Ischemia and reperfusion-induced damage of the isolated mouse heart : involvement of type IIA secretory phospholipase A2

Ischemia and reperfusion-induced damage of the isolated mouse heart

Involvement of type IIA secretory phospholipase A_2

Proefschrift

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Chapter 1

Introduction

Clinical relevance of ischemia/reperfusion induced myocardial injury

Coronary heart disease is a wide-spread disorder in the western industrialized world. Although a downward trend is observed in mortality rates due to coronary heart disease, it remains the most important cause of death (O Rourke *et al.* 1993, Uemura *et al.*, 1988, Widdershoven *et al.*, 1997). The most common clinical manifestations of the disorder include myocardial ischemia, unstable angina pectoris, cardiac arrhythmias and sudden cardiac arrest. Myocardial ischemia is often caused by an atherosclerotic stenosis of coronary arteries, while sudden cardiac syndromes such as acute myocardial infarction generally results from a thrombotic occlusion of the coronary vessel. Prolonging myocardial ischemia will eventually result in myocardial cell death by necrotic and apoptotic processes or a combination thereof (Schaper *et al.*, 1988, MacLellan *et al.*, 1997). Currently, clinical treatment mainly consists of techniques to re-open the thrombotic occlusion of a coronary vessel (reperfusion) by intracoronary thrombolysis and percutaneous transluminal coronary angioplasty. Although reperfusion is a prerequisite to prevent inevitable death of the flow-deprived cardiac muscle cells, indications exist that reperfusion itself may induce additional myocardial cell damage (Piper *et al.*, 1990, Fox *et al.*, 1992). In spite of intensive research over the past 3 decades, knowledge of the mechanisms of ischemia and reperfusion-induced cardiac cell injury is still limited and an expansion of our understanding of the causal events behind this particular disorder may have substantial therapeutic potentials.

Ischemia and reperfusion-induced cell damage

Impaired blood supply to cardiac tissue leads to a range of functional, biochemical and ultrastructural changes in cardiomyocytes (Jennings *et al.*, 1978, Schaper *et al.*, 1979, Reimer *et al.*, 1983, Post *et al.*, 1985, Van der Vusse *et al.*, 1992). Interruption of blood flow will immediately result in a lack of oxygen supply to the cardiac muscle cell. As a result mitochondrial respiration ceases and ATP generation by oxidative phosphorylation is hampered. Glycolysis, the conversion of glucose to lactate, will subsequently become the major process by which the cardiac cell can generate ATP. A negative side-effect of acceleration of anaerobic glycolysis is acidification of the oxygen deprived cells. Since ATP generation through glycolysis is insufficient to cover myocardial energy demand (Jennings *et al.*, 1978), the tissue content of phosphocreatine and ATP will decline, despite the fact that contractile activity is slowed or even stopped (Ganote *et al.*, 1975).

Cellular energy depletion will manifest itself in impaired cellular ion homeostasis, which leads to a rise in intracellular Na^+, through activation of the Na^+/H^+ exchanger. To compensate for the intracellular increase in Na^+, the Na^+/Ca^{2+} exchanger is then activated, resulting in intracellular Ca^{2+} accumulation (Peng *et al*,. 1980, Li *et al.*, 1988, Allshire *et al.*, 1989). The increase in intracellular Ca^{2+} most likely results in activation of Ca^{2+}-dependent proteases that degrade the cytoskeleton and cytoskeletal membrane connections (Schwartz *et al.*, 1984). Furthermore, activation of Ca^{2+}-dependent phospholipases might result in accumulation of membrane-destabilizing factors like lysophospholipids and fatty acids. Collectively, these processes will weaken the (intracellular) membranes of the sarcolemma, sarcoplasmatic reticulum and

mitochondria (Van der Vusse *et al.*, 1982, Chien *et al.*, 1984, Van Bilsen *et al.*, 1989). Loss of lysosomal integrity during ischemia will result in the release of lysosomal proteases and phospholipases, which will also contribute to the degradation of cellular structures (Decker *et al.*, 1990).

Prolongation of the ischemic duration eventually leads to cardiac cell death by a combination of the above mentioned processes. If restoration of blood flow takes place soon after oxygen deprivation, complete structural and functional recovery of the cardiac muscle cell is possible. If the duration of the ischemic episode is extended beyond a certain point, ischemic injury becomes irreversible and will lead to cell death (Schaper *et al.*, 1988, MacLellan *et al.*, 1997). Although restoration of flow to the weakened myocardium is necessary to alleviate the detrimental impact of ischemia, reperfusion itself may induce additional microvascular and cellular damage (the so-called reperfusion-injury; Piper *et al.*, 1990, Fox *et al.*, 1992). Whether reperfusion per se inflicts cellular damage or that the reperfusion phenomenon is merely a manifestation of ongoing destruction of the cardiac muscle cell already started during the preceding ischemic interval, is still matter of continuous debate (Miura *et al.*, 1990, Fox *et al.*, 1992).

Restoration of blood flow to the ischemic region of the heart results in enhanced supply of, among others, water, oxygen calcium ions and oxidizable substrates to the previously weakened cardiomyocytes. Due to the accumulation of low-molecular weight substanses, such as lactate, degradation products of adenine nucleotides and inorganic phosphate, reperfused cells readily take up water from the microvascular compartment during the initial reperfusion phase, which imposes an osmotic load on the affected cardiac muscle cell (Steenbergen *et al.*, 1985). Moreover, the continuing increase in intracellular Ca^{2+} may result in substantial accumulation of these ions in cardiac muscle cells (Peng *et al.*, 1980, Schwartz *et al.*, 1984, Allshire *et al.*, 1989). The increase in supply of O_2 and oxidizable substrates will result in enhanced production of oxygen free radicals by mitochondria, which may be partially damaged during the preceding period of flow deprivation (Ceconi *et al.*, 1982). Moreover, the decline in activity of a set of enzymes involved in oxygen free radical scavaging may add to the damaging effect of oxygen free radicals (Ferrari *et al.*, 1988).

The nature of the relationship between increased osmotic load, elevated intracellular Ca^{2+} levels and enhanced production of oxygen free radicals on the one hand, and loss of cellular viability on the other, is most likely complex. For example, the weakening of membranes by physical forces and the hydrolytic activity of Ca^{2+}-dependent proteases and Ca^{2+}-dependent phospholipases (membrane phospholipids damaged by oxygen free radicals are more vulnerable for hydrolysis; Weglicki *et al.*, 1973, Dan *et al.*, 1996) may contribute to the loss of cardiac cell integrity during the initial reperfusion phase.

Role of phospholipase A_2 in phospholipid degradation during ischemia and reperfusion

A variety of studies suggest that membrane phospholipid degradation is a critical event in the development of irreversible myocyte injury in flow-deprived cardiac tissue (Van der Vusse *et al.*, 1982, Chien *et al.*, 1984, Van Bilsen *et al.*, 1989, Van der Vusse *et al.*, 1992). Net degradation of phospholipids under

pathophysiological conditions is thought to destabilize cardiac membranes and to result in loss of function of the semi-permeable barrier as evidenced by the release of macromolecules into the extracellular environment (Van Bilsen *et al.*, 1989). The mechanism behind ischemia-induced phospholipid degradation is unclear, but may be due to the enhanced activity of endogenous phospholipase A_2 in the heart (Van der Vusse *et al.*, 1989, Van Bilsen *et al.*, 1995), alone or in combination with impaired resynthesis of phospholipid molecules (Van Bilsen *et al.*, 1989). The observation that arachidonic acid, which under normoxic conditions is predominantly esterified in the cardiac phospholipid pool (Van der Vusse *et al.*, 1992), readily accumulates during ischemia suggest degradation of membrane phospholipids (Van der Vusse *et al.*, 1982, Chien *et al.*, 1984, Van Bilsen *et al.*, 1989). Interestingly, accumulation of arachidonic acid proceeds even faster following restoration of flow to the previously ischemic myocardium (Van Bilsen *et al.*, 1989).

Experimental data suggest that especially cardiac type IIA phospholipase A_2, a low molecular mass member of the phospholipase A_2 family, is a likely candidate to be involved in ischemia-induced degradation of membrane phospholipids and subsequent cellular damage (Van der Vusse *et al.*, 1989, Van Bilsen *et al.*, 1995). The observation that in addition to arachidonic acid a whole set of long-chain fatty acids accumulate during prolonged myocardial ischemia and reperfusion of the isolated rat heart strongly suggests a predominant role of the aspecific type IIA phospholipase A_2 (Van Bilsen *et al.*, 1995). The notion that reperfusion is accompanied by a calcium overload of the ischemic cardiac muscle cell supports a predominant role of the calcium-dependent type IIA phospholipase A_2 (Van der Vusse *et al.*, 1992, Van Bilsen *et al.*, 1995). Finally, in a number of ischemia/reperfusion and anoxia/reoxygenation studies membrane phospholipid degradation and release of cytoplasmic proteins into the extracellular milieu could be efficiently blocked by pharmacological inhibition phospholipase A_2 and by antibodies raised against type IIA related phospholipases A_2 (Van Bilsen *et al.*, 1990, Prasad *et al.*, 1991, Kikuchi-Yanoshita *et al.*, 1993).

Aims of the present study

The present study specifically focusses on the hypothesis that hydrolytic activity of type IIA secretory phospholipase A_2 is involved in ischemia/reperfusion-induced phospholipid degradation. Testing of this hypothesis required an integration of molecular biological, biochemical and physiological techniques. Accordingly, the first aim was to establish the presence, abundance and cellular localization of type IIA secretory phospholipase A_2 in cardiac muscle by means of molecular biological and immunochemical techniques. The second aim was to develop and characterize an ex vivo, antegradely perfused mouse heart model, which allows detailed analysis of the functional and metabolic changes, with special emphasis on changes in phospholipid metabolism, during cardiac ischemia and reperfusion. Such an ex vivo model system would be helpful for the evaluation of cardiac function in genetically modified mice. The third and final aim of this thesis was to determine the role of type IIA secretory phospholipase A_2 in ischemia/reperfusion induced cellular damage by means of mutant inbred mouse

strains that lack functional type IIA secretory phospholipase A_2 and by creating transgenic mice that overexpress this phospholipase isoform in a cardiac-specific manner.

General outline of the thesis

The theoretical aspects of cardiac phospholipid homeostasis under (patho)physiological conditions are described in *Chapter 2*. In addition, an overview is given of the currently described mammalian phospholipase A_2 types that may play a role in myocardial ischemia and reperfusion-induced cellular injury. *Chapter 3* describes the cloning of the full length cDNA and molecular characterization of rat heart type IIA secretory phospholipase A_2. In *Chapter 4* the expression and purification of recombinant rat heart type IIA phospholipase A_2 is described. In addition, the ability of a polyclonal antisera raised in the rabbit against this purified recombinant enzyme was compared with several anti-type IIA secretory phospholipase A_2 antibodies to detect type IIA secretory phospholipase A_2 at the protein level. In *Chapter 5*, the development and characterization of the isolated, left ventricular ejecting mouse heart model is described. The stability of the mouse heart in this experimental model under normoxic conditions was characterized in terms of functional and metabolic performance. In *Chapter 6* the ischemia tolerance of the antegradely perfused wildtype mouse heart was determined by investigating functional and metabolic recovery following various periods of ischemia and subsequent reperfusion. In *Chapter 7* the ischemia tolerance of hearts derived from heterozygous IGF-1 knockout mice is compared to their wildtype littermates. In addition, the functional recovery and extent of irreversible cell injury following global ischemia and reperfusion is related to the tissue content of arachidonic acid in the reperfused ventricles. The study on heterozygous IGF-1 knockout mice was included to test the sensitivity of the isolated working mouse heart preparation to detect small differences in ischemia/reperfusion-induced cardiac injury. In *Chapter 8* this isolated murine heart model is applied to determine the role of type IIA secretory phospholipase A_2 in ischemia/reperfusion damage in more detail by making use of genetically closely related inbred mouse strains, one of which is wildtype and the other contains two mutated type IIA secretory phospholipase A_2 alleles, resulting in a non-functional protein product. In *Chapter 9*, prelimenary results are given about generation of transgenic mice with a cardiac-specific overexpression of type IIA secretory phospholipase A_2. In the general discussion (*Chapter 10*) the data obtained in this thesis are discussed in a broader spectrum and future directions for research are delineated. The thesis is preceded by an *Introduction*.

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Chapter 2

Phospholipase A_2-mediated hydrolysis of cardiac phospholipids: a review of literature.

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Abstract

Under pathophysiological conditions, like myocardial ischemia and reperfusion, cardiac phospholipid homeostasis is severely disturbed, resulting in a net degradation of phospholipids and the accumulation of degradation products, such as lysophospholipids and (non-esterified) fatty acids. The derangements in phospholipid metabolism are thought to be involved in the sequence of events leading to irreversible myocardial injury. The net degradation of phospholipids as observed during myocardial ischemia may result from increased hydrolysis and/or reduced resynthesis, while during reperfusion hydrolysis is likely to prevail in this net degradation. Several studies indicate that the activation of phospholipases A_2 plays an important role in the hydrolysis of phospholipids. In this review current knowledge regarding the potential role of the different types of phospholipases A_2 in ischemia and reperfusion-induced damage is being evaluated. Furthermore, it is indicated how recent advances in molecular biological techniques could be helpful in determining whether disturbances in phospholipid metabolism indeed play a crucial role in the transition from reversible to irreversible myocardial ischemia and reperfusion-induced injury, the knowledge of which could be of great therapeutic relevance.

Introduction

All living cells are surrounded by membranes, consisting of phospholipids, cholesterol and proteins. The cell membrane (plasmalemma) serves as a selective barrier to create and maintain an internal environment to allow the cell to fulfil its specific functions. In addition to its function as physical barrier, the plasmalemma is also an important source of phospholipid-derived bioactive lipids, like lysophospholipids and fatty acids, such as arachidonic acid (Van der Vusse *et al.*, 1992). Arachidonic acid can subsequently serve as a substrate for the production of auto/paracrine factors, including prostaglandins and thromboxanes (Karmazyn *et al.*, 1989, Engels *et al.*, 1990).

The integrity of the membrane is a prerequisite for proper functioning of the cell. Therefore, the major constituents of the plasmalemma, the phospholipids, are subjected to a continuous turnover process to enable the cell to synthesize any required phospholipid and to regulate the fatty acyl composition of the phospholipids (Van der Vusse *et al.*, 1992). In this way, the cellular membrane adjusts its physico-chemical properties in response to changes in the extracellular environment. The turnover of phospholipids requires the liberation of fatty acids from phospholipids (deacylation), as well as the reincorporation of fatty acyl moieties into the phospholipid pool (reacylation). These phospholipid turnover cycles are also operative in cardiomyocytes (Van der Vusse *et al.*, 1992).

During myocardial ischemia/reperfusion a net phospholipid degradation has been observed, either caused by increased activity of phospholipid hydrolyzing enzymes, such as phospholipase A_2, or caused by an impairment of the resynthesis of phospholipids (Van Bilsen *et al.*, 1989, Van der Vusse *et al.*, 1990, Van der Vusse *et al.*, 1992, Van Bilsen *et al.*, 1995). Therefore, detailed knowledge of cardiac phospholipid homeostasis and derangements therein under pathophysiological conditions can be of great therapeutic value, as ischemic heart diseases as a result of the occlusion of a coronary artery are still by far the most common cause of death in western industrialized countries.

In this review we will focus on cardiac phospholipase A_2-mediated phospholipid hydrolysis and its possible role in the derangements in phospholipid homeostasis as a crucial event in the transition from reversible to irreversible cell damage as a result of myocardial ischemia and reperfusion. Although not the subject of this review, it is important to note that derangements in the resynthesis of phospholipids may also be involved, especially under energy-deprived conditions like ischemia. In addition, the usefulness of sophisticated molecular biological techniques in combination with an appropriate murine heart model in the assessment of the role of phospholipases A_2 during myocardial ischemia and reperfusion will be discussed.

Cardiac phospholipids

Myocardial membranes consist of a variety of phospholipid species, differing in chemical composition of the hydrophylic alcoholic headgroup, or of the hydrophobic tail that is composed of two long-chain fatty acyl residues connected to

the glycerol backbone. The phospholipid subtypes are classified by their hydrophylic alcohol headgroup. The hydrophylic alcohol is attached to the third (*sn-3*) carbon atom of the glycerol moiety via an inorganic phosphate (Figure 1). The hydrophobic part of phospholipids is formed by the fatty acyl chains bound to the first (*sn-1*) and second (*sn-2*) carbon atom of the glycerol backbone (Figure 1). The *sn-1* and *sn-2* fatty acyl residues are generally a saturated and (poly-)unsatured fatty acid, respectively. The number of carbon atoms of the long-chain fatty acyl residues commonly ranges from 14 to 24. The number of unsaturated bonds of the *sn-2* fatty acyl chain may vary between zero and six (Van der Vusse *et al.*, 1992).

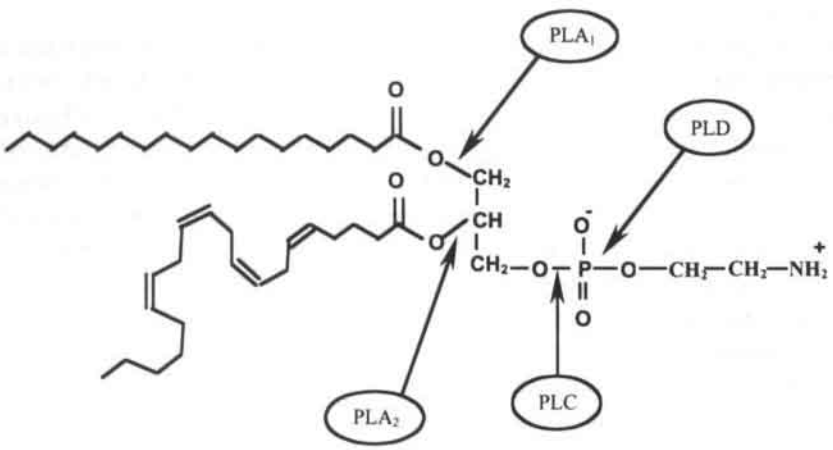

Figure 1. Chemical structure of 1-palmitoyl, 2-arachidonoyl phosphatidylethanolamine and the cleavage site of different phospholipases. The arrows point to the covalent bond hydrolyzed by phospholipase A_1 (PLA_1), phospholipase A_2 (PLA_2), phospholipase C (PLC) and phospholipase D (PLD).

The fatty acyl residues at the *sn-1* and *sn-2* position are generally O-acyl-residues, i.e., they are connected to the glycerol backbone via an ester linkage. In membrane phospholipids, however, a certain proportion of the fatty acyl residues at the *sn-1* position is connected to the *sn-1* carbon atom of glycerol via a vinyl ether linkage (O-[1-alkenyl]-residues). These phospholipids are commonly referred to as plasmalogens. The plasmalogen content in membranes is tissue-dependent and varies between cardiac membranes of different animal species, ranging from ~ 5% in the rat heart to ~ 40% in the rabbit heart (Hatch *et al.*, 1989).

Because of their shape and amphipathic nature, phospholipids readily form lipid bilayers in an aqueous environment. In case of the sarcolemma, the hydrophilic headgroups of the inner leaflet point towards the intracellular space and those of the outer leaflet towards the extracellular space. The fatty acids tails are burried in the inner part of the bilayer.

