPLC method and plasma isoprostane (Iso-P) by ELISA. For markers of inflammation, plasma tumour necrosis factor (TNF)α, plasma interleukin (IL)-6 and IL-8 were analysed by ELISA. Serum high-sensitive C-reactive protein (hs-CRP) was measured by an ultra-sensitive particle-enhanced immunoturbidimetric assay. CKMB and TnT, both from serum, were determined by standard procedures for the regular sampling indicating myocardial damage.

MRI

A 1.5-T system (Magnetom Vision, Siemens, Erlangen, Germany) or a 1.5-T system (Philips Intera CV, Philips, Best, NL) was used. T1-weighted images short- and long-axis images covering the left ventricle were acquired.

Materials and methods Paper IV

A mixture of female Swedish landrace, Pigham and Yorkshire pigs, weighing around 40 kg was used. The pigs were sedated before general anaesthesia and ventilated by a respirator.

Ischemia induction

An angioplasty balloon was used for inducing ischemia in the myocardium area supported by the LAD, distal to the first diagonal branch. The balloon occlusion time was 45 minutes and restitution of coronary flow was controlled with angiography. All pigs were reperfused for 120 minutes.

Microdialysis

A sternotomy was performed and microdialysis catheters were inserted into the beating myocardium in the anterior wall, to be exposed to ischemia, as well as in the lateral wall, which would serve as a control. The protocol for sampling consisted of three time periods, equilibrium for 60 minutes, occlusion for 45 minutes and then reperfusion for 120 minutes.

Cell cultures

HL-1 cells were exposed to six hours of hypoxia. As controls, HL-1 cells were studied after six hours of normoxic conditions.

Materials and methods Paper V

HL-1 cells were incubated under hypoxic (1%) or hypoxic, energy-depleted and acidic (HEDA) with or without ROS influence environment simulating ischemia for six, 12, 18 and 24 hours, respectively. The cell cultures were also undergoing six hours of reestablishment of physiologic (REPH) conditions simulating reperfusion. Cells exposed for normoxia for each observed time served as controls. Total cell proteins were collected and cytosolic contents of FKBP12 and FKBP12.6 were analysed by Western blot. RT-PCR was performed for mRNA expression of FKBP12 and FKBP12.6 for demonstration of production. Viability was estimated by using regular biomarkers for myocardial cell damage, such as lactate dehydrogenase (LDH) and high-sensitive TnT, and by means of the trypan blue exclusion method for each occasion with the automated instrument Vi-cell (Beckman Coulter Vi-Cell XR™).

Statistics

All material was considered as normally distributed. Standard statistical methods were used. Values are presented as mean ± standard error. For Papers I-IV: statistical calculations were performed with the two-tailed Student t-test for comparison between results at various time points and defined baseline. In Paper V: A statistical analysis of all groups with respect to time of exposure to intervention and kind of intervention for the viability including the biomarkers and the Iso-P ELISA results were made with a two-

way ANOVA including post hoc analysis with a test of least square difference (LSD). For the results of Western Blot analysis and RT-PCR within-group comparisons were performed for each exposure time with a one-way ANOVA and also with post hoc analysis according to Dunnett's two-sided t-test in which all interventions were compared with normoxia. The significance level was set at $p< 0.05$.

Results

Paper I:

The marker for ROS production, P-MDA, showed a significant decrease analysed with HPLC in patients treated with pPCI for AMI. No significant changes in plasma TBARS levels were observed. No significant correlation between the plasma concentrations of MDA and TBARS was demonstrated. All patients had angiographically verified reperfusion with TIMI flow III post-pPCI.

Paper II:

Markers for inflammation, neutrophil activation, myocardial remodelling and ROS activation were analysed. Results are shown in table 1. Inflammation -markers increased over time as expected, though with a late pattern of TNFα and hs-CRP. MPO, considered as a marker for neutrophil activation, was at maximum at occluded vessel, and decreased significant during the first day. The biomarker indicating myocardial remodelling MMP-9 peaked between 1.5 and 3 hours. For markers of ROS activation were the MDA results similar to the results obtained in Paper I with a significant decrease during the observed period. P-Iso-P showed no significant change comparing baseline to all given time points. All patients had angiographically verified reperfusion with TIMI flow II-III post-pPCI.

Table 1

Marker	Unit	Baseline	1.5h	3h	24h
P-MPO	ng/mL	13.0±1.5	10.9±1.0	7.6±0.6*	8.4±1.0*
S-NGAL	ng/mL	124.4±10.2	109.1±8.1	97.8±7.5	113.4±8.4
S-MMP-9	ng/mL	287.3±45.6	438.1±68.8**	551.9±75.8**	412.1±44.5
P-MDA	μmol/L	1.10±0.08	1.06±0.08	0.96±0.08	0.80±0.06**
P-Iso-P	pg/mL	199.7±40.8	217.7±29.7	164.8±22.5	222.6±68.1
P-IL-6	ng/mL	8.2±2.1	20.4±4.4**	28.4±7.6*	29.7±4.4**
P-IL-8	ng/mL	1.3±0.4	3.7±0.8**	6.1±1.4**	7.5±1.1**
P-TNFα	ng/mL	1.3±0.1	1.3±0.2	1.3±0.2	1.5±0.1**
S-hsCRP	mg/L	5.9±1.8	7.5±2.6	8.1±4.0	43.1±8.8**

Values are given as mean± standard error. *p<0.05, **p<0.01.

Paper III:

Peak values of CKMB and cTnT occur between 3 and 12 hours after acute reperfusion. The values correlate well infarct size measurements with DE-MRI, and may be used for the estimation of myocardial infarction size post-pPCI. All analysed patients showed angiographically verified reperfusion with TIMI flow III post-pPCI.

Paper IV:

FKBP12 and myoglobin (a marker of myocardial cell damage) were identified as being released during ischemia and reperfusion in the porcine microdialysates. RT-PCR analysis on the porcine biopsies exposed to ischemia revealed a non-significant increase in mRNA expression of FKBP12, and a significant increase in mRNA expression of FKBP12.6. Further investigation on cell lysates from HL-1 cells exposed to six hours of hypoxia demonstrated an increase of FKBP12 and a significant increase in mRNA expression of FKBP12.6, which was interpreted as an increased production/ upregulation of FKBP12.6 due to hypoxia.

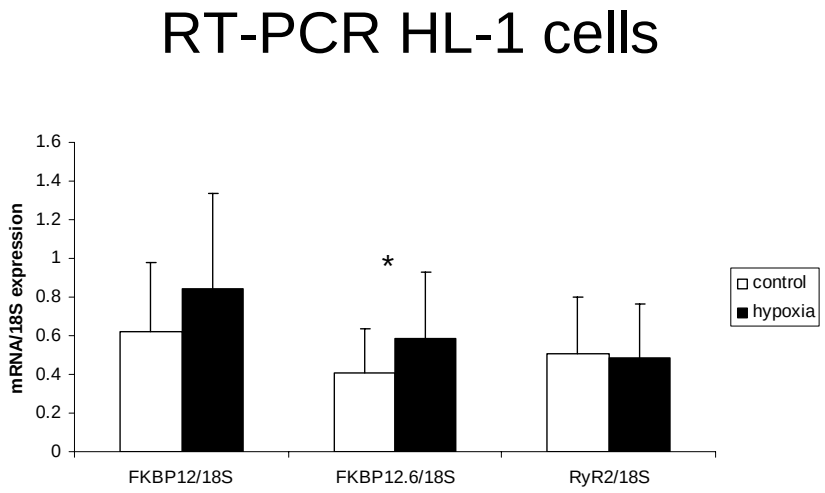

Figure 10. The increase in mRNA-expression of FKBP12 and FKBP12.6 in relation to 18S at six hours of hypoxic conditions. The mRNA-expression of RyR2 was not changed during the hypoxia.

Paper V:

The HL-1 cell model was investigated with regard to viability parameters and with particular interest in the release of FKBP12 and FKBP12.6 during different oxygen levels, energy depletion and acidosis, with and without ROS activation, as well as with and without REPH. Our results showed significant decreases of the parameters concerning viability as expected following duration and ischemic/energy depleted environments. A cut-off level after 18 hours of ischemic conditions could be shown.

Our findings concerning the proteins FKBP12 and FKBP12.6 revealed that the content of both FKBP12 and FKBP12.6 after simulated ischemia increased, and this increase was accompanied by a depressed production measured as mRNA expression. The increase of protein contents was shown at groups exposed to simulated ischemia with conditions simulating reperfusion and at groups exposed for ROS activation for longer exposure times.

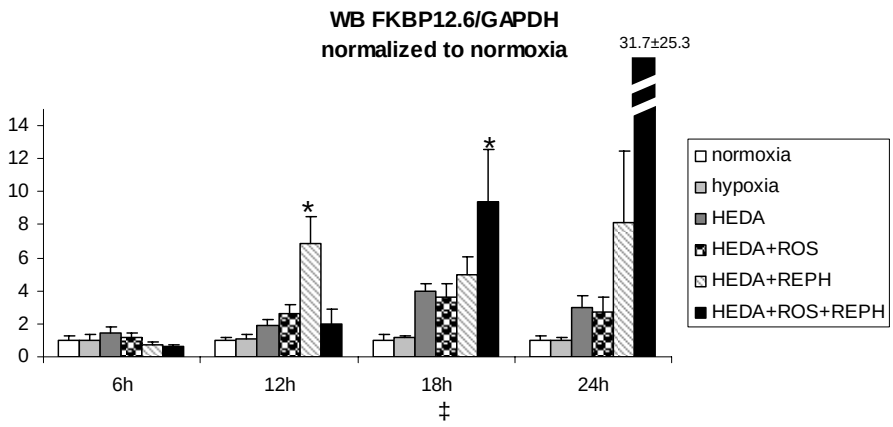

Figure 11. The contents of FKBP12.6 related to GAPDH and then normalized to normoxia. *= $p<0.05$ indicating increase of FKBP12.6 in cell lysate. ANOVA ‡= $p<0.05$ between groups.