The various phospholipid classes in the sarcolemmal bilayer are asymmetrically distributed. The outer leaflet is relatively enriched with phosphatidylcholine and sphingomyelin, while the negatively charged

phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol are located almost exclusively in the cytoplasmic leaflet. This phenomenon results in a more negatively charged inner leaflet as compared to the outer sarcolemmal leaflet. To compensate for differences in fluidity between the leaflets, the cholesterol/phospholipid ratio is higher in the outer leaflet of the sarcolemma (Post *et al.*, 1988).

Phospholipid homeostasis in the normoxic situation

Under normoxic conditions the amount of intracellular (non-esterified) fatty acids is very small, indicating that the pool size of these fatty acids is well controlled (Van der Vusse *et al.*, 1992). Apparently a strict balance exists between the activity of enzymes that control the liberation of fatty acyl moieties from phospholipid molecules and that of enzymes that control the rate of incorporation of fatty acyl residues into the phospholipid pool.

The cardiomyocyte is equipped with a set of enzymes required for the resynthesizing part of phospholipid turnover as well as for the hydrolytic part of the phospholipid turnover cycle. *De novo* synthesis of phospholipids takes place in the sarcoplasmic reticulum. Newly synthesized phospholipids are transported to the sarcolemma through the cytosol (Van der Vusse *et al.*, 1992). For *de novo* synthesis of phoshatidylcholine the condensation of 1,2-diacylglycerol and CDP-choline is achieved by the action of choline-phosphotransferase. When the substrate is a lysophoshatidylcholine, the incorporation of the second fatty acyl moiety is dependent on the activity of the enzymes acyl-CoA synthetase and lysophosphatidylcholine acyltransferase (Figure 2).

Among the enzymes involved in the hydrolytic part of the phospholipid turnover process are phospholipase A_1 (PLA_1) and phospholipase A_2 (PLA_2), which hydrolyze the ester bond between the fatty acyl unit at the *sn-1* and *sn-2* carbon atom of glycerol, respectively. In case of a plasmalogen, a plasmalogen-specific type PLA_2 is present in the cardiomyocyte to remove fatty acyl chains from the *sn-2* position (Hazen *et al.*, 1991). The vinyl ether bond at the *sn-1* position is hydrolyzed by a specific plasmalogenase (Arthur *et al.*, 1985). Phospholipase C (PLC) breaks the covalent bond between the polar headgroup and the carbon atom at the *sn-3* position of glycerol and phospholipase D (PLD) the bond between phosphate and the alcohol unit that together form the polar headgroup (Figure 1). The final products of phospholipase A_1 and A_2 activity are lysophospholipids and fatty acids. In turn, the remaining fatty acyl chain of lysophospholipids can be removed by lysophospholipase. It has been hypothesized that in cardiac cells the balance between the activities of lysophospholipid acyltransferase and phospholipase A_2 determines the actual level of unesterified arachidonic acid (Irvine, 1982, Van der Vusse *et al.*, 1992).

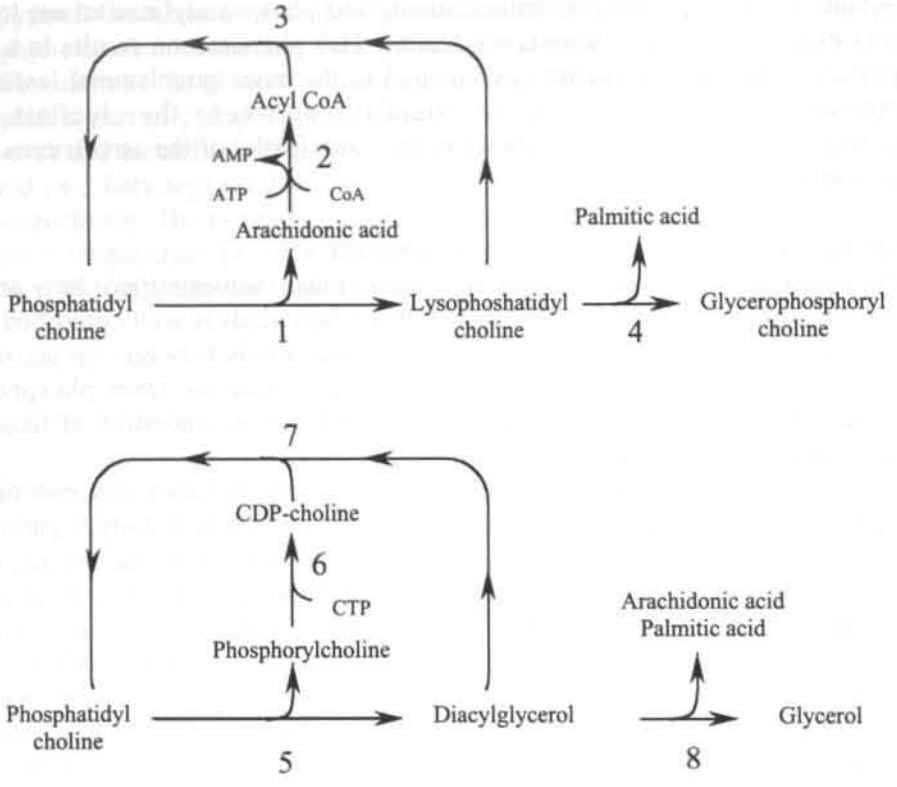

Figure 2: Synthesis and degradation of 1-palmitoyl, 2-arachidonoyl phosphatidylcholine. *Pathway I* (upper panel) describes the deacylation-reacylation pathway for this phospholipid. *Pathway II* depicts the turnover of the hydrophylic headgroup of phsphatidylcholine. Numbers refer to the enzymes involved in the pathways: (1) phospholipase A_2, (2) fatty acyl-CoA synthetase, (3) lysophosphatidylcholine acyltransferase, (4) lysophospholipase, (5) phospholipase C, (6) CTP:phosphocholine cytidyltransferase, (7) phosphocholine transferase, (8) diacylglycerol + monoacylglycerol lipases. [Reprinted with permission from Van der Vusse *et al.* 1992]

Studies with radiolabeled substrates show that the cardiomyocyte is able to change the polar headgroup as well as the fatty acyl units of the membrane phospholipids (Van der Vusse *et al.*, 1992). Detailed knowledge about phospholipid turnover rate in cardiomyocytes, however, is still lacking. *In vitro* measurements revealed a maximal myocardial total phospholipase activity of about 3 µmol phosholipid . h^{-1}. g^{-1} tissue in rat heart (Termin *et al.*, 1987). Based on the presence of approximately 30 µmol total phospholipid . g^{-1} tissue and assuming that degradation of phospholipids keeps pace with resynthesis, complete turnover of all sarcolemmal phospholipids could be accomplished in about 10 hours. However, it cannot be excluded that phospholipid species in different subcellular compartments are subjected to different turnover rates (Miyazaki *et al.*, 1987, Miyazaki *et al.*, 1990).

The heart contains at least three different types of phospholipases A_2. Recently, a member of type IIA secretory PLA_2 (type IIA $sPLA_2$) was cloned in our laboratory (De Windt *et al.*, 1996). All mammalian type II PLA_2s are small molecular weight acylhydrolases of about 14 kD with a slight preference for phosphatidylserine and phosphatidylethanolamine over phosphatidylcholine, but they have no specificity for the type of fatty acyl moiety at the *sn-2* position. Type IIA $sPLA_2$s have been found to be associated with membranes like the plasmalemma of guinea pig spermatozoa (Garcia *et al.*, 1991), the outer contact sites and the inner membrane of mitochondria (Levrat *et al.*, 1992), or the matrix of α-granules in platelets (Aarsman *et al.*, 1989). Furthermore, type IIA $sPLA_2$ can be secreted from rat platelets upon stimulation, due to the presence of a putative eukaryotic signal sequence for secretion at the N-terminal end of the enzyme (Horigome *et al.*, 1987). Whether in cardiomyocytes type IIA $sPLA_2$ is present in a membrane-associated form or can be secreted remains to be elucidated. Circumstantial evidence for a membrane-associated localization of type IIA $sPLA_2$ in cardiomyocytes was provided by a cytochemical study by Kriegsmann and coworkers, in which they showed that a monoclonal antibody against bee venom type II $sPLA_2$ bound to antigens at the level of the sarcolemma (Kriegsman *et al.*, 1993).

The knowledge of mechanisms involved in the regulation of type IIA $sPLA_2$ activity is gradually increasing; mechanisms of short-term regulation of the enzyme activity and those involved in long-term regulation of enzyme content can be distinguished (Van Bilsen *et al.*, 1995). Short-term regulation is based on the requirement of (sub)millimolar calcium concentrations for maximal activity of the enzyme. This raises questions about the probability of its intracellular activity under normoxic conditions, as in the cardiomyocyte the overall intracellular calcium concentration oscillates from 0.15 μM during diastole to 2.0 μM during systole. However, Langer and colleagues have demonstrated that in specific regions close to the sarcolemma calcium concentrations up to 600 μM may be reached during systole, postulating the existence of multiple compartments with different calcium concentrations within the cytosol (Langer, 1994). Accordingly, the calcium concentration in the subsarcolemmal space might be sufficient to allow intracellular activity of type IIA $sPLA_2$ under physiological conditions.

As far as the short-term regulation of extracellular activity is concerned evidence has been provided that type IIA $sPLA_2$ activity can be modulated by the association of the enzyme via its putative C-terminal heparin-binding domain to the proteoglycans of the extracellular matrix. The association of type IIA $sPLA_2$ with the sulfated polysaccharides results in changes of enzyme activity on membrane phospholipids (Sartipy *et al.*, 1996). This mechanism of regulation could be of importance for the heart, if cardiac type IIA $sPLA_2$ is associated to the extracellular part of the sarcolemma, where calcium concentrations are no longer a limiting factor for the activity of the enzyme. Finally, it appears that type IIA $sPLA_2$ activity can be modulated by accessory proteins, like phospholipase A_2-activating protein (PLAP) which has a stimulatory effect, or uteroglobins and annexins which are putative inhibitors of type IIA $sPLA_2$ activity. The exact mechanism of action and

the physiological significance of these accessory proteins on type IIA $sPLA_2$ activity are still a matter of debate (Van Bilsen *et al.*, 1995, Jans *et al.*, 1995).

Long-term regulation of type IIA $sPLA_2$ activity is accomplished by adjusting the level of gene transcription. In several cell types type IIA $sPLA_2$ expression is induced by inflammatory cytokines like interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor α (TNFα), or by cAMP-elevating substances like forskolin (Schalkwijk *et al.*, 1992, Pfeilschifter *et al.*, 1993, Konieczkowski *et al.*, 1993). These observations were substantiated by the demonstration of the presence of cAMP and cytokine responsive elements in the 5 - flanking region of the type IIA $sPLA_2$ gene (Ohara *et al.*, 1990). In contrast, the expression of this enzyme is downregulated by a variety of substances like glucocorticoids, transforming growth factor β (TGFβ), aspirin, and tetranactin (Schalkwijk *et al.*, 1992, Van den Bosch *et al.*, 1992, Vervooldeldonk *et al.*, 1996, Walker *et al.*, 1996). Recently it was shown that activation of the nuclear transcription factor NF-κB is an essential component of the cytokine signalling pathway responsible for type IIA $sPLA_2$ gene regulation (Vervoordeldonk *et al.*, 1996). That this mechanism of regulation is also operative in cardiac cells is supported by the observation that type IIA $sPLA_2$ mRNA levels increase in cultured rat neonatal cardiomyocytes upon stimulation with TNFα (De Windt *et al.*, 1996).

The recent discovery of a high-molecular mass phospholipase A_2 (85-110 kD) or type IV cytosolic PLA_2 (type IV $cPLA_2$) led to a different view of the activation of *sn-2* acylhydrolysis through receptor mediated signal transduction (Scharp *et al.*, 1993). This enzyme selectively cleaves arachidonoyl residues at the *sn-2* position of membrane phospholipids. Type IV $cPLA_2$ is equally active against ethanolamine- and choline containing phospholipids, and hydrolyzes both 1-acyl-2-arachidonoyl phospholipids, and 1-alkenyl-2-arachidonoyl phospholipids. Type IV $cPLA_2$ has a predominantly cytosolic localization and requires Ca^{2+} in the micromolar range for translocation to its site of action, the phospholipid bilayer. Because of these features, the high molecular mass cytosolic phospholipase A_2 is a likely candidate for the acute liberation of arachidonic acid from membrane phospholipids in cardiac cells under physiological conditions, e.g. by alterations in the cellular Ca^{2+} concentration as a result of ligand-receptor interactions. This notion is substantiated by the observation that type IV $cPLA_2$ itself is a substrate for mitogen-activated protein kinases (MAPK), which serve as important mediators for a variety of receptor-mediated signal transduction pathways (Lin *et al.*, 1993).

The third type of phospholipase A_2 present in the myocardium was first reported in 1985 by Gross and coworkers (Wolf *et al.*, 1985). It has a molecular mass of ~ 40 kD and was shown to be Ca^{2+}-independent for its activity. Although it has a preference for *sn-2*-arachidonoyl plasmalogens, it is also capable of hydrolyzing diacyl-phospholipids or plasmalogens with other fatty acids at the *sn-2* position, albeit at lower rate (Diez *et al.*, 1994). Ca^{2+}-independent, plasmalogen specific type V phospholipase A_2 (type V $iPLA_2$) is localized in the cytosol and can translocate to the cell membrane by interacting with the glycolytic enzyme phosphofructokinase (PFK), forming a large 400 kD protein complex, indirectly regulating the activity of type V $iPLA_2$ via allosteric modulation of PFK by cytosolic ATP levels (Hazen *et al.*, 1993). Recently a novel regulatory mechanism

was identified whereby the Ca^{2+}-independent type V $iPLA_2$ is modulated indirectly by calcium ions, i.e., through alterations in the interaction of the phospholipase complex with calmodulin (Wolf *et al.*, 1996).

To summarize, at least three different types of phospholipase A_2 activities have been identified in the heart: a 14 kD Ca^{2+}-dependent type IIA $sPLA_2$, type V $iPLA_2$ and type IV $cPLA_2$. Recently, Chen and colleagues cloned another low molecular mass Ca^{2+}-dependent phospholipase A_2 from rat heart that showed only limited homology to type IIA $sPLA_2$ (Chen *et al.*, 1994). Dennis and coworkers demonstrated that type IIA $sPLA_2$, type IV $cPLA_2$ and type V $iPLA_2$ were involved in the release of arachidonic acid following stimulation of $P388D_1$ macrophages (Balsinde *et al.*, 1996). Interestingly, type IIA $sPLA_2$ was accountable for 60-70 % of the AA release in this particular model system. As far as cardiomyocytes are concerned the contribution of the different phospholipase A_2 types in the ligand-receptor-induced hydrolysis of phospholipids remains to be established.

Effect of ischemia and reperfusion on cardiac phospholipids

Ischemic heart diseases are predominantly caused by impaired coronary perfusion. If the ischemic period has a limited duration, structural and functional recovery of the myocardium is possible by timely restoration of blood flow. If the ischemic period is prolonged, however, injury becomes irreversible, eventually leading to cell death and cardiac dysfunction. During myocardial ischemia phospholipid homeostasis is disturbed. The net degradation of phospholipids results in an increase of their degradation products, mainly fatty acids and lysophospholipids (Weglicki *et al.*, 1973, Van der Vusse *et al.*, 1982, Chien *et al.*, 1984, Van Bilsen *et al.*, 1989). The sarcolemma appears to be the primary target for accelerated phospholipid degradation in oxygen and energy-depleted cardiomyocytes (Van der Vusse *et al.*, 1992). Although timely restoration of blood flow is required to save the myocardium from irreversible damage, an even more dramatic rise in the tissue levels of lysophospholipids and fatty acids has been observed during the reperfusion phase. Whether this reperfusion-induced injury is a manifestation of cell damage which was already present during ischemia or is a caused by the re-introduction of blood flow *per se* to the previously ischemic myocardium, is still a matter of debate (Van der Vusse *et al.*, 1989, Van der Vusse *et al.*, 1994).

Currently there are two hypotheses concerning the mechanism involved in the increased phospholipid degradation of the sarcolemma during ischemia and reperfusion (Van der Vusse *et al.*, 1989, Van der Vusse *et al.*, 1994). The first hypothesis implies that the integrity of the sarcolemma is lost due to physical forces, before the phospholipids are hydrolysed. Mechanical stress will be imposed on the sarcolemma of energy-deprived cells by adjacent non-ischemic, contracting myocytes or by hypercontracture of the ischemic cells themselves. In addition, low-molecular weight substances such as lactate, protons and inorganic phosphate, will accumulate inside ischemic myocytes. This will lead to a shift of water from the extracellular to the intracellular space, imposing an increased osmotic force on the cell membrane. Together with weakening of the anchoring of the cytoskeleton to the sarcolemma, this might result in enhanced membrane fragility. When the

sarcolemma is unable to withstand the physical forces anymore the sarcolemma will rupture, after which intracellular structures will become accessible to the extracellular fluid. This will lead to the activation of, amongst others, Ca^{2+}-dependent proteases and phospholipases, which will start to digest cellullar components. According to the physical forces hypothesis, degradation of phospholipids is merely an epiphenomenon, reflecting post-mortem autolysis of cardiac cells (Van der Vusse *et al.*, 1989, Buja *et al.*, 1991).

In the second hypothesis, the loss of integrity is explained, among others, by a imbalance between hydrolysis and resynthesis of the membrane phospholipids. As indicated before, this may result from increased activity of hydrolyzing enzymes, like phospholipases, or impaired resynthesis due to loss of catalytic activity of the enzymes involved. Acyl-CoA synthetase, an enzyme involved in phospholipid resynthesis, requires ATP as cofactor and is inhibited by AMP and adenosine. Therefore, the ischemia-induced decrease in cellular ATP and elevated levels of AMP and adenosine might lead to impairment of resynthesis (Van Bilsen *et al.*, 1989). In this light it is also of interest to note that the activity of lysophosphatidyl acyltransferase was found to be compromized in the ischemic pig heart (Das *et al.*, 1986) while in several studies PLA_2 activity was found to be increased in the ischemic and reperfused heart (Van der Vusse *et al.*, 1992). The latter hypothesis implies that the increased degradation of membrane phospholipids plays a pivotal role in the transition to irreversible injury of cardiomyocytes during myocardial ischemia (Buja *et al.*, 1991, Van der Vusse *et al.*, 1992).

Role of PLA_2 in ischemia/reperfusion induced phospholipid hydrolysis

Several studies indicate that the activation of especially PLA_2 plays an important role in the transition from reversible to irreversible ischemic myocardial injury. First, in ischemic myocardium various phospholipid degradation products accumulate, including arachidonic acid and lysophospholipids. As arachidonic acid is predominantly (99 %) located in the esterified form at the *sn-2* position in phospholipids, its liberation is conceivably due to PLA_2 activity (Van der Vusse *et al.*, 1992). Second, chemical inhibitors of PLA_2 have been reported to protect the cardiomyocytes against ischemia, anoxia, or energy-deprivation (Van Bilsen *et al.*, 1990, Atsma, 1996).