The depressed production was demonstrated with analysis of RT-PCR of each FKBP12 and FKBP12.6. Previous results from Paper IV showing an increase of production of FKBP12 and FKBP12.6 at hypoxic conditions after six hours was demonstrated again, but the pattern following longer exposure times was different. Interestingly, simulated ischemia and ROS activation depressed the production of both proteins significantly.

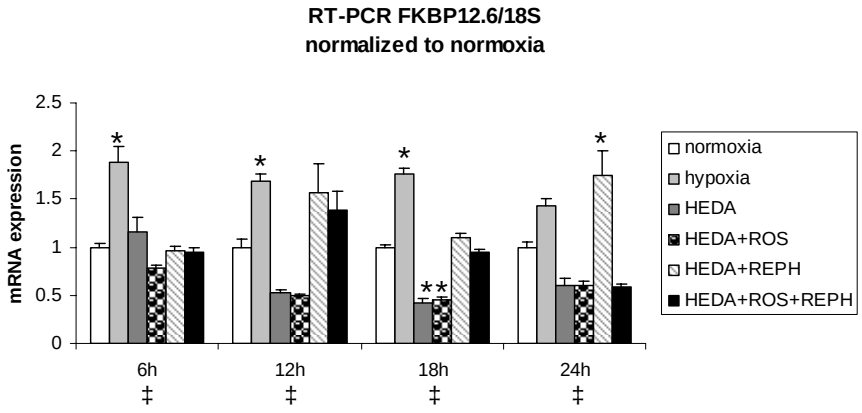

Figure 12. The mRNA-expression of FKBP12.6 in relation to 18S and then normalized to normoxia. *= $p<0.05$ indicating significant change of mRNA expression in cell lysate, ANOVA ‡= $p<0.05$ between groups.

Discussion

Myocardial reperfusion injury: a reality?

For several decades myocardial reperfusion injury has been a subject of discussion among clinicians and scientists regarding its true existence. Animal experiments with different mammal species, such as mice, rats, rabbits, pigs and dogs, have shown convincing results that both reversible and irreversible myocardial reperfusion injury truly exists. Still, concerns are raised whether this injury may be an important issue in the human clinical setting. Firm conclusive support about its existence has been the subject of numerous investigations.

During myocardial ischemia and infarction, a substantial loss of viable muscular bed occurs, which may be either temporary or definite. This situation is apparent for the clinician when patients present with symptoms such as severe acute heart failure and sometimes malignant arrhythmias, even if rapid restoration of the coronary perfusion is achieved (reperfusion). Coronary reperfusion is the current treatment of choice according to large clinical trials and worldwide guidelines.

From a clinical perspective, the reversible injury is important for patients that are at highest risk with minimal functional margins, i.e. patients with complications such as concomitant cardiogenic shock, presenting with severe acute heart failure with severe hypotension, causing cerebral hypoperfusion and ceased kidney circulation. These patients have an acute mortality of around 50%, even if they are treated with current optimal medical and mechanical treatment (e.g. intra-aortic balloon pump or intraventricular support devices). If the identification and diagnosis of this reversible injury situation was possible, and if there was some kind of treatment against it, this would be of tremendous use and even life-saving for some patients. With regards to irreversible reperfusion injury, decreasing the extension of the injury would diminish the imminent acute as well as the chronic complications. These might involve the development of chronic heart failure, a disease with a pessimistic prognosis and high mortality; despite modern medical treatment it is estimated at 5-10% per year, and for the

most complicated cases at late stages of the disease at 40-50% in Sweden [Läkemedelsverket 2006].

Myocardial reperfusion injury is, and has been, a difficult issue to study. Many aspects and ideas regarding the mechanisms have been tested in both experimental and human settings with mixed results. The hitherto known pathophysiology behind the underlying causes in regard to AMI and reperfusion has revealed that several biochemical pathways (both protective and damaging, and even serving in both directions) are activated or blocked by several diverse agents. The time spectrum for the different agents to act differs, as well as the clinical outcome for the patients.

At the present time there are more animal experiments than human trials performed on this subject [Yellon 2007]. The advantages of studying animal experimental settings are many compared with an acute human clinical situation. The most obvious advantage is that the experimental animal infarction models result in controlled myocardial damage, which has the ability to be measured and evaluated, with regard to the intensity and extension of the induced infarct. It is also possible to study the physiological and biochemical reactions directly in the myocardium within the animal models, which is not considered to be an option in patients. Another advantage is that in an animal model it is possible to evaluate the effects of presumed therapeutic interventions. These interventions in the animal models may be administered exactly at the right occasion, as the timing of occlusion and reperfusion is strictly controlled.