Earlier attempts to identify the PLA_2 type involved in the enhanced phospholipid hydrolysis during myocardial ischemia and reperfusion have focussed on the plasmalogen-specific, Ca^{2+}-independent type V $iPLA_2$. Hazen and coworkers reported that in the rabbit heart the activity of type V $iPLA_2$ is greatly enhanced already 2 min after the onset of ischemia (Hazen *et al.*, 1991). Moreover, halo-enol-lactone suicide substrate (HELSS), a specific inhibitor of type V $iPLA_2$ was found to reduce cell death in cultured neonatal cardiomyocytes exposed to chemical anoxia (Atsma *et al.*, 1996). However, several other observations raise questions as to whether cardiac type V $iPLA_2$ plays an important role in the ischemia/reperfusion-induced phospholipid degradation. First, in cardiac homogenates the diacyl-form of phosphatidylethanolamine is hydrolyzed rather than the plasmalogen-form of phosphatidylethanolamine (Kikuchi-Yanoshita *et al.*,

1993). In line with the latter finding Davies and coworkers observed that the levels of lysoplasmenylcholine and lysoplasmenylethanolamine did not significantly increase in isolated rat hearts subjected to ischemia (Davies *et al.*, 1992). Secondly, the time course of arachidonic acid accumulation during reperfusion does not coincide with that of type V iPLA$_2$ activation. The enzyme activity quickly goes down to normal levels after ischemia, whereas arachidonic acid continues to accumulate (Hazen *et al.*, 1991, Van der Vusse *et al.*, 1992). Thirdly, Vesterqvist and colleagues reported a marked decrease in the activity of type V iPLA$_2$ in isolated rabbit hearts subjected to a prolonged period of global ischemia (Vesterqvist *et al.*, 1996). In their study they used intact isolated sarcolemmal membranes as substrate to measure enzyme activity, while Hazen and colleagues used exogenously added phospholipid substrates (Hazen *et al.*, 1991).

In our opinion type IIA sPLA$_2$ is a likely candidate to be involved in ischemia/reperfusion-induced degradation of membrane phospholipids, because not only arachidonic acid, but also other (un)satured fatty acids accumulate during prolonged myocardial ischemia (Figure 3). This argues in favor of the involvement of an aspecific PLA$_2$ rather than an arachidonoyl-, plasmalogen-specific PLA$_2$ (Buja, 1991, Van der Vusse *et al.*, 1994). Secondly, the observation that cardiac fatty acid levels, including arachidonic acid, continue to rise in the reperfusion phase points toward a role for type IIA sPLA$_2$ in the process. Reperfusion is known to be accompanied by a substantial increase in intracellular calcium concentration, so that the activation of calcium-dependent phospholipases can be anticipated. Furthermore, during reperfusion peroxidation of membrane phospholipids, as a result of enhanced oxygen free radical production, has been demonstrated (Van der Vusse *et al.*, 1992). It is of interest to note that peroxidation-damaged phospholipids are more vulnerable for type IIA sPLA$_2$ attack (Dan *et al.*, 1996). Furthermore, in a study by Prasad and colleagues it was shown that pretreatment of isolated hearts with antibodies raised against a snake venom type II sPLA$_2$ effectively blocked the degradation of membrane phospholipids and mitigated the release of cytoplasmic proteins in the acute reperfusion phase (Prasad *et al.*, 1991). Anti-type IIA sPLA$_2$ antibodies were also found to decrease the phospholipid degradation in homogenates of rat hearts that had been previously subjected to a period of hypoxia and reoxygenation (Kikuchi-Yanoshita *et al.*, 1993). Finally, increased expression levels of type IIA sPLA$_2$ have been found in rat brain after severe forebrain ischemia (Lauritzen *et al.*, 1994) and in rat small intestinal mucosa after ischemia and revascularization (Otamiri *et al.*, 1987). On the basis of the above, it is tempting to speculate that cardiac type IIA sPLA$_2$ expression levels will also increase during myocardial ischemia and/or reperfusion.

Alternatively, the observation that type II PLA$_2$ activity can be greatly enhanced by members of the inflammatory cytokine family, might be of relevance to the process of ischemic injury. First of all it has been shown that serum levels of inflammatory cytokines, like IL-1, IL-6 and TNFα, are increased in various pathophysiological conditions, including myocardial ischemia (Maury *et al.*, 1989, Guill n *et al* ., 1995).

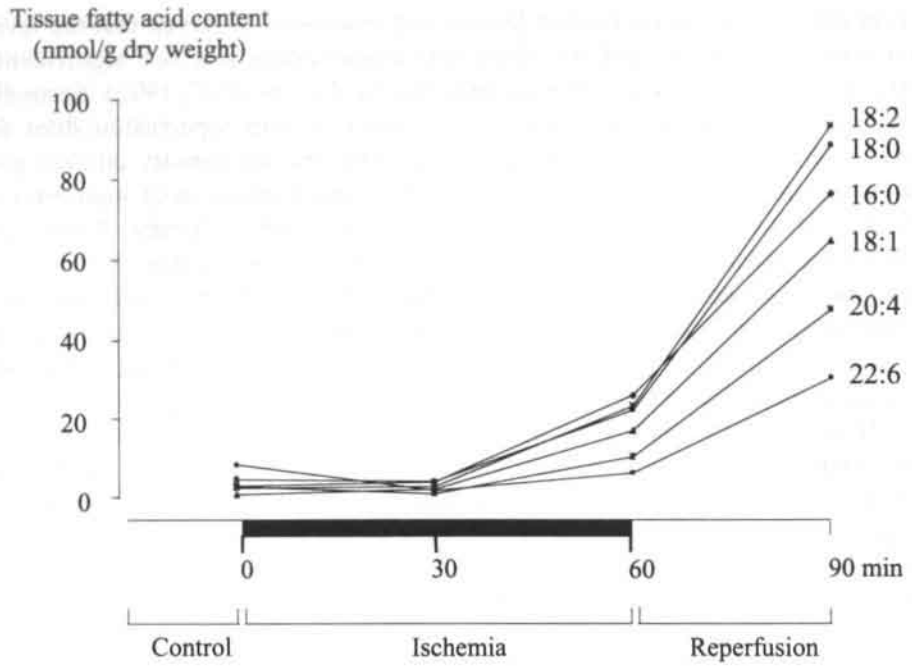

Figure 3: Effects of ischemia and reperfusion of isolated rat hearts on the tissue content of individual fatty acid species. Values are give in nmol.g^{-1} dry weight and represent the mean of 6-10 experiments. 16:0 refers to palmitic acid, 18:0 to stearic acid, 18:1 to oleic acid, 18:2 to linoleic acid, 20:4 to arachidonic acid, and 22:6 to docosahexaenoic acid. [Adapted from Van Bilsen, thesis 1989].

Moreover, for other cell types it has been shown that cytokine stimulation caused a very rapid (within 5 minutes) increase of type IIA $sPLA_2$ activity (Bomalski *et al.*, 1992). Finally, it has been demonstrated that cardiomyocytes can produce TNFα under hypoxic stress (Yamauchi-Takihara *et al.*, 1995), and that stimulation of cultured rat neonatal cardiomyocytes with TNFα resulted in increased type IIA $sPLA_2$ mRNA levels (De Windt *et al.*, 1996). Therefore, it can be hypothesized that cytokines secreted by cardiomyocytes could indirectly influence sarcolemmal stability, by increasing the cellular level of type IIA $sPLA_2$ in an autocrine manner.

Future directions: the use of molecular and transgenic techniques

As indicated above, no definitive proof has been provided that enhanced phospholipid hydrolysis and the induction of irreversible cell damage are causally related. It is also uncertain which type of PLA_2 would be accountable for the ischemia and reperfusion-induced hydrolysis of membrane phospholipids. Studies performed with phospholipase inhibitors lack specificity, which hampers an unequivocal interpretation of the results obtained. For example, in an isolated rat heart preparation subjected to a period of global ischemia followed by reperfusion, the phospholipase inhibitor mepacrine was able to reduce the phospholipid degradation, but also was shown to exert a negative inotropic effect (Van Bilsen *et al.*, 1989) and to interfere with transsarcolemmal calcium fluxes (Philpson *et al.*, 1985). Accordingly, current conventional pharmacological and/or physiological

approaches do not provide conclusive answers as to the role of PLA_2 in ischemia/reperfusion injury.

The advent of molecular biological techniques and transgenic technology allows the generation of mice with either an overexpression (gain of function) or the absence (loss of function) of specific genes. By using transgene technology it has been possible to study the role of a specific gene of interest and to dissect complex cardiovascular phenotypes involving several gene products. This approach has been demonstrated to be effective in creating murine models resembling human myocardial diseases, such as cardiac hypertrophy/failure, congenital heart diseases, and hypertension, just to mention a few (see reviews by Field, 1993 and Chien, 1994). The transgene technology has also been successfully applied in studies related to ischemia and reperfusion damage. For example, the contribution of oxygen free radical to ischemia/reperfusion damage was confirmed by the subjecting the hearts of transgenic mice overexpressing superoxide dismutase to transient ischemia (Chen *et al.*, 1996). In addition, transgenic mice overexpressing HSP70 were found to be less vulnerable against ischemic injury, clearly indicating the protective effect of HSP70 in this setting (Plumier *et al.*, 1995, Marber *et al.*, 1995).

In our laboratories we are currently applying transgene technology in order to modulate type IIA $sPLA_2$ activity in the heart. With this approach mechanisms underlying disturbances in phospholipid homeostasis as a result of cardiac ischemia and reperfusion will be studied. To achieve this, transgenic mice have been generated that harbor the recently cloned cDNA of rat heart type IIA $sPLA_2$ downstream to the proximal fragment (250 bp) of the promoter of the myosin light chain-2 (MLC-2) gene. Recent studies indicate that this part of the promoter is sufficient to mediate cardiac-specific expression (Lee *et al.*, 1992). In this way, several lines of transgenic mice, differing in copy number and, hence, in the level of cardiac type IIA $sPLA_2$ activity, will become available for analysis.

The hearts of transgenic and control mice will be subjected to ischemia and reperfusion *ex vivo* to assess the causal relationship, if any, between the level of type IIA $sPLA_2$ and the extent of phospholipid hydrolysis on the one hand and cellular damage and functional recovery on the other. We are currently scaling down the technique of isolated rat heart perfusion (Neely *et al.*, 1967), according to "the assisted mode perfusion", to the level of the mouse. This model will allows us to study functional recovery by parameters like the recovery of left ventricular developed pressure and cardiac output. The development of this isolated left ventricular ejecting mouse heart model will not only be of interest for this particular problem, but will be of great value for future studies to evaluate cardiac function of transgenic mice, whatever the transgene they are harboring.

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Chapter 3

Cloning and cellular distribution of type IIA secretory phospholipase A_2 expressed in the heart.

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Abstract

Phospholipase A_2 has been considered to play a role in physiological membrane turnover in cardiac tissue and in the degradation of membrane lipids under pathophysiological conditions, such as ischemia and reperfusion. We report the cloning of a cDNA encoding a member of the Ca^{2+}-dependent, low molecular mass type IIA secretory phospholipase A_2 (type IIA $sPLA_2$) present in rat heart. The cDNA predicts a mature protein of 146 amino acid residues including a 21 amino acid sequence at the N-terminal end, which has the features characteristic of eukaryotic secretory signal peptides. The deduced amino acid sequence constitutes an enzyme of the type II class of $sPLA_2$s, and resembles type IIA $sPLA_2$s from other mammalian sources. A Northern blot analysis performed to determine the tissue distribution showed that rat ileum contains the largest amount of the type IIA $sPLA_2$ transcript among the tissues examined, a weaker signal was present in heart, spleen, and soleus muscle, and no signal could be detected in EDL muscle, stomach, liver, kidney, brain, and lung. Northern blot analysis and RT-PCR techniques indicate the presence of this enzyme in neonatal and adult rat cardiomyocytes and in a cultured rat cardiac fibroblast-like cell line, but not in rat cardiac-derived endothelial cell lines. Transcription levels of rat heart type IIA $sPLA_2$ in isolated neonatal rat cardiomyocytes were found to increase after stimulating the cells with tumor necrosis factor-α or the α_1-adrenergic agonist phenylephrine.

Introduction

Phospholipases A_2 (PLA_2s) (phosphatide 2-acylhydrolase; EC 3.1.1.4) hydrolize the *sn-2* fatty acyl ester bond of phospholipids giving yield to fatty acids and lysophospholipids. PLA_2s have been implicated in such diverse activities as membrane turnover, signal transduction, and inflammation (Van Bilsen and Van der Vusse, 1995). Furthermore, myocardial ischemia and reperfusion are associated with disturbances in phospholipid homeostasis. The net loss of membrane phospholipids and the concomitant accumulation of lysophospholipids and fatty acids are likely to compromise cellular integrity (Van der Vusse *et al.*, 1992). Several lines of evidence support the notion that the degradation of phospholipids during ischemia/reperfusion is due to the activation of myocardial PLA_2.

In mammalian cells three types of PLA_2s have been distinguished, i.e., an ubiquitously expressed high molecular mass (85 kDa) PLA_2 with a high specificity for arachidonic acid-containing phospholipids (Sharp *et al.*, 1991), a 40 kDa Ca^{2+}-independent enzyme that prefers *sn-2* arachidonoyl plasmalogens as substrate (Hazen *et al.*,1990, Clark *et al.*, 1991), and the low molecular mass (13-15 kDa), Ca^{2+}-dependent PLA_2s (Van Schaick *et al.*, 1993, Komada *et al.*, 1989, Ishizaki *et al.*, 1989).

At present, it is unclear which type(s) of PLA_2 is (are) involved in the degradation of phospholipids during ischemia and reperfusion. The findings in a number of studies indicate that a calcium-dependent non-specific PLA_2 may play an essential role in the hydrolysis of myocardial phospholipids under these conditions. This notion is substantiated by the finding that antibodies raised against a snake venom type II sPLA$_2$ were able to inhibit phospholipase activities in the isolated rat heart after an ischemic insult (Prasad *et al.*, 1991). Further indications for the presence of a membrane-associated type IIA sPLA$_2$ in rat heart myocytes were provided by a histochemical study of Kriegsmann and colleagues (1993) using a monoclonal antibody against bee venom PLA_2.

To allow a more specific analysis of the (patho)physiological role of type IIA sPLA$_2$ within the heart, the availability of its cDNA would be very helpful. Because of the presence of marked differences in subcellular localization and subtle differences in DNA sequence between type IIA sPLA$_2$s that were cloned from various rat tissues (Van Schaick *et al.*, 1991, Komada *et al.*, 1989, Ishizaki *et al.*, 1989), we have chosen for a strategy with conventional cloning techniques to identify the cDNA of type IIA sPLA$_2$ that might be present in the heart. In addition, we wanted to determine the tissue distribution of this PLA_2 and its cellular distribution within the heart. Furthermore, it was investigated whether in cardiomyocytes the transcription level of this PLA_2 is subject to regulation by cytokines similar to what has been found in other cell types, like rat vascular smooth cells (Nakano *et al.*, 1990), rat hepatoma cells (Crowl *et al.*, 1991), and rat mesangial cells (Pfeilschifter *et al.*, 1989).

Materials and Methods

Isolation of adult and neonatal cardiomyocytes

Adult cardiomyocytes (CMC) were isolated from the ventricles of rats (Wistar-Kyoto) as described previously by Linssen *et al.* (1993). Briefly, after perfusion of the isolated heart with a collagenase solution, the ventricles were chopped and incubated in Modified Krebs Ringer buffer containing 2.5 mM $CaCl_2$, 0.06% collagenase type I (Gibco BRL, Breda, the Netherlands) and 1% bovine serum albumin (BSA). Cells were centrifuged at 25×g for 90 s. The cells were plated on tissue culture dishes in CMC culture medium (i.e., M199, 2 mM L-glutamine, 13 mM $NaHCO_3$, 10 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 4% fetal calf serum). The immortal cardiac-derived endothelial cell lines RHEC-50 and RHEC-116, and the cardiac-derived fibroblast-like cell line CFLC-122 (cell lines described by Linssen *et al.*, 1990 and 1993) were cultured in DMEM (Gibco 42430) supplemented with 10% fetal calf serum (Gibco BRL).

Neonatal ventricular myocytes were isolated as described by Iwaki *et al.* (1990) with slight modifications. Briefly, hearts were removed from decapitated 1 to 3 days old Wistar-Kyoto rats. Atria were trimmed off and ventricular tissue was cut into pieces, transferred to a spinner flask and digested to single cells with collagenase type I (Gibco BRL) and pancreatin (Gibco BRL) in Ads-buffer (pH 7.35), consisting of 116 mM NaCl, 20 mM HEPES, 0.9 mM NaH_2PO_4, 5 mM glucose, 5.4 mM $MgSO_4$. The cell suspension was loaded on a discontinuous gradient of Percoll (Sigma Chemical Co., St. Louis, USA) with two different densities (1.059 and 1.082 g/ml, respectively) to separate the cardiomyocytes from non-myocytes, mainly fibroblasts and endothelial cells. The cardiomyocytes were allowed to adhere to tissue culture dishes coated with 1% gelatin type B (Sigma G-9382) in a 4:1 mixture of DMEM (Gibco 42430) and M199 (Gibco 31153), supplemented with 10% horse serum (Gibco 16050), 5% newborn calf serum (Sera-Lab, Sussex, UK), and antibiotics (pennicilin 100 U/ml, streptomycin 100 µg/ml, P/S, Gibco BRL).

RT-PCR

Total RNA from adult rat heart (Lewis strain), isolated myocytes, and the above described cardiac-derived cell lines, was prepared using TRIzol Reagent (Gibco BRL). Reverse transcription (RT) was performed with Superscript™-II RNAse H⁻ reverse transcriptase (Gibco BRL) according to the instructions of the supplier. Polymerase chain reaction (PCR) was performed on a DNA thermal cycler (Perkin Elmer Co., Norwark, USA) using Thermus Aquaticus (Taq) polymerase (Gibco BRL). After an initial incubation of 5 min at 95 °C, the reactions were cycled 30 times between 55 °C, 72 °C, and 95 °C, all for 1 min. An additional extension reaction at 72 °C for 10 min was included after cycle 30. Samples were size-fractionated by agarose gel electrophoresis and visualized after staining with ethidium bromide. RT-PCR and PCR reactions were performed with combinations of primers designated PLA-F1 [5'-GAGTTTGGGCAAATGATTC-3'], PLA-R1 [5'-CAGCTTTATCGCACTGGCA-3'], and PLA-R2 [5'-AGTCCTGGTTTGTAGAGCAG-3'] (all purchased from Pharmacia, Uppsala, Sweden). PLA-F1 and PLA-R1 correspond to bp 73-91 and bp 328-346 of the sequence of rat spleen type IIA $sPLA_2$ (Ishizaki *et al.*, 1989). PLA-R2 is a nested

primer in the rat heart PLA$_2$ cDNA sequence corresponding to bp 291-311 in Figure 2. The subsequently found 273 bp RT-PCR product was subcloned in a pBluescript vector. The fragment was excised as a Xho I/Pst I fragment and radioactively labeled using [^{32}P]-dCTP (3000 Ci/mmol, NEN DuPont, Dordrecht, the Netherlands) by random priming using a commercial kit (rediprime™, Amersham, Buckinghamshire, UK). The probe was designated PLA-probe.

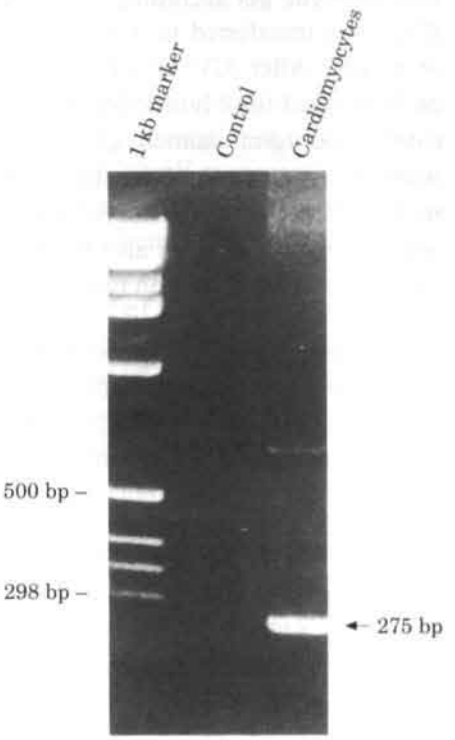


Figure 1
PCR amplification of RT-RNA from adult rat myocytes. The PCR products generated with primers PLA-F1 and PLA-R1 on RT-RNA from adult rat cardiomyocytes were resolved by gel electrophoresis and stained with ethidium bromide. Control refers to PCR reaction to which H_2O was added instead of template (negative control). An 1 kb ladder (Gibco BRL) was used as molecular weight marker.