Nevertheless, even if many reactions and the physiological response tend to be correct, animal models are not always possible to translate directly to a clinical situation. Humans suffering from cardiovascular disease, such as AMI, mostly have other medical conditions as well, e.g. diabetes mellitus, hypercholesterolemia, hypertension, or inflammatory diseases, which the animals are free of. The above mentioned co morbidities are also subjects for treatment, e.g. hypertension, and hypercholesterolemia which today are treated with angiotensin-converting enzyme inhibitors (ACEi), angiotensin II receptor blockers (A2 blockers), and statins which in themselves are considered to contain several cardiomyoprotective effects, both activating and blocking [Wang 2002, Hausenloy 2007].

Another difference that needs to be mentioned is that the STEMI appearance of an AMI is the result of an occlusive thrombus covering a rupture of a vulnerable coronary plaque. This is absent in animal models, which have healthy (non-atheromatotic) vessels occluded by instrumentation or ligated for establishing the disruption of coronary flow. The timing of ischemia varies greatly among patients as does the timing of when complete myocardial reperfusion is established. These variations may influence the outcome of protection against reperfusion when given in the human clinical setting.

Working with cell culture models is another possibility for studying biochemical and molecular reactions during myocardial cell hypoxia, ischemic-like and reperfusion-like conditions. With this instrument many of the pathways and cross-roads may be elucidated, but unfortunately the cell culture models do not include all cellular components that human AMI patients with co morbidities present with. One important aspect is the lack of neutrophil leukocytes and their inflammatory response.

Hitherto, no complete solution has even come close to reducing the mortality of AMI. It remains a challenge to find an applicable explanation which may lead to a useful and applicable cardioprotection, especially in the AMI situation. So, how to perform studies of reperfusion injury in a human clinical setting? Which factors need to be considered and avoided?

First, biomarkers for detecting reactions appearing at reperfusion injury need to be reliable. The markers need to be specific and sensitive, not just presenting results which may arise as well as other unspecific reactions as end products of chain reactions. In the practical situation, when dealing with patients, biomarkers usually have to be obtained from the peripheral venous blood. If ROS are considered to be an important actor, better biomarkers need to be discovered. ROS are extremely short-lived with half-lives of 10^{-6}-10^{-9}s and therefore impossible to detect directly in humans with the methods available today [Jeroudi 1994]. The biomarker that may reflect ROS production may be an end product from any other reaction, alternatively the turnover time from the blood flow, from the origin in the myocardium to the venous system, will result in unspecified effects. Our results have been disappointing using the most commonly employed and accepted markers for ROS production, namely MDA and Iso-P. Both these markers

showed inverse reactions or no changes at all when they were obtained from patients with AMI treated with pPCI. Previous studies, when AMI was treated pharmacologically with thrombolytic drugs, showed the opposite result [Davies 1990, Young 1993, Öhlin 1995]. Our finding poses the question whether the MDA and Iso-P markers at all reflect the reaction of lipid peroxidation attained at reperfusion with pPCI. Another issue may be that the pPCI procedure in itself or together with the anti-thrombotic, anti-platelet drugs protects against reperfusion injury.

Secondly, accurate detection methods of intensity and extension of myocardial infarction with the practical possibility of reiterations are required. Today, tools such as contrast enhanced MRI, echocardiography, ECG changes (ST-elevation, depression or inversion of T-waves) and biomarkers for myocardial damage, such as CKMB or cTnT, which follow the time course of an AMI are commonly available.

Third, for performing a clinical trial studying myocardial reperfusion injury and proposed treatments, a structured choice of clinical endpoints has to be made. Both earlier and present studies have demonstrated a diversity of endpoints, making the studies unfeasible to interpret and to translate to the everyday coronary care setting.

The outline of this thesis is as follows

1. The first part is a clinical section that deals with:
    i. MDA and isoprostanes as biomarkers for oxidative stress from patients with STEMI and treated with pPCI.
    ii. Markers for inflammation and their time-course from patients with first-time STEMI and treated with pPCI.
    iii. Biomarkers for infarct size estimation compared with MRI, from patients with first-time STEMI and treated with pPCI.
2. The second part is an experimental section with animal (pigs, Sus scrofa) and HL-1 mouse cardiomyocyte cell cultures used as models for investigating specific protein releases participating with the Ca^{2+} ion regulation in vivo and in vitro during ischemia and reperfusion.

Clinical section

Oxidative stress

In the first part of the thesis, the clinical studies were performed on patients presenting with STEMI and verified occluded coronary artery. They all achieved a fully controlled, angiographically verified reperfusion by pPCI in combination with anti-thrombotic and anti-coagulant drugs. In the first study, we used two common and accepted markers for the detection of ROS production, namely MDA and Iso-P. MDA is relatively unspecific as a marker for the production of ROS since it merely reflects a cascade reaction such as lipid peroxidation, and is one of its end products [Öhlin 1988]. MDA is also formed during the activation of platelets, which also occur in acute coronary syndrome. It has been proposed that Iso-P would be more specific as a marker for ROS production [Delanty 1997, Reilly 1997, Sakamoto 2002]. Another indirect marker for ROS production is the consumption of scavengers, also called anti-oxidants, such as tocopherol (vitamin E), retinol (vitamin A), and glutathion with the GSSG-GSH switch [Ferrari 1998]. All these markers do not directly quantify the amount of ROS but illustrate the consequences of ROS activation through the pattern of consumption.

Previous clinical studies, from our group and others, have investigated MDA as a marker for ROS production after medical treatment for AMI with thrombolytic therapies such as streptokinase, r-TPA, or recombinant plasminogen activator (rPA). The results demonstrated an increase of plasma MDA [Davies 1990, Young 1993, Öhlin 1995]. This was interpreted as an increase of lipid peroxidation which would reflect an enhanced ROS production caused by the medically attained reperfusion. Following the new guidelines for treatment of AMI by pPCI representing a mechanical approach, combined with anti-thrombotic and anti-coagulant drugs, we performed two studies on this new regimen. Our results demonstrated the opposite, with a significant decrease of MDA in two separate settings. Concerning Iso-P, which has been considered a more specific marker for ROS production [Delanty 1997, Reilly 1997, Sakamoto 2002], no significant change could be demonstrated [Paper I, Paper II]. This gave rise to the question whether these markers, especially MDA, really reflect oxidative stress in this clinical setting, or if

they are too unspecific, reflecting other reactions occurring simultaneously. If this is the case, the pPCI procedure, including the adjunctive medical treatment, may in itself have an anti-oxidative effect scavenging ROS. On the other hand, this regimen does not activate oxidative stress in a similar way to medical thrombolytic therapy. Further studies on MDA after systematic variation of the incorporated components may eventually identify factors that, by themselves, have the ability to decrease its levels. A larger supply of markers for oxidative stress would therefore be of great value for further investigations.

ROS work by directly damaging cellular membranes. They disturb cellular integrity, disintegrate the cellular structure, and induce cell death. ROS also act on intracellular elements by damaging receptors localized on membranes, i.e. ion-exchange pumps such as the NHE and the RyR. RyRs are located on the SR and regulate the intracellular Ca^{2+} ion balance, which is vital for electrical stability and contractility especially in the cardiomyocyte. The function of the RyR is closely connected to the binding of FKBP12 and FKBP12.6, and this protein-receptor complex governs the outflow of Ca^{2+} from the SR to the cardiomyocyte cytosol. As shown in the experimental part of this thesis, explicitly in Paper V, HEDA and HEDA with ROS activation enhanced the amount of FKBP12 and FKBP12.6 in HL-1 cell lysates. This indicates the importance of ischemia and ROS influence and the part of the reperfusion injury that is connected to intracellular calcium homeostasis.

The real existence of ROS during ischemia and reperfusion is no longer a matter of theoretical discussion, but strong clinical evidence is still pending. ROS appear to act in several positions, and at several time points. The initial burst of free electrons during early reperfusion might arise from the early opening of the MPTPs. ROS may also arise from an accumulation of neutrophil leukocytes attracted to the injured area by biological chemoattractants such as interleukins and complement factors, resulting in inflammation. This production of ROS fits better into the late phase of reperfusion injury. ROS also function as cardioprotection by interfering with the RISK pathway, stimulating protective signals [Hausenloy 2007, Yellon 2007].

Another perspective concerning reperfusion injury is the appearance of inflammation. This condition is well established, intensively explored, and considered necessary regarding the process of wound healing [Engler 1986, Go 1988, Dreyer 1991, Vinten-Johansen 2004]. However, inflammation is induced by necrosis, and at the AMI a substantial amount of the cardiac muscle tissue has converted into a necrotic mass. The inflammatory response initiates further production of ROS, which may aggravate the original injury by auxiliary extension. This has been shown in experimental animal models [Bolli 1991]. Previous studies of neutrophil activation have shown an increase in plasma levels of such markers after medical thrombolytic therapy of AMI [Bell 1990, Randalayan 1991, Sylvén 1992]. We, on the other hand, showed an inverse course, with the markers for neutrophil activation, similar to the MDA results after pPCI at AMI levels, decreasing gradually [Paper II]. Today, rapid restoration of coronary flow is mandatory as a treatment of myocardial ischemia. It is also possible that reperfusion itself may decrease the activation/infiltration of neutrophils, despite the generally held notion of reperfusion as a trigger for neutrophil activation. An alternative explanation is that less platelet activation takes place during the pPCI procedure compared with thrombolysis, thus giving the different results and might explain why markers for ROS not are comparable in trials. Another explanation may be that thrombolysis by itself induces an activation of neutrophils or that the pPCI with its adjunctive medical treatment contains an anti-neutrophil effect. Here, as well, may be an issue for further systematic variation of components participating in the treatment to identify factors that may influence the neutrophil activation, i.e. by studying different GPIIb/IIIa receptor blockers and direct thrombin inhibitors with and without the concomitant administration of unfractionated heparin. An interesting finding was made by Li et al, where patients with stable angina pectoris treated with PCI showed transient elevations of MPO levels when treated with unfractionated heparin and the GPIIb/IIIa receptor blocker eptifibatide compared with treatment with the direct thrombin bivalirudin. The conclusion of this study was that the elevation of MPO, as a marker of neutrophil activation, was a direct consequence of the actions of heparin upon leukocytes [Li 2007].