RACE-RT-PCR

5'-RACE-RT-PCR (rapid amplification of cDNA-ends-reverse transcription polymerase chain reaction) was applied to generate the full length cDNA from adult rat cardiomyocyte total RNA, using a kit from Gibco BRL. Primer PLA-R1 was used for first round amplification, while the supplied anchor primer and the nested primer PLA-R2 were used for the second round of amplification, according to the supplier's instructions.

Library screening

1.2×10^6 clones from an adult rat heart UNI-ZAP™ XR-cDNA library (Stratagene, La Jolla, California, USA) were lifted in duplicate onto nylon filters. The filters were denatured, baked at 80 °C for 1 h, prehybridized for 2 h in 6× SSPE, 1×Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), 100 µg/ml salmon sperm DNA at 58 °C, and hybridized overnight in the same solution with 10^6 cpm/ml of ^{32}P-labeled PLA-probe. Final washing conditions were 0.1×SSC at 60 °C for 15 min.

Filters were exposed to X-ray film for 24 h. After three subsequent rounds of duplicate filter lifting in order to obtain a purified clone, 12 potential positive clones were found and subjected to an In Vivo Excision reaction of the insert, using the ExAssist/SolR system (Stratagene), thereby directly cloning the insert into a

pBluescript vector. Candidate clones were subjected to restriction enzyme digest analysis and sequence reactions using the T7 sequence kit (Pharmacia).

Northern blot analysis

Total RNA from various rat tissues and cells was isolated using TRIzol Reagent (Gibco BRL) and the RNA (10 µg) was size-fractionated on a 1% agarose, 18% formaldehyde gel according to the method described in Sambrook *et al.* (1989). The RNA was transferred to a nylon filter (Hybond-N, Amersham) by capillary transfer overnight. After UV crosslinking and baking at 80 °C for 2 h, the filters were prehybridized for 2 h in 6×SSPE, 1×Denhardt s solution, 0.5% sodium dodecyl sulfate (SDS), 100 µg/ml salmon sperm DNA at 58 °C and hybridized in the same solution with 10^6 cpm/ml of ^{32}P-labeled PLA- probe. Final washing conditions were 1×SSC with 0.1% SDS at 50 °C for 30 min. To correct for possible differences in loading and transfer, the filters were also hybridized with an ^{32}P-labeled 18S probe. Filters were exposed to X-ray film with two intensifying screens.

Stimulation of neonatal cardiomyocytes

For these experiments, after isolation from the hearts, 2×10^6 neonatal rat cardiomyocytes were allowed to adhere to 10 cm tissue culture dishes coated with 1% gelatin type B in a 4:1 mixture of DMEM (Gibco 42430) and M199 (Gibco 31153), supplemented with 10% horse serum (Gibco 16150), 5% newborn calf serum, and P/S. After 24 h the cells were rinsed twice with sterile PBS and serum free medium was added (a 4:1 mixture of DMEM and M199 with P/S). After 24 h tumor necrosis factor-α (TNF-α, final concentration 1 nM) or phenylephrine (PE, final concentration 10 µM) were added to the cells. After a 16 h incubation unstimulated cells, TNF-α-stimulated, and PE-stimulated cells were harvested for RNA isolation using TRIzol reagent (Gibco). The RNA was subjected to Northern blot analysis according to the method described above. The Northern blots were exposed to storage phosphor screens and subsequently scanned and quantified using a PhosphorImager and the ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

Results

RT-PCR on isolated myocytes

In order to investigate the presence of type IIA $sPLA_2$ in cardiac myocytes, two primers, designated PLA-F1 and PLA-R1, were generated corresponding to bp 73-91 and bp 328-346 of the sequence of rat spleen type IIA $sPLA_2$ (Ishizaki *et al.*, 1989). PCR amplification using these primers on reverse transcribed (RT) RNA from isolated adult rat heart myocytes, produced several bands among which one prominent band of the expected size of 273 bp (Figure 1). These fragments were subcloned into the pBluescript vector and sequenced in both directions. Sequence comparison was performed using the GenBank data base. This confirmed only the 273 bp PCR-product to be part of a type IIA sPLA2 cDNA. The 273 bp fragment was found to be nearly identical to the corresponding part of rat spleen and platelet type IIA $sPLA_2$. Only at one position (bp 126 in Figure 2/Figure 3, Panel A) a thymidine residue in the

spleen/platelet type was found to be be replaced by a cytidine residue in the heart cDNA.

The 273 bp PCR-product was subcloned in the pBluescript vector and isolated as a Xho I/Pst I fragment and labeled with [^{32}P]-dCTP. The fragment was designated PLA-probe and used as a probe for the screening of a cDNA library and in RNA blot analysis.

Screening of rat heart cDNA library.

In order to obtain the full length cDNA of the PLA$_2$, 1.2×10^6 clones of an adult rat heart cDNA library were screened, using the radioactively labeled PCR-fragment as a probe. Twelve potential candidates were identified after three sequential rounds of plaque purification. After using the ExAssist/SolR system supplied with the cDNA library to allow efficient excision of the pBluescript phagemid, the candidates were subjected to restriction analysis and Southern blot analysis. Based on these analyses, only one of the twelve candidates proved to contain a part of a type IIA sPLA$_2$ cDNA. This was affirmed after sequencing the pBluescript plasmid containing the fragment of approximately 600 bp. This revealed that the clone contained a large part of the coding region, starting from basepair 104, and the complete 3 untranslated region ending in a poly(A) tail (Fig. 2). However, the 5 end of the coding region was missing.

5'-RACE-PCR

In order to procure the missing part of the coding region, the 5'-rapid amplification of cDNA ends-PCR technique was applied. Total RNA from adult rat heart myocytes was isolated, and a first round PCR amplification was performed, using primer PLA-R1 to produce a crude 5'-rat heart PLA$_2$-cDNA sample. This product was tailed with a supplied anchor primer at its 5' end and amplified using a supplied complementary anchor primer and the nested PLA$_2$ primer, designated PLA-R2. The product obtained was electrophorized on an agarose gel and visualized after staining with ethidium bromide. A very faint PCR-fragment of approximately 400 bp was isolated from the gel via the Qiaex gel extraction procedure (Qiagen GmbH, Hilden, Germany), and re-amplified with the last set of primers that was applied in the 5'-RACE-PCR reaction. This product was cloned into a PCR-II vector of the TA cloning kit (InVitrogen Corp., San Diego, USA). Sequencing reactions using the primers PLA-F1 and PLA-R2 revealed the product as being a part of a type IIA sPLA$_2$ cDNA and containing the missing 5 end of the rat heart type IIA sPLA$_2$ cDNA.

Cloning of full length PLA$_2$

The first 404 basepairs of the PLA$_2$ 5'-RACE-PCR fragment and the clone isolated by screening the cDNA library were combined, using internal BamHI sites at bp 164 and bp 610 in de cDNA sequence of type IIA sPLA$_2$ and an EcoRI site in de anchor primer used in the 5 RACE-PCR reaction, which resulted in the full length PLA$_2$ cDNA in pBluescript. In this sequence an open reading frame (438 basepairs) was found, predicting a protein of 146 amino acids, containing a short sequence composed of 21 amino acid residues at the N-terminal end, which has the features characteristic of eukaryotic secretory signal peptides. Furthermore, the enzyme contains a carboxyl-terminal extension of six amino acids (KGKTPSC) and misses the elapid loop present

in type I $sPLA_2$, both features being characteristic for type II $sPLA_2$ enzymes (Davidson *et al.*, 1990, Figure 2/Figure 3, Panel A).

```
-93                                                                    TCCTGGTTTGTAGA  -79

-78   GCATTGGGAGTATAGGAAAAACAAGGCAGGCCCTTGAACAAGAAGCCATACCACCATCCCATCCAAGAGAGCTGACAGC    0

      Met Lys Val Leu Leu Leu Leu Ala Val Val Ile Met Val Phe Gly Ser Ile Gln Val Gln
 1    ATG AAG GTC CTC CTG TTG CTA GCA GTT GTG ATC ATG GCC TTT GGC TCA ATT CAG GTC CAG   60

          ↓
      Gly Ser Leu Leu Glu Phe Gly Gln Met Ile Leu Phe Lys Thr Gly Lys Arg Ala Asp Val
61    GGG AGC CTT CTG GAG TTT GGG CAA ATG ATT CTG TTT AAG ACA GGA AAG AGA GCT GAT GTT  120

      Ser Tyr Gly Phe Tyr Gly Cys His Cys Gly Val Gly Gly Arg Gly Ser Pro Lys Asp Ala
121   AGC TAC GGC TTC TAC GGT TGC CAT TGT GGT GTG GGT GGC AGA GGA TCC CCC AAG GAT GCC  180

      Thr Asp Trp Cys Cys Val Thr His Asp Cys Cys Tyr Asn Arg Leu Glu Lys Arg Gly Cys
181   ACA GAT TGG TGC TGT GTG ACT CAT GAC TGT TGT TAC AAC CGT CTG GAG AAA CGT GGA TGT  240

      Gly Thr Lys Phe Leu Thr Tyr Lys Phe Ser Tyr Arg Gly Gly Gln Ile Ser Cys Ser Thr
241   GGC ACA AAG TTT CTG ACC TAC AAG TTC TCC TAC CGA GGG GGC CAA ATC TCC TGC TCT ACA  300

      Asn Gln Asp Ser Cys Arg Lys Gln Leu Cys Gln Cys Asp Lys Ala Ala Ala Glu Cys Phe
301   AAC CAG GAC TCC TGC CGG AAA CAG CTG TGC CAG TGC GAT AAA GCT GCC GCT GAA TGT TTT  360

      Ala Arg Asn Lys Lys Ser Tyr Ser Leu Lys Tyr Gln Phe Tyr Pro Asn Lys Phe Cys Lys
361   GCC CGG AAC AAG AAA AGC TAC AGT TTA AAG TAC CAG TTC TAC CCC AAC AAG TTT TGC AAA  420

      Gly Lys Thr Pro Ser Cys
421   GGG AAG ACG CCC AGT TGC TGAAAGAGACATCTTCTGAAACATCCAGACATCCTCTAACACCTCTCCTAGCCCA  493

494   ACCAAGTTCCCCAGTGATCAAGAAAACACCCCTCTCCTAGAAGCAGGCGGGCCCTTCTGTCTTCACCCAGAAGGAGCCG  572

573   CTGAAGCCTGATCTTTCCCCAACACTCCACAGCCTTGGATCCGCCCACTTTTCCCTTGGCATCCAACTTCCTGCTGCGT  651

                                        *******
652   AGTACCTAAGAGGGTCCTGAGAGCCTCTCGCAAGTAAAGCAATTCATCAACAAAAAAAAAAAAAAAAAA            759
```

Figure 2
Nucleotide sequence of the cDNA of rat heart type IIA secretory phospholipase A_2 (type IIA $sPLA_2$).The cloned nucleotide sequence and deduced amino acid sequence of the largest found open reading frame (ORF) of rat heart type IIA $sPLA_2$ is shown. The start codon (position +1) and stopcodon are indicated in bold. The putative polyadenylation signal in the 3 -untranslated region is indicated in bold. The first 21 amino acids residues that form the eukaryotic signal peptide for protein secretion are indicated in bold. The respective position of the primers PLA-F1, PLA-R1 and PLA-R1 are underlined, and the predicted cleavage site for signal peptidase (↓) is indicated.

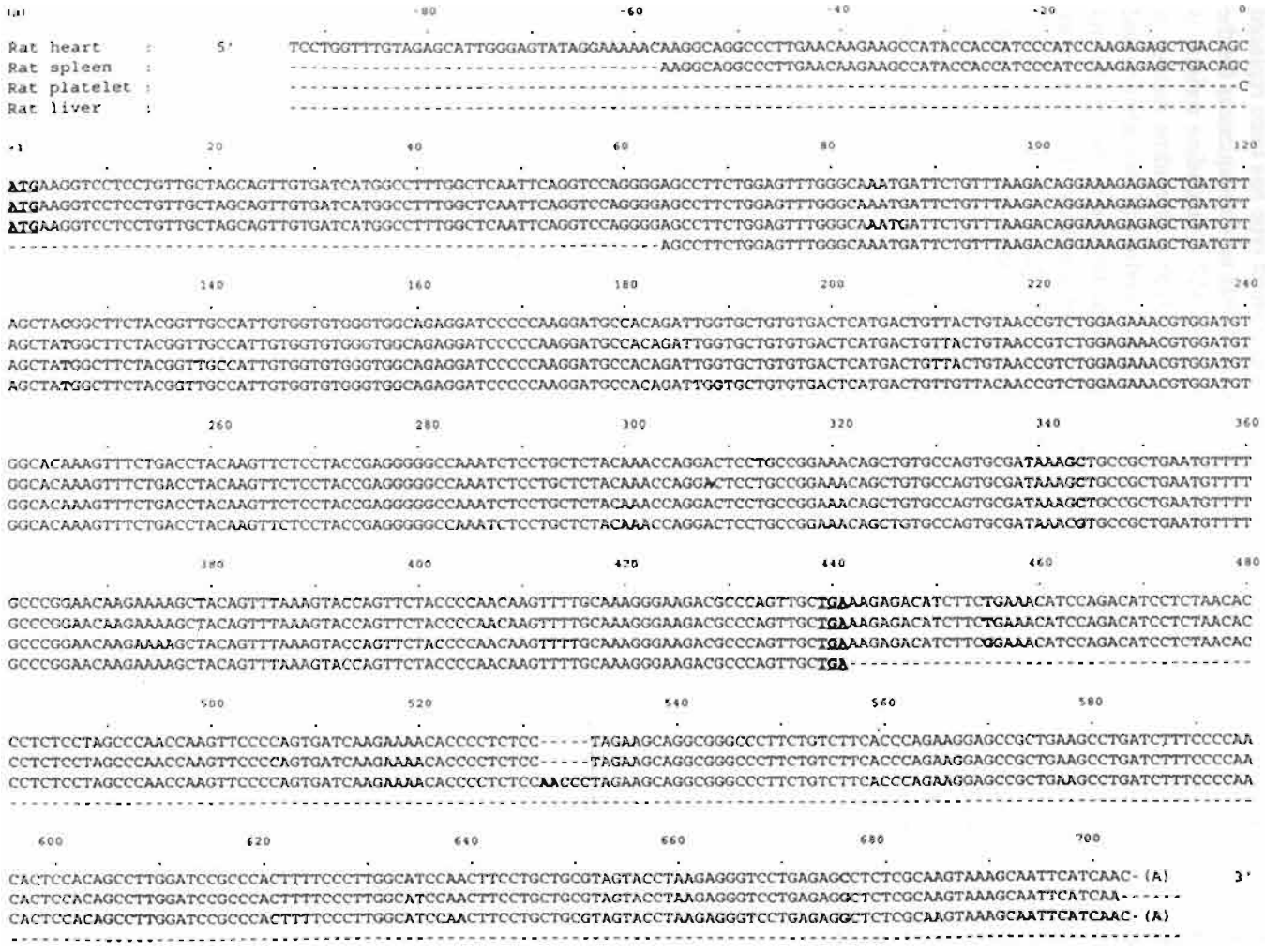
At the DNA level the differences with the sequence isolated from spleen by Ishizaki *et al.* (1989), and from platelets by Komada *et al.* (1989) are minimal. Within the coding region at bp 126 a thymidine residue in the spleen and platelet type has been replaced by a cytidine residue in the heart cDNA. This replacement has no consequences for the amino acid sequence. In the 3 untranslated region at bp 457 a guanidine residue is present in the platelet type, while in the heart and spleen type a thymidine residue is present. Furthermore, the platelet cDNA contains a short insertion of two adenine and three cytidine residues at bp 531 and at bp 676 a guanidine residue in the spleen and platelet type is substituted by a cytidine residue in the heart sequence. However, more differences in the coding region can be observed between the PLA$_2$ cDNAs cloned from rat heart and rat liver (Van Schaick *et al.*, 1989). In addition to the basepair difference in the coding region (bp 126), the rat liver cDNA shows a cytidine residue at bp 344 and a guanidine residue at bp 345, resulting in an arginine at position 115 in the amino acid sequence, whereas an alanine residue is found in the heart, spleen, and platelet type IIA sPLA$_2$ (Figure 3, Panel A and B).

Tissue and cellular distribution

Northern blot analysis showed this PLA$_2$ transcript to be present in a number of rat tissues and to correspond to a 0.9 kb signal (Figure 4). In rat ileum a very strong signal was found, and a relatively weaker signal in heart, spleen, and soleus muscle. No signal could be detected in rat EDL muscle, stomach, kidney, brain and lung tissue (Figure 4).

In addition it was investigated whether the type IIA sPLA$_2$ was present in isolated adult cardiomyocytes and several cardiac-derived cell lines (Figure 5). A positive signal was observed in adult rat ventricular myocytes and in the cardiac-derived fibroblast-like cell line CFLC-122. No hybridization signal was found in the cardiac-derived endothelial cell lines RHEC-50 and RHEC-116 (Linssen *et al.*,1993b), not even after prolonged exposure of the film. Relative to the signal obtained in total heart (100%) the signals in the cardiomyocytes and cardiac-derived fibroblast-like cell line amounted to 105% and 130%, respectively (Figure 5).

To check whether type IIA sPLA$_2$ was indeed absent in the endothelial cell lines, RT-PCR was applied because of the greater sensitivity levels that are intrinsic to this technique. RT-PCR was performed on total RNA isolated from rat ileum, total heart, isolated adult cardiomyocytes and from the two cardiac-derived endothelial cell lines and the cardiac-derived fibroblast-like cell line, using PLA-F1 and PLA-R1 as primers (Figure 6). As expected, PCR-fragments of expected size (273 bp) were found in ileum and total heart. Furthermore, a similar signal was obtained in isolated adult cardiomyocytes and the cardiac-derived fibroblast-like cell line. In contrast, no signal was detected in either one of the cardiac-derived endothelial cell lines, thereby validating the Northern blot data shown above.


Rat heart : 5' TCCTGGTTTGTAGAGCATTGGGAGTATAGGAAAAACAAGGCAGGCCCTTGAACAAGAAGCCATACCACCATCCCATCCAAGAGAGCTGACAGC
Rat spleen : ----------AAGGCAGGCCCTTGAACAAGAAGCCATACCACCATCCCATCCAAGAGAGCTGACAGC
Rat platelet : ----------C
Rat liver : ----------
ATGAAGGTCCTCCTGTTGCTAGCAGTTGTGATCATGGCCTTTGGCTCAATTCAGGTCCAGGGGAGCCTTCTGGAGTTTGGGCAAATGATTCTGTTTAAGACAGGAAAGAGAGCTGATGTT
AGCTACGGCTTCTACGGTTGCCATTGTGGTGTGGGTGGCAGAGGATCCCCCAAGGATGCCACAGATTGGTGCTGTGTGACTCATGACTGTTACTGTAACCGTCTGGAGAAACGTGGATGT
GGCACAAAGTTTCTGACCTACAAGTTCTCCTACCGAGGGGGCCAAATCTCCTGCTCTACAAACCAGGACTCCTGCCGGAAACAGCTGTGCCAGTGCGATAAAGCTGCCGCTGAATGTTTT
GCCCGGAACAAGAAAAGCTACAGTTTAAAGTACCAGTTCTACCCCAACAAGTTTTGCAAAGGGAAGACGCCCAGTTGCTGAAAGACATCTTCTGAAACATCCAGACATCCTCTAACAC
CCTCTCCTAGCCCAACCAAGTTCCCCAGTGATCAAGAAAACACCCCTCTCC-----TAGAAGCAGGCGGGCCCTTCTGTCTTCACCCAGAAGGAGCCGCTGAAGCCTGATCTTTCCCCAA
CACTCCACAGCCTTGGATCCGCCCACTTTTCCCTTGGCATCCAACTTCCTGCTGCGTAGTACCTAAGAGGGTCCTGAGAGCCTCTCGCAAGTAAAGCAATTCATCAAC-(A) 3'

(b)

```
        +1          10          20          30          40          50
        .           .           .           .           .           .
Heart   MKVLLLLAVVIMAFGSIQVQGSLLEFGQMILFKTGKRADVSYGFYGCHCG
Spleen  MKVLLLLAVVIMAFGSIQVQGSLLEFGQMILFKTGKRADVSYGFYGCHCG
Platelet MKVLLLLAVVIMAFGSIQVQGSLLEFGQMILFKTGKRADVSYGFYGCHCG
Liver   ----------------------SLLEFGQMILFKTGKRADVSYGFYGCHCG

                60          70          80          90          100
                .           .           .           .           .
        VGGRGSPKDATDWCCVTHDCCYNRLEKRGCGTKFLTYKFSYRGGQISCST
        VGGRGSPKDATDWCCVTHDCCYNRLEKRGCGTKFLTYKFSYRGGQISCST
        VGGRGSPKDATDWCCVTHDCCYNRLEKRGCGTKFLTYKFSYRGGQISCST
        VGGRGSPKDATDWCCVTHDCCYNRLEKRGCGTKFLTYKFSYRGGQISCST

                110         120         130         140
                .           .           .           .
        NQDSCRKQLCQCDKAAAECFARNKKSYSLKYQFYPNKFCKGKTPSC
        NQDSCRKQLCQCDKAAAECFARNKKSYSLKYQFYPNKFCKGKTPSC
        NQDSCRKQLCQCDKAAAECFARNKKSYSLKYQFYPNKFCKGKTPSC
        NQDSCRKQLCQCDKRAAECFARNKKSYSLKYQFYPNKFCKGKTPSC
```

Figure 3
Comparison of type IIA sPLA$_2$ primary structures. ***A***, The cDNA sequences of type IIA sPLA$_2$ from rat heart, spleen, platelet and liver are depicted. Alignment is based on maximizing homology using Clustal W 1.60 Multiple sequence alignment software. The start codon (position +1) and stop codon are underlined. Differences between the cDNA sequences are given in bold and are depicted within boxes. ***B***, the amino acid sequences of rat heart, spleen, platelet and liver type IIA sPLA$_2$ are depicted in one letter code. Alignment is based on maximizing homology using Clustal W 1.60 software. The first amino acid residue in the rat heart enzyme (position +1) is indicated. Differences between the sequences are given in bold and are depicted whithin boxes. The putative secretory signal peptide is also given in bold.

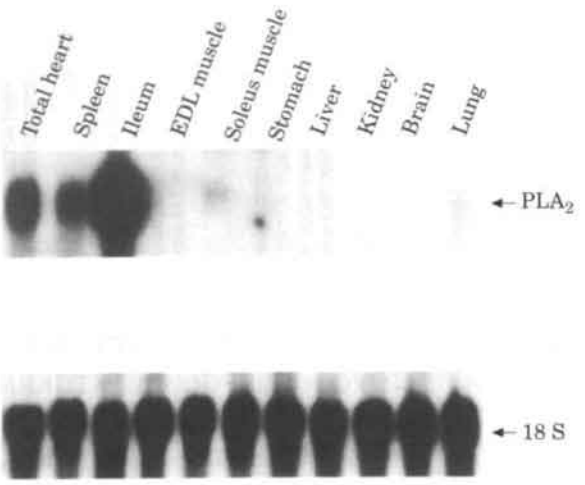


Figure 4
Northern blot analysis of different rat tissues. Total RNA (10 µg) isolated from heart, spleen, ileum, extensor digitorum longus (EDL) muscle, soleus muscle, stomach, liver, kidney, brain and lung was analyzed. A 273 bp ^{32}P-labeled fragment of rat heart type IIA $sPLA_2$ cDNA was used as a probe. The blot was stripped and reprobed for 18S ribosomal RNA to check whether comparable amounts of RNA had been loaded.

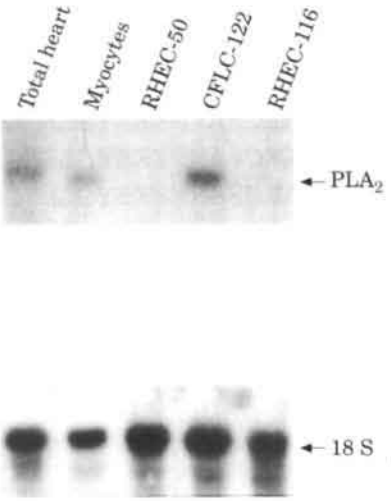


Figure 5
Northern blot analysis of different rat cardiac cell types. Total RNA (10 µg) isolated from total heart, adult myocytes, the cardiac-derived fibroblast-like cell line CFLC-122 and two cardiac-derived stable endothelial cell lines RHEC-50 and RHEC-116 was analyzed. As probe, the ^{32}P-labeled fragment of rat heart type IIA $sPLA_2$ cDNA was used. The blot was stripped and reprobed for 18S ribosomal RNA to correct for differences in RNA loading. Picture depicts one of two independent Northern blot analyses with similar findings.

Stimulation of neonatal myocytes

To monitor whether type IIA $sPLA_2$ transcription can be induced by cytokines in cardiomyocytes, similar as to what has been shown for several other cell types (Pfeilschifter *et al.*, 1989, Nakano *et al.*, 1990, Crowl *et al.*, 1991), primary cultures of neonatal ventricular myocytes were stimulated with TNF-α (1 nM). In addition, the myocytes were stimulated with the α_1-adrenergic agonist phenylephrine (10 μM). The results show a five fold upregulation of type IIA $sPLA_2$ transcript after TNF-α stimulation and a nearly two fold increase after addition of phenylephrine to the culture medium of neonatal rat cardiomyocytes (Figure 7).

Discussion

The significance of type IIA $sPLA_2$ diversity

In this study we describe the cloning of the cDNA sequence of a type IIA $sPLA_2$ present in rat cardiomyocytes and in the cardiac-derived fibroblast-like cell line CFLC-122. It appears to be a low-molecular mass enzyme, the presence of which has been previously described in rat spleen, platelets, and liver (Ishizaki *et al.*, 1989, Komada *et al.*, 1989, Van Schaick *et al.*, 1993).

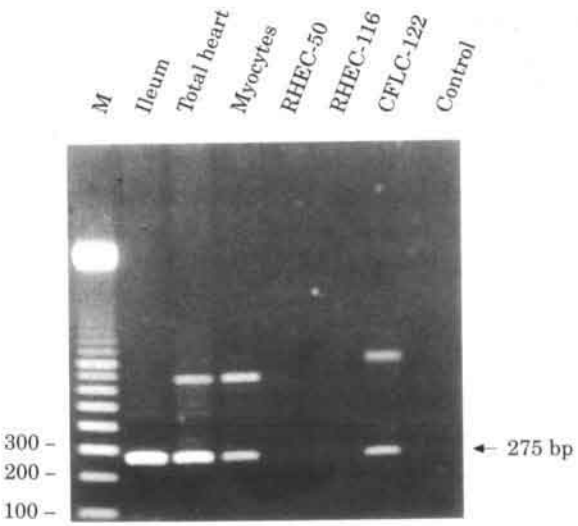


Figure 6

Determination of cellular distribution by RT-PCR amplification. The presence of type IIA $sPLA_2$ in different cardiac cell types was assessed by means of PCR with primers PLA-F1 and PLA-R1 on reverse transcribed RNA from adult myocytes, cardiac-derived endothelial cell line RHEC-50 and RHEC-116, and the cardiac-derived fibroblast-like cell line CFLC-122. As positive controls RT-PCR amplifications were performed on RT-RNA from ileum and total heart. A PCR reaction without template was also included (control). *Lane M*, molecular weight standard (100 bp ladder, Gibco BRL.)

At the DNA level the differences with the spleen and platelet sequences are minimal and have no consequences for the amino acid composition. The differences with the cDNA sequence of type IIA $sPLA_2$ isolated from liver were more pronounced and resulted in the substitution of one amino acid residue. The question could be raised whether the differences between the previously reported rat type IIA $sPLA_2$s and the one we cloned from rat heart represent cloning/sequencing artefacts, strain differences, or are the result of differential splicing of one or more related genes. Firstly, it should be noted that the sequence of a part of the cDNA that shows

differences with the platelet and spleen type IIA $sPLA_2$, has been obtained in three independent ways, i.e., after sequencing (i) the 273 bp product of the PCR reaction on reverse-transcribed myocyte RNA, (ii) the fragment picked up from the rat heart cDNA library, and (iii) the product obtained after performing the 5 RACE-PCR. Therefore, the occurrence of cloning or sequencing artefacts of the cardiac PLA_2 cDNA can be ruled out. Hence, it can concluded that the basepair differences are genuine.

With respect to strain differences, it is noteworthy that in various inbred mouse strains a naturally occurring thymidine insertion within the coding region of the mouse type IIA $sPLA_2$ gene has been observed (Kennedy *et al.*, 1995, MacPhee *et al.*, 1995). In addition, the amino acid sequence deduced from platelet type IIA PLA_2 cDNA (Komada *et al.*,1989) was found to be different from the previously determined amino acid sequence of the purified platelet enzyme as published by the same research group (Hayakawa *et al.* 1988). In this case, the discrepancy between the two sequences was also explained on the basis of strain differences. Accordingly, it could be taken into consideration that the few basepair differences between the sequence cloned from the heart in the present study, on the one hand, and the sequences cloned from spleen and platelets, on the other, are due to the use of different rat strains. Indeed, the rat heart type IIA $sPLA_2$ cDNA of the present study was cloned from a heart cDNA library of Wistar-Kyoto rats, while the platelet type IIA $sPLA_2$ cDNA was cloned from a megakaryocyte cDNA library of Sprague-Dawley rats (Komada *et al.*, 1989).

The gene encoding rat type IIA $sPLA_2$ has been cloned (Komada *et al.*, 1990) and Southern blot analysis of genomic DNA revealed that only one single copy exists, which would imply that type IIA $sPLA_2$ cDNA s cloned from various cell types or tissues should be identical or derived by differential splicing. However, Chen and coworkers recently published cDNA sequences of two new, low molecular mass PLA_2s, one cloned from a rat genomic library and relatively abundantly present within the heart and to a lesser extent within the lung (Chen *et al.*, 1994a), and the other cloned from a rat brain library, the mRNA of which was detected in rat testis only (Chen *et al.*, 1994b). These sequences exhibit a limited homology to the type IIA enzymes mentioned in the present study and was therefore designated type V $sPLA_2$. For instance, the cDNA of rat heart type IIA $sPLA_2$ shows a homology of 64.5% and 43.8% at the DNA and protein level, respectively, with the sequence found by Chen and coworkers (1994a) in the heart. For the testis enzyme these values are even lower (59.7% and 29.5%, respectively). In addition, when screening a rat megakaryocyte cDNA library for the cDNA of platelet type IIA $sPLA_2$, Komada and coworkers (1989) found two highly homologous sequences that differed at various positions, which was taken as an indication for the presence of two or more type IIA isoforms. The latter findings point to the existence of several isoforms of type II PLA_2s in mammalians. Accordingly, based on the available data no conclusive answer can be given as to whether the observed minimal differences in the type IIA $sPLA_2$ cloned from various rat tissues/cells are due to strain differences and/or to the existence of splice variants.

Tissue/cellular distribution

Via Northern blotting analysis we examined the tissue distribution of the type IIA sPLA$_2$, yielding high levels of mRNA in the ileum, and lower levels in spleen, heart, and soleus muscle, while no signal could be detected in EDL muscle, liver, lung, brain, stomach, and kidney. More interestingly, the mRNA of type IIA sPLA$_2$ was present in isolated cardiomyocytes and cardiac-derived fibroblast-like cells, but not in the immortalized cardiac-derived endothelial cell lines RHEC-116 and RHEC-50 as assessed by Northern blotting and RT-PCR, which points to the absence of a type IIA sPLA$_2$ in the endothelial compartment of the heart. However, to definitively establish whether type IIA sPLA$_2$ is not expressed in endothelial cells under *in vivo* conditions, *in situ* hybridization experiments on myocardial tissue are required.

The low intensity of the hybridization signal in total heart and in the different cardiac cell types suggests that the type IIA sPLA$_2$ is present at relatively low abundance. This notion is supported by the finding that only one positive clone was picked up after screening more than a million plaques of the commercial rat heart cDNA library.

The presence of an eukaryotic secretory signal peptide at the N-terminal end of the type IIA sPLA$_2$ raises the question as to whether this enzyme will be secreted by cardiac myocytes. Information about the localization of the other members of the type IIA sPLA$_2$ does not give a conclusive answer. In platelets the type IIA sPLA$_2$ is localized in secretory granula and is secreted after certain stimuli, the spleen type was found to be membrane-associated, whereas the liver form was purified from the mitochondrial fraction (Aarsman *et al.*, 1989). Some insights into the possible localization of the type IIA sPLA$_2$ in the cardiac myocyte is provided by an immunohistochemical study of Kriegsmann and colleagues (1993). Using a monoclonal antibody raised against bee venom type II PLA$_2$ they observed cross-immunoreactivity at the level of the sarcolemma of cultured neonatal rat cardiac myocytes, which could indicate that the enzyme cloned in the present study is also membrane-associated.

(Patho)physiological role of type IIA sPLA$_2$

Myocardial ischemia and reperfusion are associated with disturbances in phospholipid homeostasis. The net loss of membrane phospholipids and concomitant accumulation of lysophospholipids and fatty acids are likely to compromise cellular integrity (Van der Vusse *et al.*, 1992). Earlier findings support the notion that the degradation of phospholipids is directly due to the activation of myocardial phospholipases A$_2$, although an impairment of resynthesis processes cannot be excluded (Chien *et al.*, 1984, Van Bilsen *et al.*, 1989).

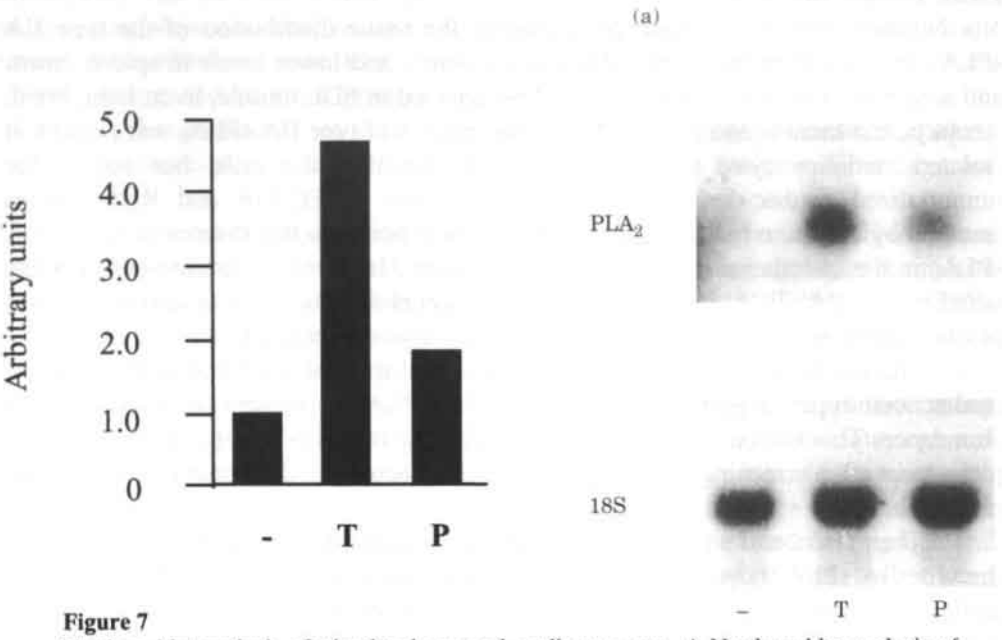


Figure 7
Northern blot analysis of stimulated neonatal cardiomyocytes. ***A***, Northern blot analysis of type IIA $sPLA_2$ mRNA in neonatal cardiomyocytes cultured for 16 h in serum-free designated (-), in the presence of TNF-α (final concentration 1 nM) designated (T), or phenylephrine (final concentration 10 μM) designated (P). The signals in the RNA samples obtained with the ^{32}P-labeled probe for rat heart type IIA $sPLA_2$ and the 18S probe are designated. ***B***, Quantitative presentation of Northern blot data. The PLA_2 signal was normalized to the 18S signal. The PLA_2/18S ratio in control cells was set at 1.0. The Northern blot data of two independent cell cultures are shown. In the other experiment the degree of induction was comparable (4.3- and 1.5-fold for TNF-α and phenylephrine, respectively).

Several lines of evidence suggest that type IIA $sPLA_2$ plays a crucial role in the hydrolysis of myocardial phospholipids under these pathological conditions. Firstly, the composition of the fatty acids accumulating in the isolated rat heart after a period of ischemia and reperfusion argue in favor of the involvement of an aspecific PLA_2, rather than an arachidonoyl-specific PLA_2 (Van Bilsen *et al.*, 1989). Secondly, it has been known that reperfusion is associated with the influx of massive amounts of calcium, so that the activation of calcium-dependent phospholipases has to be anticipated. Finally, some immunological evidence exists for a role of type IIA $sPLA_2$ in ischemia/reperfusion induced membrane phospholipid degradation. In a study by Prasad and colleagues (1991) it was shown that pretreatment of isolated rat hearts with antibodies raised against a snake venom type IIA $sPLA_2$ effectively inhibited the degradation of phospholipids and the release of cytoplasmic proteins during the acute reperfusion phase. Furthermore, antibodies against a type II $sPLA_2$ were found to decrease the rate of phospholipid hydrolysis in homogenates of rat hearts that had been subjected to a period of hypoxia and reoxygenation (Kikuchi-Yanoshita *et al.*, 1993).

All these observations point to a role of type IIA sPLA$_2$ in phospholipid degradation and subsequent membrane destabilization after a period of ischemia and reperfusion.

Besides its requirement for calcium, very little is known about the regulation of type IIA sPLA$_2$s. The presence of a putative IL-6 responsive element in the 5' flanking region of the type IIA sPLA$_2$ gene has been demonstrated (Crowl *et al.*, 1991). Upon stimulation with lipopolysaccharides or cytokines, such as IL-1, IL-6 or tumor necrosis factor-α, a rapid increase in type IIA sPLA$_2$ mRNA and enzyme activity has been observed in rat vascular smooth muscle cells (Nakano *et al.*, 1990), in hepatoma cells (Crowl *et al.*, 1991), and in renal mesangial cells (Pfeilschifter *et al.*, 1989). Northern blot data presented in this paper suggest that also in rat cardiac myocytes cytokines stimulate the transcription of the type IIA sPLA$_2$ gene. In addition, the present findings indicate that the transcription level of type IIA sPLA$_2$ enzymes is upregulated in response to the α_1-adrenergic agonist phenylephrine, suggesting that type IIA sPLA$_2$ present in rat cardiac myocytes may be involved in the signalling pathways leading to cardiac hypertrophy. It should be noticed, however, that at the concentration used in the present study (10 μM), phenylephrine may also exert some β-adrenergic effects. In this respect, it is worth mentioning that in neonatal rat cardiomyocytes β-adrenergic receptor stimulation was also coupled to a rise in phospholipase A$_2$ activity (Wallukat *et al*, 1991).