Over time, different methods have been used for the estimation of infarct size, such as MRI, echocardiography, serial measurement of biomarkers and changes in the ECG. This size measurement has its value when performing clinical studies of infarcts and estimated extension possibly due to reperfusion injury. We studied the relation between routine biochemical markers for AMI and DE-MRI [Paper III]. Our findings revealed a good correlation with infarct size measured with DE-MRI and peak values of serial measurements of routine biochemical markers such as CKMB and TnT. These markers are useful for further clinical trials of reperfusion injury, at least if large studies are planned.

Experimental section

The experimental section was initially conducted with animals (pigs) with an open-chest balloon-induced myocardial infarction with reperfusion, elaborately worked out at our institution and well-established and reproducible. With help of the microdialysis technique, myocardial interstitial tissue fluids were obtained and then analysed with proteomic methods. Our results from the microdialysis samples showed a release of a protein, FKBP12, which was significantly higher in the infarcted area compared with samples obtained from a control non-ischemic area from the same heart. This finding was then further explored with RT-PCR analysis of porcine cardiac muscular biopsies and then with HL-1 cell cultures [Paper IV]. FKBP12 and its homologue FKBP12.6 have been demonstrated to be central regulators of intracellular calcium handling by interactions with receptors such as the RyR on the outer SR membrane [Wehrens 2005]. Within the heart, these interactions have important effects on cellular contractile function and arrhythmias. Disturbances in these functions are well in line with what is known of the clinical representation of ischemia and reperfusion injury in the heart [Kloner 1993].

An additional study investigating the cell culture model, using HL-1 cells, was performed with the aim of investigating the impact of HEDA, ROS activation and REPH conditions simulating ischemia and reperfusion. HL-1 cells are murine cardiomyocytes derived from an atrial tumour line and are well designed for research purposes [Claycomb 1998, White 2004].

In our cell model, it was obvious that only hypoxia in itself did not cause cell injury on top of some loss of cell viability over time. This was also observed in normoxic cells, as has been previously described by Andersen et al [Andersen 2009]. We could demonstrate a drop in survival in cells exposed to simulated ischemia for 18 and 24 hours, thus defining a threshold for survival. Another important finding was that, restitution of normoxia, normal pH and culture medium with nutritional components did not add on any decrease of viability, which would have been assumed as a sign of reperfusion injury. However, the HL-1 cell cultures were harvested directly after the decided observational times for protein extraction and physiological signs of disturbances, as stunning or electrical instability (interpreted as arrhythmias) could consequently not be evaluated.
We were able to confirm the previous results from Paper IV with an increase of FKBP12.6 during hypoxic conditions.
Our findings regarding FKBP12 and FKBP12.6 indicate that the content increased after 12 hours of simulated ischemia, and this increase was accompanied by a depressed production measured as mRNA expression. The results may be interpreted as signs of dissociation of these proteins from intracellular receptors to which they bind, i.e., the Ca^{2+} flow regulating receptors, the RyR2 and the IP_3R. A result of this dissociation, not previously described in association with ischemia and reperfusion, may be increased and uncontrolled Ca^{2+} flow from the SR to the cytosol contributing to Ca^{2+} overload and cell injury. This finding may indicate a possible link between ischemia, ROS and FKBP12 and FKBP12.6 in terms of cellular protection systems.

As discussed in my studies, many different mechanisms contributing to reperfusion injury have been investigated. The need for reliable and reproducible biomarkers and newer approaches to identify this event may be a matter of great importance. The increasing number of acute coronary interventions in patients with AMI, of whom many suffer from severely depressed left ventricular function, which may turn into a chronic condition, underlines the need for studies on the pathophysiological mechanisms of reperfusion injury and pharmacological therapies directed against it. My intention with this work has been to try to understand the pathophysiology and to possibly identify some of the

mechanisms contributing to this injury. In the future, this understanding and recognition might lead to the treatment or prevention of reperfusion injury.

Does reperfusion injury exist? Yes, definitely, from my point of view.