In summary, a cDNA encoding the type IIA sPLA$_2$ present in cardiac tissue was cloned, predicting an enzyme that, on the basis of the presence of an eukaryotic signal peptide, theoretically could be secreted from myocytes. Analysis of its tissue distribution shows it to be expressed at high levels in ileum, and at lower levels in heart, spleen, and soleus muscle. Within the heart it is present in cardiomyocytes and fibroblasts, but probably not in endothelial cells. Stimulation of cultured rat neonatal myocytes with TNF-α and PE shows an increase in the cellular level of mRNA coding for this type IIA sPLA$_2$, suggesting its involvement in cellular signal transduction. The demonstration of the presence of a type IIA sPLA$_2$ in cardiomyocytes and its cloning allow a more detailed analysis of the biological role of PLA$_2$s in the heart.

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Chapter 4

Immunological determination of type IIA secretory phospholipase A_2

Abstract

In the present study the cDNA coding for type IIA phospholipase A_2 (type IIA $sPLA_2$) cloned from rat myocardium was used to generate and purify a recombinant 6×His-tagged type IIA $sPLA_2$ from *E.Coli* via a single step chromatographical procedure. The yield of purified recombinant type IIA $sPLA_2$ typically amounted to 1 mg/liter *E.Coli.* Against this purified fraction a polyclonal antibody was raised in rabbit. The ability of this antibody to detect type IIA $sPLA_2$ in a number of cell types and tissues, including rat and mouse myocardium, via Western blotting procedures was compared with four other anti-small molecular mass PLA_2 antibodies. It was found that all five antibodies tested were able to detect purified recombinant type IIA $sPLA_2$. In addition, the detection level of a monoclonal antibody raised against type IIA $sPLA_2$ present in rat liver was limited to a purified rat platelet fraction which contains relatively high amounts of type IIA $sPLA_2$. Finally, a commercially available polyclonal antibody originally raised against recombinant human type IIA $sPLA_2$ present in synovial fluid specifically detected the protein not only in human and rat platelets, but also in rat ileum. None of the antibodies tested, however, was able to detect type IIA $sPLA_2$ in cardiac tissue via Western blotting procedures. These results provide strong indication that under physiological conditions type IIA $sPLA_2$ protein levels in rat and mouse myocardium are relatively low and, hence, can not be detected by Western blotting using the techniques preesently available.

Introduction

Phospholipase A_2 (PLA_2) catalyzes the hydrolysis of phospholipids to generate fatty acids and lysophospholipids. To date, three major types of mammalian PLA_2s can be distinguished: high molecular mass cytoplasmic PLA_2 (type IV cPLA_2), 40 kD Ca^{2+}-independent plasmalogen specific PLA_2 (type VI iPLA_2), and the Ca^{2+}-dependent low molecular mass (14 kD) PLA_2 (Van Bilsen *et al.*, 1995, Tischfield *et al.*, 1997, De Windt *et al.*, 1998). The mammalian 14-kD PLA_2s are subdivided into a type I or pancreatic PLA_2, type IIA or secretory PLA_2 and type V PLA_2. Type IIA secretory phospholipase A_2 (type IIA sPLA_2) is present at high concentrations in rat, mouse and human intestine (Ishizaki *et al.*, 1989, Nevalainen *et al.*, 1995, De Windt *et al.*, 1997) and in the secretory granula of platelets (Aarsman *et al.*, 1989), while varying amounts have been detected in most other tissues, among which cardiac tissue (Ishizaki *et al.*, 1989, De Windt *et al.*, 1997).

Type IIA sPLA_2 has been considered to play a role in the physiological deacylation-reacylation cycle of phospholipids (Van Bilsen *et al.*, 1995, De Windt *et al.*, 1998) and in signal transduction pathways involving the liberation of membrane phospholipid-derived arachidonic acid in a number of cell types (Van Bilsen *et al.*, 1995). Furthermore, type IIA sPLA_2 activity may play a crucial role in the degradation of membrane phospholipids leading to cellular injury as observed during ischemia/reperfusion in the small intestine (Otamiri *et al.*, 1987), the brain Lauritzen *et al.*, 1994) and the heart (Van der Vusse *et al.*, 1989, Van Bilsen *et al.*, 1995, De Windt *et al.*, 1998). To study the pathophysiological role of type IIA sPLA_2 in the heart during ischemia and reperfusion the development of Western blotting analyses to quantitate type IIA sPLA_2 protein levels will be instrumental. Furthermore, the availability of specific anti-sPLA_2 antibodies will also enable future immunohistochemical localization studies at the light or electron microscopical level to determine the (sub)cellular localization of sPLA_2 in the heart and potential changes thereafter during ischemia and reperfusion.

A first attempt was made to detect cardiac type IIA sPLA_2 in immunoblotting analyses by using a previously described monoclonal anti-rat liver type IIA sPLA_2 antibody (De Jong *et al.*, 1987, Van Schaick *et al.*, 1993, Kriegsmann *et al.*, 1993). This particular antibody, however, only detected type IIA sPLA_2 in rat platelets among the various tissues tested. Therefore we decided to raise antibodies against type IIA sPLA_2 ourselves, making use recombinant rat heart type IIA sPLA_2 and a bacterial expression system.

In the present study a recombinant rat heart type IIA sPLA_2 was produced and purified from *E. Coli* to near homogeneity via a single step chromatographic purification procedure. A polyclonal antibody was raised against the recombinant rat heart type IIA sPLA_2 in the rabbit. The presence of rat heart type IIA sPLA_2 in rat and mouse heart homogenates and various rat and mouse tissues was investigated by immunoblotting using the polyclonal anti-rat heart type IIA sPLA_2 antibody. The applicability of this antibody to detect type IIA PLA_2 in Western blotting experiments was compared to that of a number of monoclonal and polyclonal anti-14 kD type II PLA_2 antibodies recently developed by other research

groups (Van Schaick *et al.*, 1993, Kriegsmann *et al.*, 1993, Murakami *et al.*, 1998) or commercially available.

Materials and Methods

Chemicals

Bovine serum albumin (BSA) was purchased from Boehringer Mannheim (Germany). Tween-20, glycine, imidazole and glycerol were purchased from Merck (Darmstadt, Germany). Urea and guanidine hydrochloride were purchased from Sigma (St. Louis, MO, USA) and ICN Biochemicals (Cleveland, OH, USA), respectively. Ni-NTA Agarose and Polyacrylamide were obtained from Qiagen (Leusden, The Netherlands) and Bio-Rad Laboratories (Hercules, CA, USA), respectively. Goat-anti-rabbit IgG/HRP complex (GARPO) and rabbit-anti-mouse/HRP complex (RAMPO) were both purchased from Dako A/S (Glostrup, Denmark). Monoclonal antibody against rat liver $sPLA_2$ was a kind gift of Dr. Van den Bosch. Monoclonal antibody against bee venom PLA_2 was a kind gift of Dr. Kriegsmann. Rabbit polyclonal antibody against rat platelet $sPLA_2$ was a kind gift of Dr. Murakami. *Naja Naja* venom PLA_2 and porcine pancreatic PLA_2 were obtained from Sigma and Boehringer Mannheim, respectively. All other chemicals used were of analytical grade and purchased from Merck.

Construction of 6×His-tagged rat heart type IIA $sPLA_2$

For the generation and purification of the recombinant rat heart type IIA $sPLA_2$ an N-terminal 6×His extension was applied, which allows rapid purification of target proteins from *E. Coli* using a metal-chelating resin (*The QIAexpressionist*, Qiagen, Leusden, The Netherlands, Bush *et al.*, 1991, Hofmann *et al.*, 1991). To produce N-terminal 6×His-tagged rat heart type IIA $sPLA_2$ in *E. Coli*, a vector containing the recently cloned full length rat heart type IIA $sPLA_2$ cDNA (pCRII-PLA_2) was digested with Bcl I to release a 479 bp fragment containing the complete rat heart type IIA $sPLA_2$ coding region except for the first 10 N-terminal amino acids (De Windt *et al.*, 1997). The Bcl I fragment was ligated into BamH I linearized pQE9, pQE10 and pQE11 vectors, which were subsequently designated pQE9-PLA_2, pQE10-PLA_2 and pQE11-PLA_2, respectively. These vectors allow generation of N-terminal 6×His-tagged recombinant heterologous proteins in *E. Coli* (Figure 1). Candidate clones were subjected to restriction analysis and sequenced using the T7 sequence kit (Pharmacia, Uppsala, Sweden). As expected, only pQE11-PLA_2 contained the type IIA $sPLA_2$ coding region in the correct reading frame (Figure 1). To achieve high levels and tight regulation of protein expression, pQE11-PLA_2 was transformed to the K12 derived *E. Coli* strain M15[pREP4] (Qiagen).

Induction and purification of 6×His-tagged rat heart type IIA $sPLA_2$ from E. Coli

LB-broth (1 liter) containing 100 mg/ml ampicillin and 25 mg/ml kanamycin was inoculated with *E. Coli* M15[pREP4](pQE11-PLA_2) and grown at 37 ...C under vigorous shaking until a OD_{600} of 0.7 was reached. To induce recombinant 6×His-tag type IIA $sPLA_2$ production in *E. Coli*, IPTG was added to the culture at a final

concentration of 2 mM and the culture was continued to grow overnight. The next day cells were harvested by centrifugation at 5,000 g for 10 min at 4 ...C. All subsequent handlings were performed at room temperature, unless indicated otherwise. The pellet was resuspended in 6 M guanidine hydrochloride, 0.1 M NaH_2PO_4, 0.01 M Tris (pH 8.0) in one tenth of the original culture volume and cells were lysed by gentle vortexing and subsequent mixing for 30 min. After centrifugation at 5,000 g for 10 min, 2 ml of Ni-NTA resin was added to the supernatant and mixed gently for 30 min. To pellet the resin, the suspension was centrifuged at 1,500 g for 5 min. The recombinant protein was allowed to renaturate while immobilized to the Ni-NTA resin by repeated resuspension and recollection of the resin by centrifugation in 500 mM NaCl, 20 % glycerol, 0.1 mM NaH_2PO_4, 0.01 mM Tris-HCl, pH 8.0, containing consecutively 8.0, 7.0, 6.0, 5.0, 4.0, 3.0, 2.0, 1.0, 0.5, and 0.25 M Urea.[18] The complete renaturation procedure lasted approximately 2 h. After renaturation the proteins were eluted from the Ni-NTA resin by addition of 2 column volumes of 0.25 M Urea, 500 mM NaCl, 0.1 mM NaH_2PO_4, 0.01 mM Tris-HCl and 250 mM imidazole (pH 8.0). The eluted proteins were extensively dialysed against PBS for 8 h at 4 ...C, aliquoted, and stored at —70 ...C until further use. Typically, this procedure yielded 1 mg 6×His-tag type IIA sPLA$_2$ per liter of *E. Coli* M15[pREP4](pQE11-PLA$_2$) culture.

Antibody preparation

Polyclonal antibodies were raised against purified recombinant soluble 6×His-tag rat heart type IIA sPLA$_2$ in a Flemish giant rabbit by subcutaneous immunization with 250 µg purified recombinant 6×His-tag type IIA sPLA$_2$ in PBS dissolved in Specol (Leenarts *et al.*, 1995; Specol:PBS 1:1 v/v). The injection fluid (2 ml) was divided equally over a number of injection sites along the backside of the rabbit. After four and five weeks the rabbit was boosted by intracuteneous injection of 250 µg 6×His-tag type IIA sPLA$_2$ in Specol (1:1 v/v). Six weeks after the first immunization, the rabbit was boosted intravenously with 250 µg 6×His-tag type IIA sPLA$_2$ in Specol and bleeded two days after the final boosting. Collected blood (approximately 100 ml) was allowed to cloth on ice, centrifuged at 1,500 g for 10 min and the serum was stored at —70 ...C until further use. Before the first immunization (0-serum) and two days after each boost, 2 ml of blood was collected in glass tubes and allowed to cloth on ice. After centrifugation at 1,500 g for 10 min, the resulting serum was stored at —70 ...C until further use. The whole porcedure was approved by the Institutional Animal Care and Use Committee of the Maastricht University.

The titer of the antisera was assessed as described previously (Vork *et al.*, 1991). Briefly, serial dilutions of the antisera collected (0-serum, 4-, 5-week boosting, and final antisera) were added to a microtiterplate, previously coated with 10 ng of purified recombinant 6×His-tag type IIA sPLA$_2$. Detection was performed by incubation of GARPO diluted 1:2,500 in phoshate buffered saline containing 0.34 mM NaCL, 0.1 % BSA and 0.05 % Tween-20, pH 7.4 (PBT) and visualized using the TMP Enzymatic Kit (Roche Diagnostic Systems Inc., Somerville, NJ, USA) according to the supplier s instructions. The titer of the antisera was defined as the antiserum dilution at which the measured extinction amounted to 50 % of the

maximal extinction and amounted to 0, 300, 800 and 1500 for the 0-serum, 4-, and 5-week boosting and final antiserum, respectively.

Tissue/subcellular homogenate and sample preparation

Tissues were homogenized in SET buffer (mM sucrose, 10 mM Tris-HCL, 2 mM EDTA, 1 mM PMSF, pH 7.4; 1:10 v/v) on ice using a Ultra-Turrax T25 (IKA Labortechnik, Staufen, Germany). Thereafter the homogenate was briefly sonicated (Soniprep 150, Beun-De Ronde, Abcoude, The Netherlands). Platelets were isolated from heparinized murine, rat and human blood by a one step centrifugation procedure at 160 g for 15 min and brief sonification on ice. Liver and heart subcellular fractions were obtained as described previously (Aarsman *et al.*, 1989). Briefly, subsequent centrifugation steps of liver and heart homogenates at 600 g for 10 min, 3,600 g for 10 min, 10,000 g for 10 min, 26,500 g for 20 min and 104,000 g for 60 min, resulted in fractions relatively enriched with nuclei and cell debris, heavy, intermediate, and light mitochondria, a microsomal fraction and a cytosolic fraction, respectively. Protein concentration of the homogenates was determined using the Micro BCA Protein Assay (Pierce, Rockford, IL, USA) following the manufacturer s instructions. Prior to sodium dodecyl sulfate - polyacrylamide gelelectrophoresis (SDS PAGE) an identical volume of sample buffer (125 mM Tris-HCl, 10 % glycerol, 10 % SDS, 5 % β-mercaptoethanol , pH 6.8) was added to the samples and the electrophoresis samples were heated to 100 ...C for 5 min. For non-reducing SDS PAGE β-mercaptoethanol was omitted from the sample buffer.

SDS-PAGE and Western blotting

Proteins were separated by electrophoresis in 12 or 15 % polyacrylamide gels in the presence of 0.1 % SDS. The separated proteins were either stained with Coomassie Brilliant Blue, or were electrophoretically transferred to nitrocellulose membrane (Protan, Schleicher & Schuell, Dassel, Germany). For Western blot analyses, non-specific protein binding on the nitrocellulose filter was blocked by incubation with phosphate-buffered saline (PBS; 10 mM phosphate, 154 mM NaCl, pH 7.4) containing 1 % BSA, either for 2 h at room temperature or overnight at 4 ¡C under continuous gentle shaking. First and secondary antibody incubation were performed in PBT at room temperature under gentle shaking for 2 and 1 hrs, respectively. First and secondary antibody preparations and their dilutions used in Western blotting analyses are presented in Table I. After antibody incubations the filter was washed four times with PBT under gentle shaking, treated with ECL Western blotting detection system (Amersham, Buckinghamshire, UK) according to the manufacturer s instructions and exposed to hyperfilm (Amersham).

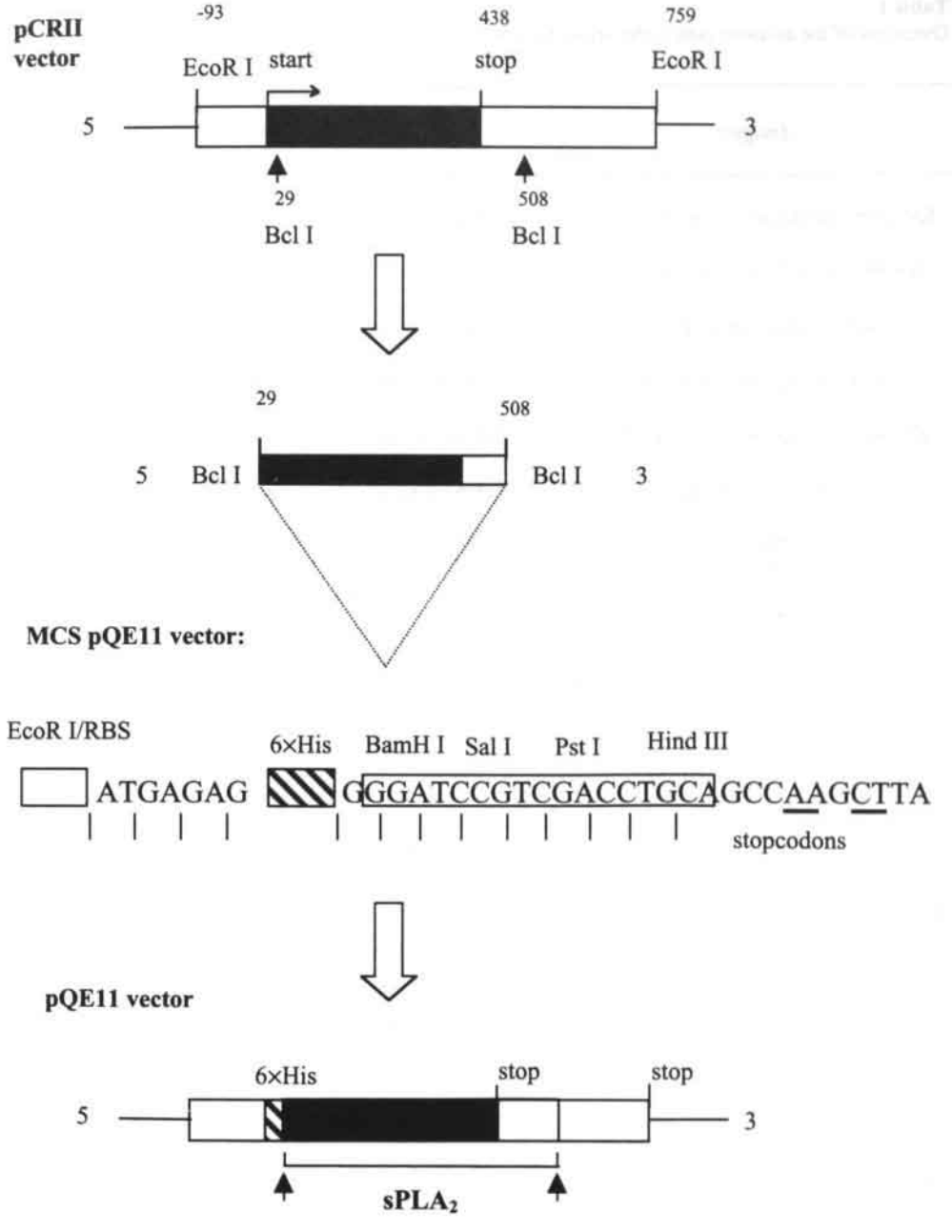

Figure 1
Overview of cloning strategy resulting in the construction of the bacterial expression vector pQE11-PLA$_2$, which allows production of 6×His-tagged sPLA$_2$. In the upper panel the outline of the pCRII-PLA$_2$ plasmid is designated, containing the full length cDNA of rat heart sPLA$_2$. Digestion of pCRII-PLA$_2$ with Bcl I released a 479 bp fragment containing the major part of the coding region and 80 bp of the 3 -untranslated region of rat heart sPLA$_2$. Ligation of the latter fragment into the BamH I site of the multiple cloning site of the pQE11 vector resulted in the production of a N-terminal 6×His-tagged rat heart sPLA$_2$ in *E.Coli*.

Table I
Overview of the antisera used in the present study.

Antigen	*Subclass*	*Dilution*	
Rat liver mitochondrial type IIA $sPLA_2$	Monoclonal, IgG	1:5,000	12
6 × His-tag rat heart type IIA $sPLA_2$	Polyclonal	1:100	*
Bee venom type II $sPLA_2$	Monoclonal, IgG	1:20	15
Rat platelet type IIA $sPLA_2$	Monoclonal, IgG	1:3,000	16
Recombinant human type IIA $sPLA_2$	Polyclonal, IgG	1:1,000	17
RGD 6 × His-tag	Monoclonal, IgG	1:1,000	**
Rabbit IgG	Gaot polyclonal, IgG	1:2,500	***
Mouse IgG	Rabit polyclonal, IgG	1:2,500	****

*, Antibody raised against recombinant 6xHis-tag type IIA $sPLA_2$ as described in the present study; **, recognizes N-terminal 6xHis-tag protein epitope with amino acid sequence R-G-H-H-H-H-H-H (purchased from Qiagen, Westburg, Leusden, The Netherlands); ***, recognizes rabbit IgG and is complexed with horseradish peroxidase (HRP; Dako A/S, Glostrup, Denmark); ****, recognizes mouse IgG and is complexed with HRP (Dako A/S, Denmark).

Results

Application of anti-rat liver type IIA $sPLA_2$ antibody

To detect the presence of type IIA $sPLA_2$ protein in (subcellular) rat heart (fractions), a monoclonal anti-rat liver type IIA $sPLA_2$ antibody was first tested on subcellular fractions of liver homogenates, relatively enriched with cell nuclei, heavy, intermediate, and light mitochondria, microsomes and cytosol via immunoblotting procedures. In contrast to earlier studies (De Jong *et al.*, 1987, Aarsman *et al.*, 1989, Van Schaick *et al.*, 1993) using the identical antibody and technical procedures, no signals were obtained in the various liver subcellular fractions (lanes 1-6, Figure 2), despite several technical modifications, nor in a number of subcellular heart and myocyte fractions (lanes 8-12, Figure 2),. However, in all Western blot analyses a strong signal of the expected size (14 kDa) was obtained in rat platelet fraction with the anti-rat liver type IIA $sPLA_2$ antibody. Platelets were included in the experiments since type IIA $sPLA_2$ is relatively abundantly present in these cells (lane 7, Figure 2).

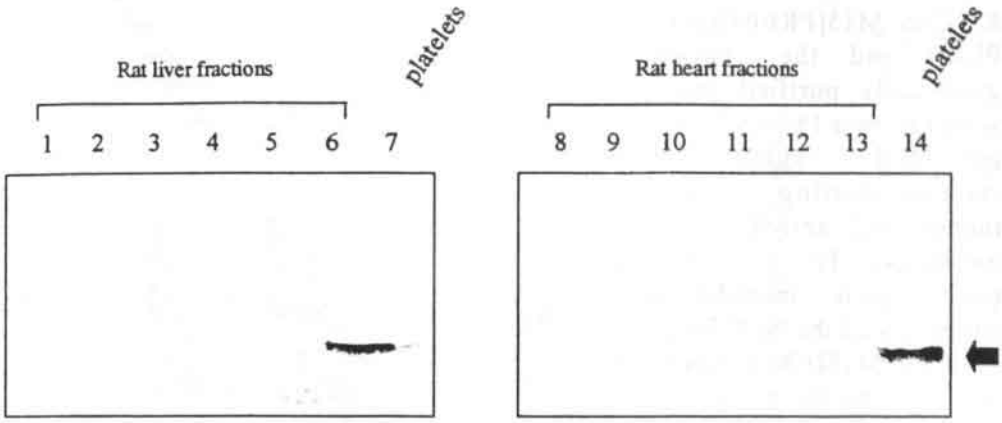

Figure 2
Result of a typical immunoblotting experiment using a monoclonal anti-rat liver type IIA sPLA$_2$ antibody on a number of rat liver subcellular fractions (left panel) and rat heart subcellular fractions (right panel). 1-6: rat liver fractions with 1, nuclei; 2, heavy mitochondria; 3, intermediate mitochondria; 4, light mitochondria; 5, microsomal fraction; 6, cytosolic fraction. 8-12: rat heart subcellular fractions with 8, nuclei; 9, heavy mitochondria; 10, intermediate mitochondria; 111, light mitochondria; 12, microsomal fraction; 13, cytosolic fraction. Lanes 7 and 14 were included as a positive controls (rat platelets). Black arrow indicates expected size of type IIA sPLA$_2$.

Production and purification of 6×His-tagged sPLA$_2$

To raise antibodies that detect rat heart type IIA sPLA$_2$ immunoblots, recombinant 6×His-tagged type IIA sPLA$_2$ was produced as described in the Materials and Methods section. After overnight IPTG induction of *E. Coli* M15[PREP4](pQE11-PLA$_2$) at 37 °C and purification using a Ni-NTA resin, a relatively pure protein fraction was obtained consisting of a prominent 17-18 kDa band as determined by Coomassie Brilliant Blue staining (solid arrow; Figure 3). In addition, two other protein bands of lower intensity were co-purified in a highly reproducible manner: a diffuse product smaller than the prominent 17-18 kDa band and one sharp band which migrated as a 29 kDa band on SDS-PAGE (open arrows; Figure 3). The slightly slower mobility of the intact 6×His-tagged type IIA sPLA$_2$ in SDS-PAGE experiments compared to native type IIA sPLA$_2$ (~ 14 kDa) might be due to the presence of the 6 N-terminal histidine residues in addition to the 21 amino acids N-terminal signal peptide of type IIA sPLA$_2$, which is normally cleaved from the mature protein.

To confirm the specific purification of a 6×His-tagged recombinant protein, non-induced and IPTG-induced *E. Coli* M15[PREP4](pQE11-PLA_2) and the chromatographically purified products were run on a 15 % SDS-PAGE gel and subjected to immunoblotting using a monoclonal anti-His epitope antibody. To control the purification method and specificity of the Ni-NTA resin, *E. Coli* M15[PREP4](pQE16) (provided by the manufacturer) was IPTG-induced to produce a 25 kDa recombinant 6×His-tagged murine dihydrofolate reductase (DHFR) and purified using similar procedures as described for the purification of 6×His-tagged rat heart type IIA $sPLA_2$.

No signals were obtained in the non-IPTG induced *E. Coli* M15 [PREP4] (pQE11-PLA_2), while in protein isolated from IPTG-induced *E. Coli* two strong signals were obtained, which electrophoresed at similar heights as the Ni-NTA agarose purified 17-18 kDa and 5-10 kDa proteins fractions.

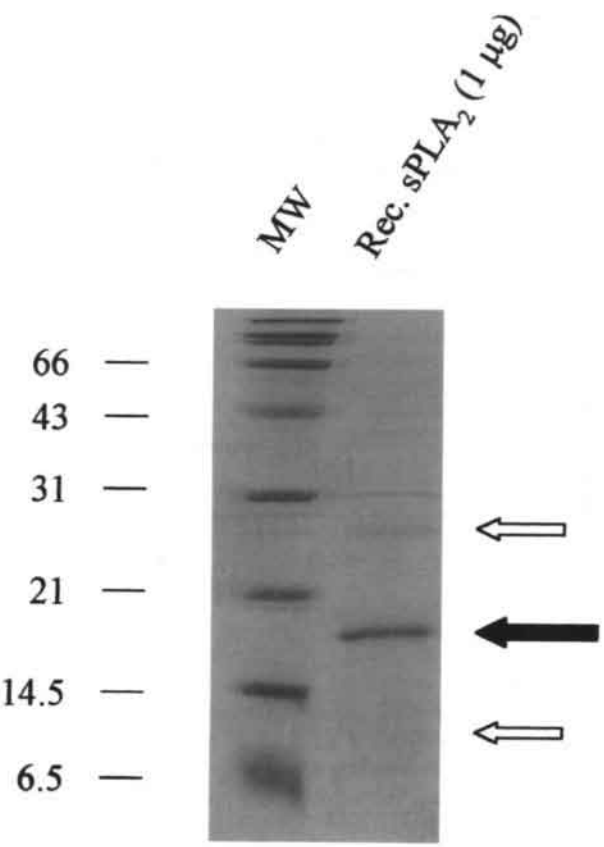

Figure 3
Coomassie Brilliant Blue staining of total protein in a 15 % SDS-PAGE gel. First lane, molecular weight marker (MW); second lane, Ni-NTA purified protein fraction of IPTG-induced *E.Coli* M15[PREP4](pQE11-PLA_2) as described in the Materials and Methods section.

In both IPTG induced *E. Coli* M15[PREP4](pQE16) crude lysates and the purified fraction, 6×His-tagged murine DHFR was specifically recognized by the monoclonal anti-6×His epitope antibody (data not shown). These results indicate the production and specific purification of a candidate recombinant 6×His-tagged type IIA $sPLA_2$ protein which migrates in SDS-PAGE electrophoresis as a 17-18 kDa protein. The detection of the smaller protein fraction of 5-10 kDa with this antibody points towards the co-purification of degradation products of 6×His-tagged type IIA $sPLA_2$.

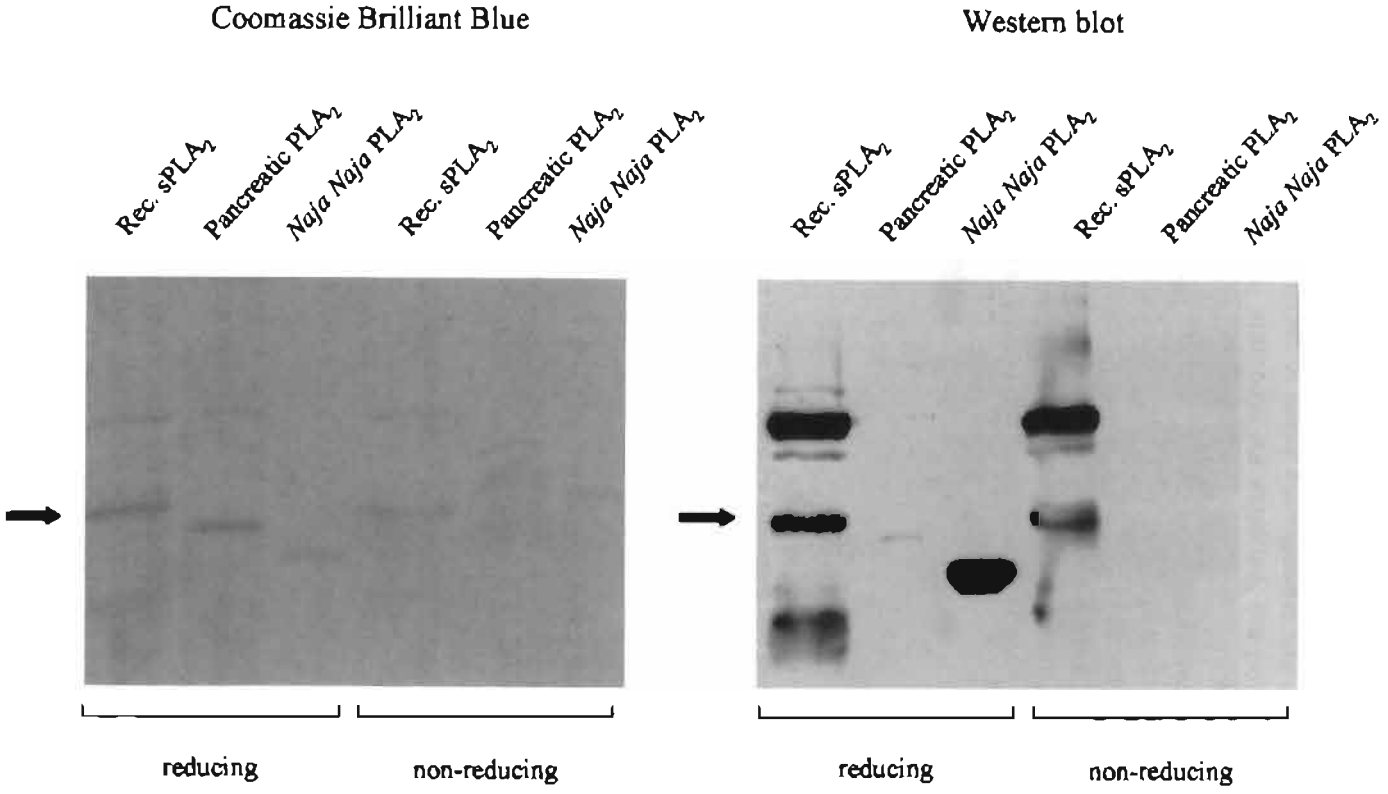

Figure 4
Coomassie Brilliant Blue total protein staining (left panel) and immunoblotting (right panel) of Ni-NTA purified 6×His-tagged type IIA $sPLA_2$, pancreatic PLA_2 and *Naja Naja* venom PLA_2 electrophoresed under reducing and non-reducing conditions. Solid arrow denotes the recombinant 6×His-tagged type IIA $sPLA_2$ product of about 17-18 kD. For immunoblotting the rabbit polyclonal anti-6×His-tagged type IIA $sPLA_2$ antibody fraction was used.

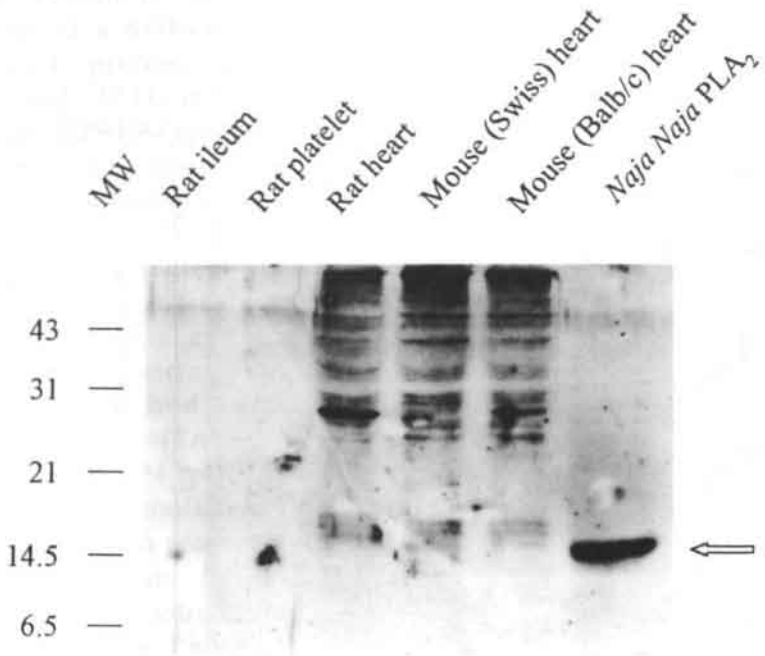

Figure 5
Result of a representative Western blot containing a number of rat and mouse tissues using the polyclonal anti-recombinant rat heart type IIA $sPLA_2$ antibody. The molecular weights of the molecular marker are indicated (MW). Naja Naja venom type PLA_2 was included as a positive control. Open arrow denotes product of expected size.

Application of polyclonal anti-6×His-tagged type IIA $sPLA_2$ antibody

After using this purified fraction to raise antibodies in the rabbit, the cross-reactivity of the polyclonal anti-6×His tagged type IIA $sPLA_2$ antibody was tested on purified recombinant 6×His tagged type IIA $sPLA_2$, pancreatic PLA_2 (type IA PLA_2) and *Naja Naja* venom PLA_2 (type IB PLA_2) in immunoblotting experiments. Figure 4 demonstrates differences in electrophoretic mobility of the three small molecular mass PLA_2s, prepared under reducing and non-reducing conditions, as demonstrated after staining the gel with Coomassie Brilliant Blue (left panel; Figure 4). Immunoblotting of an identical gel resulted in very strong signals in the recombinant type IIA $sPLA_2$ lane, a weak signal in the lane with pancreatic PLA_2 and a strong signal for *Naja Naja* venom PLA_2, following treatment of the protein samples under reducing conditions (right panel; Figure 4). In contrast, electrophoresis of the same preparations in the absence of reducing agents resulted only in a strong signal for recombinant type IIA $sPLA_2$ and no detectable signals for pancreatic and *Naja Naja* PLA_2 (right Panel; Figure 4). The latter result indicates that the polyclonal anti-6×His-tagged type IIA $sPLA_2$ antibody strongly reacts with the purified 6×His-tagged proteins under reducing as well as under non-reducing conditions. In addition, the polyclonal anti-6×His-tagged type IIA $sPLA_2$ antibody reacted to a lesser extent with reduced and non-reduced type IA and IB PLA_2s as

compared to the 6×His-tagged type IIA sPLA$_2$. A 29 kD product purified from IPTG-induced *E. Coli* M15[PREP4](pQE11-PLA$_2$) gave rise to an additional and reproducible strong signal in immunoblotting experiments, while the purified degradation products of 5-10 kD only gave a weak and diffuse signal in the immunoblot using the polyclonal anti-6×His tagged type IIA sPLA$_2$ antibody.

Figure 5 demonstrates the result of an immunoblot to detect the presence of type IIA sPLA$_2$ in a number of tissue homogenates using the polyclonal anti-6×His tagged type IIA sPLA$_2$ antibody. As expected, the polyclonal anti-6×His tagged type IIA sPLA$_2$ antibody readily detected 1 µg of *Naja Naja* PLA$_2$ (indicated by an open arrow; Figure 5). In contrast, despite many technical modifications and antibody dilutions tested no signals of the expected mass of 14 kD could be obtained in cell types and tissues which contain relatively high amounts of type IIA sPLA$_2$ such as rat platelets or rat ileum (Figure 5) or human platelets (data not shown). As could be expected from the latter result, no signals of were ever obtained in either rat or mouse heart homogenates (Figure 5).