To date however, this concept is based on observations of clinical deterioration after reperfusion rather than on firm evidence of injury caused by reperfusion itself. The incidence or prevalence of myocardial reperfusion injury is not known. One explanation may be that myocardial infarcts today have infarct sizes that are less than 20% of the left ventricle, due to accomplished reperfusion with effective treatments. This infarct size (20%) has been considered a critical threshold for the appearance of adverse symptoms such as stunning and arrhythmias. Of course there are infarcts that exceed this percentage but this group of patients is a minority (estimated at 25% of the infarct population) and perhaps not easy to identify [Downey 2009].

Many different mechanisms are proposed as causes of reperfusion injury, such as intracellular Ca^{2+} overload release, perhaps due to gate-keeping receptor failure, tissue acidosis and action of ROS mediated by activated neutrophils, and/or inflammation and the early opening of MPTPs. These are mechanisms that are probably connected in a complicated and dependent network. For achieving any effect against reperfusion injury, a concurrent attack against several of them is necessary to gain a result.

Conclusion

The general aim with this thesis was to gain knowledge about the complex pathophysiology of myocardial reperfusion injury. This has been achieved.

- Markers for the detection of ROS production, such as MDA and Iso-P, and markers of neutrophil activation decreased or remained unchanged following reperfusion accomplished by pPCI, in contrast to results from earlier studies with thrombolysis.
- Routine biomarkers for myocardial injury such as CKMB and TnT may be used as tools for infarct size estimation, which is important for future clinical studies regarding infarct size reduction and diminishing reperfusion injury.
- The intracellular Ca^{2+} flow regulating proteins FKBP12 and FKBP12.6 was released into the myocardium or showed increased production in myocardium subjected to ischemia and reperfusion in a porcine model. These findings were confirmed in cell cultures.
- A cell culture model was developed in which cell survival in response to ischemic stress during different periods could be defined. In this model we could demonstrate that FKBP12 and FKBP 12.6 increased in response to increased ischemia, while production measured as mRNA-expression was depressed. This indicates a release from Ca^{2+} regulating receptors or leakage from membranes due to cell disintegration. These proteins may play a role in intracellular response to ischemia. Dissociation of these proteins from Ca^{2+} regulating receptors may contribute to Ca^{2+} overload that is considered as an important mechanism behind ischemic and reperfusion injury.

Summary in Swedish / Svensk sammanfattning

Myokardiell ischemi och reperfusionsskada, kliniska och experimentella studier.

Vid blodpropp i ett av hjärtats kranskärl kommer en del av hjärtmuskeln att drabbas av en hjärtmuskelskada- en hjärtinfarkt. Återställandet av blodflödet (reperfusion) med antingen läkemedelsbehandling (trombolys) eller med mekanisk behandling (ballongvidgning och stent, dvs PCI) har förbättrat överlevnaden och även minskat risken för hjärtsvikt som senkomplikation till hjärtinfarkten. Vissa patienter drabbas ändå av symtom som akut övergående hjärtsvikt (nedsatt pumpförmåga hos hjärtmuskeln) och ibland av allvarliga/livshotande hjärtrytmstörningar. Detta har tolkats som om att reperfusionen skulle starta ytterligare reaktioner i hjärtmuskeln som medför en förvärrad skada- så kallad reperfusionsskada. Denna finns beskriven som reversibel, dvs övergående, och irreversibel, dvs kvarstående. Olika mekanismer har framlagts som orsak till reperfusionsskadan såsom aktivering av inflammationssystemet och frisättning av fria syreradikaler. Dessa är i sig toxiska substanser som kan trigga igång andra skademekanismer, bland annat kroppens eget försvarssystem, de vita blodkropparna,som i sig initierar inflammation och därutöver initierar ytterligare produktion av fria syreradikaler. Man har även studerat cellernas jonbalans och energiproduktion, samt membranporers funktion i cellens energiverk (mitokondrierna). Ett fokus har lagts på Ca^{2+}-regleringen i hjärtmuskelcellen, som har en stor betydelse både ur kontraktions- och arytmisynvinkel.

Syftet bakom mina studier har varit att kunna förstå och klargöra en del av de patofysiologiska mekanismerna bakom reperfusionsskadan.

I första delarbetet studerades markörer i blod av fria syreradikaler. Dessa är synnerligen kortlivade: 1 milli- till 1 nanosekund, och därför har tillgängliga mätmetoder varit begränsade till indirekta metoder, där man mätt slutprodukter i biokemiska kedjereaktioner. Hjärtinfarktpatienter, som genomgick akut behandling med PCI studerades med analyser av markören malondialdehyd. Konklusionen i arbetet blev att malondialdehyd inte kan betraktas som någon säker markör för fri radikalproduktion vid akut hjärtinfarkt som behandlas med PCI.

I delarbete 2 studerades en ny infarktpatientgrupp. Patienter med förstagångshjärtinfarkt behandlades med akut PCI. Ett flertal inflammationsmarkörer, markörer för aktivering av vita blodkroppar samt återigen markörer för fria radikaler analyserades. Resultatet blev att malondialdehyd inte kan betraktas som säker markör, att inflammationsmarkörer ökar under första dygnet som förväntat samt att vissa av markörerna för förekomst och aktivering av vita blodkroppar var högst före återställande av blodflödet till hjärtmuskeln, för att sjunka under det första dygnet.

I delarbete 3 studerades en del av samma patientmaterial som i delarbete 2. Vi riktade in analyserna på hjärtskademarkörer och korrelerade med grad av hjärtmuskelskada värderad med kontrastförstärkt magnetresonans undersökning (DE-MRI). Slutsatsen i denna studie var att maxvärden av hjärtskademarkörer tagna inom 6 till 12 timmar efter symtomdebut korrelerade väl med DE-MRI-mätt infarkt. Således kan man använda de vedertagna hjärtskademarkörerna för skadebedömning för fortsatta studier under förutsättning att de tages inom rätt tidsintervall.

Delarbete 4 var en djur- och cellodlingsstudie. Första delen av arbetet bestod av en hjärtinfarktmodell på gris, där mikrodialys utfördes direkt i hjärtmuskeln i både infarktområde och ett kontrollområde i samma hjärta, som ej var utsatt för blodbrist. FKBP12 (ett protein) kunde påvisas i infarktområdet med hjälp av proteomik. Produktion av FKBP12 och en sidoform, FKBP12.6, i myokardcellerna kunde dessutom påvisas med RT-PCR analys av biopsier tagna från riskområde vid hjärtinfarkt hos gris. Därutöver utfördes försök på cellodlingar av mushjärtceller (HL-1 celler). Cellerna i vårt försök blev utsatta för syrebrist/hypoxi i 6 timmar och därefter analyserade med RT-PCR och Western Blot (WB) som kunde verifiera produktion och förekomst av FKBP12 och FKBP12.6. FKBP12 och FKBP12.6 har en grindvaktsfunktion på ett proteinreceptorkomplex, (t.ex. ryanodinreceptorn), som är lokaliserad på sarkoplasmatiska retiklets cellmembranyta inuti hjärtmuskelcellen. Sarkoplasmatiska retiklet fungerar som en reservoar för cellens kalciumjoner. Kalciumjonerna ska vara fördelade inuti cellens plasma i reglerad mängd för att upprätthålla balans mellan cellens kontraktion och elektriska förmåga och därigenom åstadkomma en harmonisk rörelse. Hur förhållandet är vid akut ischemi/blodbrist och vid reperfusion är hittills inte helt klarlagt. Vi kunde påvisa med hjälp av olika experimentella modeller, gris och mushjärtcellodlingar, att förekomsten av detta protein FKBP12 och dess sidoform FKBP12.6 ökade inuti cellerna (även med uppreglerad produktion) samt att det läckte ut ur cellerna vid ischemi och reperfusion.
Delarbete 5 var en cellodlingsstudie. HL-1 celler utsattes för olika syreförhållanden med olika tidsperioder (6,12,18 och 24 timmar) för att simulera en modell av syrebristförhållande, dels med normalt pH och dels med sur miljö, samt även toxisk stressnivå av fria radikaler i hjärtcellen. Grupperna som var utsatta för sur och energifattig miljö, samt även de som var påverkade av fria radikaler genomgick därutöver 6 timmars simulerad reperfusion, dvs återställande av normalt cellodlingsmedium, normalt pH och normala syrgasnivåer. Vi kunde påvisa ett tröskelvärde (kring 18 timmar) för överlevnad av HL-1 cellerna som utsattes för olika längder av stressbetingelser med hypoxi i kombination med surgjort media med energibrist. Vi kunde inte påvisa att fria radikaler minskade cellöverlevnaden. Den simulerade reperfusionen åstadkom inte heller någon ökad dödlighet av HL-1 cellerna. Däremot kunde vi visa att både den simulerade ischemin med surgjord och energifattigt medium med och utan fria radikaler ökade nivån av FKBP12 och FKBP12.6 i cellysaten. Vi kunde visa samtidigt att de ischemiska cellerna inte nyproducerade något FKBP12 eller FKBP12.6. Detta tolkas som om att ischemi och fria radikaler påverkar FKBP12 och FKBP12.6 som släpper från sitt proteinkomplex, medför en imbalans av kalciumjoner i hjärtmuskelcellen som ger symtom av upphävd kontraktion och/eller elektrisk instabilitet, motsvarande de kliniska symtom som betraktas som tecken på reperfusionsskada.

De fem olika delarbetena ger en bild av hur man skulle kunna närma sig att detektera reperfusionsskadan. Denna diagnostik kan i framtiden möjliggöra en terapi gentemot reperfusionsskadan och därigenom minska hjärtmuskelskadan och minska uppkomsten av kommande hjärtsvikt.

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