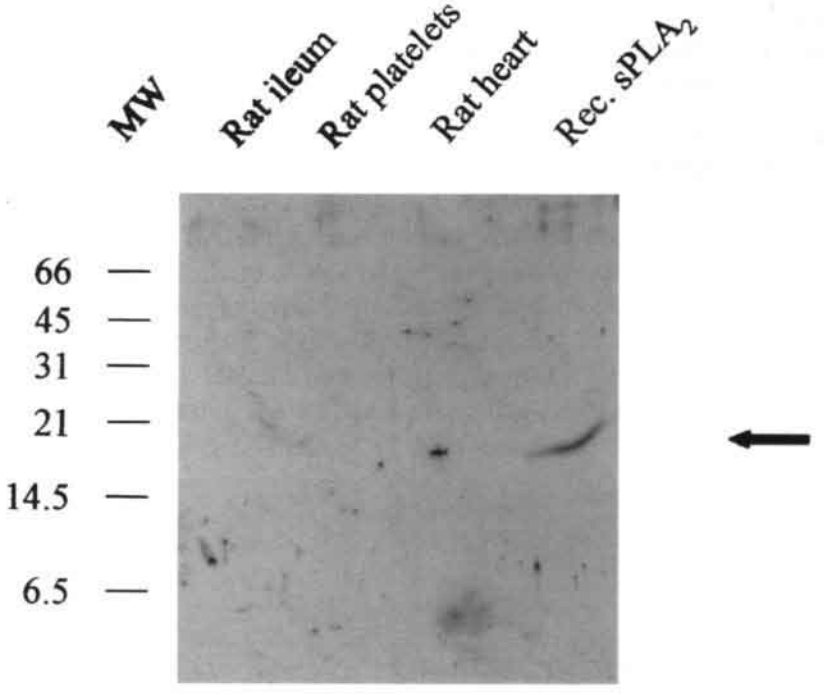

Figure 6
Representative Western blot obtained with monoclonal anti-bee venom type PLA$_2$ containing a rat ileum, rat platelets and rat heart homogenates. The molecular weights of the molecular marker are indicated (MW). Recombinant 6×His-tagged sPLA$_2$ was included as a positive control. Solid arrow denotes specific detection of a product of expected size in the 6×His-tagged sPLA$_2$ fraction.

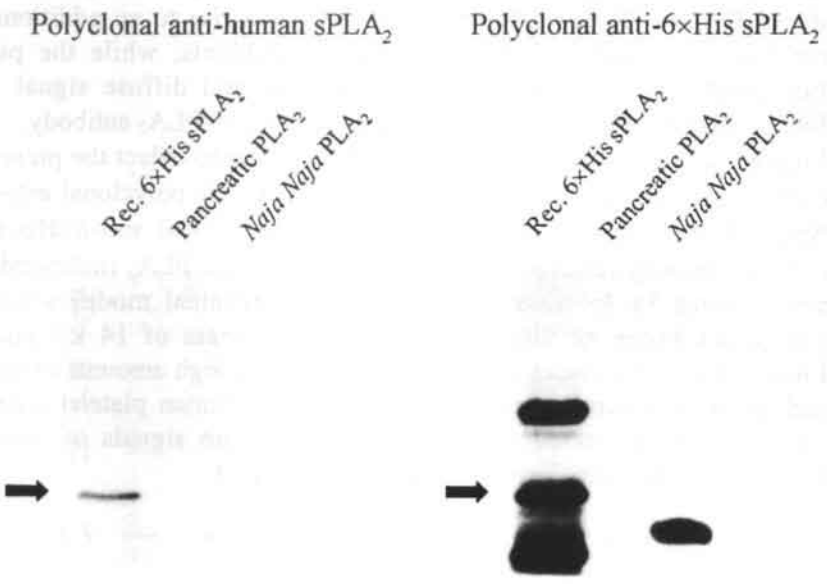

Figure 7
Immunoblots of Ni-NTA purified 6×His-tagged $sPLA_2$, pancreatic PLA_2 and *Naja Naja* venom PLA_2 electrophoresed under reducing conditions. For immunoblotting both the rabbit polyclonal anti-human synovial fluid $sPLA_2$ antibody (left panel) and rabbit polyclonal anti-6×His-tagged $sPLA_2$ antibody (right panel) were used. Solid arrow denotes the specific detection of recombinant 6×His-tagged $sPLA_2$.

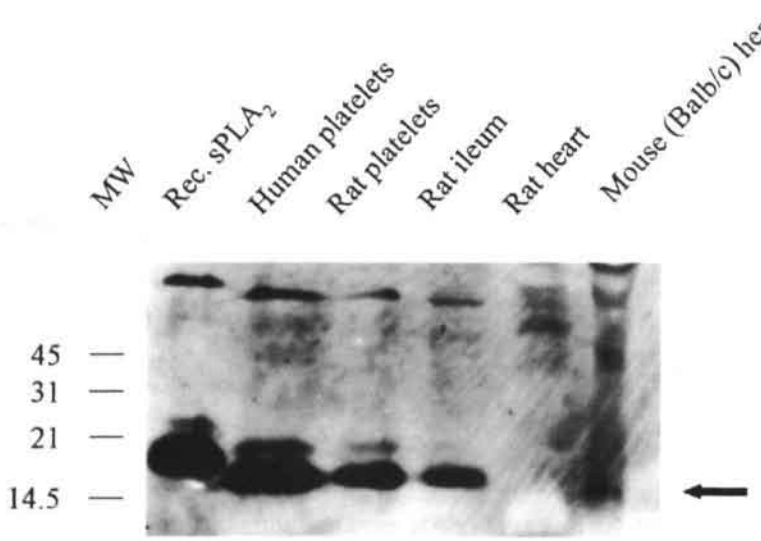

Figure 8
Representative Western blot obtained with polyclonal anti-human $sPLA_2$ antbody containing human platelets, rat platelets , rat ileum, rat heart and mouse heart homogenates. The molecular weights of the molecular marker are indicated (MW). Recombinant 6×His-tagged $sPLA_2$ was included as a positive control. Solid arrow denotes expected size of $sPLA_2$ in the various fractions.

Application of other anti-14 kDa PLA$_2$ antibodies

Due to the above negative results, we decided to test anti-14 kDa PLA$_2$ antibodies recently produced by other investigators to explore whether rat heart type IIA sPLA$_2$ is present in cardiac tissue, using Western blotting. A monoclonal anti-bee venom 14 kDa PLA$_2$ antibody (Figure 6) and a monoclonal anti-rat platelet type IIA sPLA$_2$ antibody (data not shown) were tested on purified 6×His tagged type IIA sPLA$_2$, rat ileum, rat platelet and rat or mouse heart homogenates. Figure 6 depicts a representative result obtained with monoclonal anti-bee venom PLA$_2$ in an immunoblot containing rat ileum, rat platelets, rat heart and 1 μg recombinant type IIA sPLA$_2$. The monoclonal anti-bee venom PLA$_2$ antibody readily detected 1 μg of 6×His tagged type IIA sPLA$_2$ fraction included as a positive control (Figure 6). In contrast, no signals could be obtained in any other cell type or tissue tested under conditions recommended by the suppliers.

Finally, a commercially available polyclonal anti-human synovial fluid type IIA sPLA$_2$ antibody was tested for its specificity to discriminate between different types of PLA$_2$. Figure 7 depicts two immunoblots each containing 1 μg of 6×His tagged type IIA sPLA$_2$, pancreatic PLA$_2$ and *Naja Naja* PLA$_2$, respectively, similar to the immunoblot as depicted in Figure 4. The polyclonal anti-human recombinant type IIA sPLA$_2$ antibody detected specifically the 6×His tagged type IIA sPLA$_2$ (left panel; Figure 7). As a reference, the result obtained with the polyclonal anti-6×His tagged type IIA sPLA$_2$ antibody in a similar immunoblot is depicted in the right panel of Figure 7.

The ability of the polyclonal anti-human synovial fluid type IIA sPLA$_2$ antibody to detect type IIA sPLA$_2$ in human platelets, rat platelets, rat ileum and rat and mouse total heart homogenates was tested. A strong signal was obtained both in the 6×His tagged type IIA sPLA$_2$ and human platelets fractions (lanes 1 and 2; Figure 8). The cross reactivity of this particular antibody, originally raised against human recombinant synovial type IIA sPLA$_2$, was demonstrated by the fact that signals of about 14 kD were obtained rat platelet and in rat ileum homogenates (lanes 3 and 4; Figure 8). In heart homogenates, however, no signals were observed, not even so after increasing the amounts of cardiac protein (200 μg protein/lane) and/or by increasing the concentration of the antibody 10 fold to a 100 × dilution (data not shown).

Discussion

Experimental data on the quantity and subcellular distribution of type IIA sPLA$_2$ in cardiac tissue are scarce. In the present study a number of anti-14 kDa PLA$_2$ antibodies were tested for their ability to detect type IIA phospholipase A$_2$ (type IIA sPLA$_2$) via immunoblotting procedures. Therefore, the availability of a sensitive and functional anti-type IIA sPLA$_2$ antibody would be helpful in obtaining more specific information regarding the presence and localization of type IIA sPLA$_2$ in the myocardium. Western blotting procedures would further provide a relatively straight-forward procedure to quantitate relative differences in sPLA$_2$ protein levels in cardiac tissue of transgenic mice generated with a cardiac-specific

overexpression of type IIA $sPLA_2$ in order to provide more insight into the possible participation *in vivo* of $sPLA_2$ in membrane degradation during cardiac ischemia and reperfusion.

In Table 2 the results obtained in Western blotting experiments with the antibodies tested in the present study are summarized. It was found that, in contrast to earlier studies[7,11,12], a monoclonal antibody raised against type IIA $sPLA_2$ present in rat liver mitochondria was not able to detect type IIA $sPLA_2$ in liver and heart total homogenates or subcellular fractions. This particular antibody was able to detect type IIA $sPLA_2$ in a purified rat platelets fraction, a cell type that was included in the present study as a positive control since a number of studies indicate that type IIA $sPLA_2$ protein is abundantly present in this particular cell type (Aarsman *et al.*, 1989, De Jong *et al.*, 1993, Van Schaick *et al.*, 1993, Murakami *et al.*, 1988; Table 2). However, neither in ileum, which also contains relatively high amounts of type IIA $sPLA_2$, nor in cardiac tissue a signal could be detected.

Because of these results it was decided to produce a recombinant type IIA $sPLA_2$ protein using a type IIA $sPLA_2$ cDNA recently isolated from a rat heart cDNA library (De Windt *et al.*, 1997). The cloning strategy applied to obtain recombinant type IIA $sPLA_2$ involved the bacterial expression of a N-terminal 6×Histidine tag, which allows rapid and relatively easy purification of heterologously expressed proteins (Bush *et al.*, 1991, Hofmann *et al.*, 1991). Using this strategy a 6×His-tagged type IIA $sPLA_2$ was produced and purified to near homogeneity with a typical yield of 1 mg total protein/liter *E.Coli* culture. Against this fraction a polyclonal antibody was raised in the rabbit. The resulting antibody easily detected purified 6×His-tagged type IIA $sPLA_2$ fraction and other purified types of PLA_2s, which confirms that the antibody raised against 6×His-tagged type IIA $sPLA_2$ contained antigenicity against this enzyme, but does not discriminate between the different (sub)types of small molecular weight PLA_2 enzymes. Unfortunately, this antibody did not demonstrate cross-reactivity with type IIA $sPLA_2$ present in rat or human platelet fractions or rat ileum homogenates, nor in rat or murine cardiac homogenates (Table 2).

Therefore, it was decided to test a number of other antibodies used in other studies to detect type IIA $sPLA_2$ protein, albeit in other cell types or tissues than the myocardium (Kriegsmann *et al.*, 1993, Murakami *et al.*, 1988, Dorsam *et al.*, 1995). Two monoclonal antibodies, one originally raised against 14 kDa bee venom type PLA_2 and one against rat platelet type IIA $sPLA_2$, gave similar results in Western blotting experiments. Both antibodies were able to detect 6×His-tagged type IIA $sPLA_2$, probably since this fraction contained relatively high amounts of the protein, but failed to detect type IIA $sPLA_2$ in platelets and rat ileum, both known to contain high amounts of type IIA $sPLA_2$, nor in cardiac homogenate (Table 2). This result is unexpected since the latter antibody was actually raised against rat type IIA $sPLA_2$ purified from rat platelets (Murakami *et al.*, 1988). Up till now no satisfying explanation for the negative results obtained with latter antibody fractions can be provided.

Finally, a polyclonal antibody originally raised against type IIA $sPLA_2$ from human synovial exudate was tested in immunoblotting experiments (Dorsam *et al.*, 1995). With this commercially available antibody type IIA $sPLA_2$ was readily

detected in the purified 6×His-tagged protein fraction. Moreover, signals of about 14 kD were obtained in positive control preparations such as human and rat platelets and rat ileum (Table 2). Unfortunately, no type IIA $sPLA_2$ protein could be detected in cardiac homogenates, indicating that the levels of $sPLA_2$ protein in the heart are below the detection levels of immunoblotting detection procedures. The latter finding lends further support to previously obtained circumstantial evidence of relative low abundance of type IIA $sPLA_2$ in myocardial cells (De Windt *et al.*, 1997). This notion was based on the findings that only one positive type IIA $sPLA_2$ cDNA clone was obtained after screening over one million plaques of a rat heart library. Additionally, low intensity of type IIA $sPLA_2$ hybridization signals were found in total heart and in different cardiac cell types in Northern blotting experiments (De Windt *et al.*, 1997). Moreover, results from other studies (Vervoordeldonk *et al.*, 1996, Nakano *et al.*, 1990) repeatedly indicate barely detectable type IIA $sPLA_2$ protein levels in various cell types or tissues under physiological circumstances via immunoblotting procedures, either with the antibodies of the present study or with other anti-type IIA $sPLA_2$ antibodies. Only after stimulating cells with) cytokines like TNFα and/or IL-1β or with a number of growth factors a clear upregulation of type IIA $sPLA_2$ protein levels and detection in Western blots is obtained (Vervoordeldonk *et al.*, 1996, Nakano *et al.*, 1990). These findings are in line with our own observations that type IIA $sPLA_2$ mRNA levels are over five fold upregulated in cultured neonatal rat cardiomyocytes after stimulation with TNFα or IL-1β (De Windt *et al.*, 1997). The results in the present study suggest that under physiological conditions cardiac type IIA $sPLA_2$ is present in amounts difficult to detect by means of Western blots. Future application of improved techniques, such as the use of radio-isotope imaging as an alternative detection procedure (Hunger *et al.*, 1994), might increase the sensitivity of type IIA $sPLA_2$ Western blotting to a level allowing the detection of type IIA $sPLA_2$ protein normal cardiac tissue.

In summary, a number of anti-PLA_2 antibodies were tested for their ability to detect type IIA $sPLA_2$ in non-stimulated cardiac tissue. It was found that all antibodies used in the present study were able to detect a purified, recombinantly expressed 6×His-tagged type IIA $sPLA_2$ albeit with different sensitivity. Only a monoclonal anti-rat liver type IIA $sPLA_2$ antibody and a commercially available, polyclonal anti-human synovial fluid type IIA $sPLA_2$ antibody were able to detect type IIA $sPLA_2$ in rat platelets and/or rat ileum to some extent, both preparations containing relatively high amounts of type IIA $sPLA_2$. However, none of the antibodies tested were able to detect type IIA $sPLA_2$ in non-stimulated cardiac homogenate, which indicates that relatively low amounts of type IIA $sPLA_2$ are present in cardiac tissue under physiological circumstances.

Table II
Summery of results obtained in immunoblotting experiments with five seperate antibodies in detecting $sPLA_2$ in purified fractions and in a number of tissues and cell types.

Antigen	6×His-tag $sPLA_2$	Pancreatic $sPLA_2$	*Naja Naja* venom $sPLA_2$	Rat/human platelets	Rat ileum	Rat heart
Antibodies tested						
Monoclonal anti-rat liver $sPLA_2$	+	ND	ND	+	-	-
Polyclonal anti-6×His-tag $sPLA_2$	++	+	+	-	-	-
Monoclonal anti-rat platelet $sPLA_2$	+	ND	ND	-	-	-
Monoclonal anti-bee venom $sPLA_2$	+/-	ND	ND	-	-	-
Polyclonal anti-human $sPLA_2$	++	-	-	+	+	-

ND, not determined; ++, strong specific signal obtained against antigen; +, specific signal obtained against signal; +/- weak, but specific signal obtained against antigen; -, no signal obtained against signal.

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Chapter 5

Functional and metabolic evaluation of an improved isolated, left ventricular ejecting murine heart model.

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Abstract

An improved isolated, left ventricular ejecting murine heart model is described and evaluated. Special attention was paid to the design and impedance characteristics of the artificial aortic outflow tract and perfusate composition, which contained glucose (10 mM + insulin), and pyruvate (1.5 mM) as substrates. During antegrade perfusion (preload 10 mm Hg, afterload 50 mm Hg, 2.5 mM Ca^{2+}) proper design of the aortic outflow tract provided baseline values for cardiac output (CO), left ventricular developed pressure (LVDP) and the first maximal derivative of left ventricular pressure (LV dP/dt_{max}) of 11.1 – 1.7 $ml.min^{-1}$, 83 – 5 mm Hg and 6283 – 552 mm $Hg.s^{-1}$, respectively, resembling findings in the intact mouse. During 100 min of normoxic antegrade perfusion CO decreased by less than 10 %. Varying pre and afterload resulted in typical Frank-Starling relationships with maximal CO values of 18.6 – 1.8 $ml.min^{-1}$ at pre and afterload pressure of 25 and 50 mm Hg, respectively. Left ventricular function curves were constructed at free [Ca^{2+}] of 1.5 and 2.5 mM in the perfusion medium. Significantly higher values for CO, LVDP and LV dP/dt_{max} and LV dP/dt_{min} were obtained at 2.5 mM Ca^{2+} at all loading conditions investigated. Phosphocreatine and creatine levels remained stable throughout the perfusion period. Despite a small but significant decline in tissue ATP content, the sum of adenine nucleotides did not change during the normoxic perfusion period. The tissue content of glycogen showed a statistically significant increase.

Introduction

Advances in molecular biology have made it possible to experimentally alter the mammalian genome allowing the elucidation of the functional role of specific genes in the whole animal. Transgenic or gene targeting techniques have contributed to these advances. In this respect, the mouse is the animal model of choice, because of the relative ease by which DNA can be stably introduced into the germline and the availability of embryonic stem cell lines for gene-targeting. In cardiovascular research an increasing number of genetically altered murine models are being generated, which have proven to be of great importance for a better understanding of the underlying mechanisms in complex cardiovascular disorders (Chien, 1996, Franz *et al.*, 1997).

Prerequisite for the evaluation of these cardiovascularly relevant altered murine phenotypes are adequate techniques to measure cardiac function. Efforts have been made to assess cardiac performance in the anesthetized open-chest mouse (Barbee *et al.*, 1992, Milano *et al.*, 1994, Lembo *et al.*, 1996) and in the anesthetized or conscious closed-chest mouse (Hartley *et al.*, 1995, Tanaka *et al.*, 1996, Lorenz *et al.*, 1997). In studies on cardiac function and metabolism, however, the use of isolated heart preparations is preferred, because hemodynamic performance can be assessed in a standardized way, while possible interferences such as changes in sympathetic drive, peripheral resistance, substrate supply and circulating hormone levels are avoided. For this purpose, isolated retrogradely perfused murine heart models have been developed independently by a number of investigators (Ng *et al.*, 1991, Marner *et al.*, 1995, Plumier *et al.*, 1995, Radford *et al.*, 1996, Yoshida *et al.*, 1996, Li *et al.*, 1997, Matherne *et al.*, 1997). Although these so-called Langendorff heart preparations are of use for specific applications, the antegradely perfused or left ventricular ejecting heart preparation is generally preferred, because of its more physiological resemblance and because cardiac performance can be analyzed more accurately. The development of such a model, however, is not without problems. Hemodynamic function in terms of cardiac output and contractility of the isolated ejecting mouse heart reported in previous studies (Ng *et al.*, 1991, Bittner *et al.*, 1996, Gauthier *et al.*, 1998a and 1998b) differs markedly from the performance reported in the intact mouse (Barbee *et al.*, 1992, Milano *et al.*, 1994, Hartley *et al.*, 1995, Lembo *et al.*, 1996, Lorenz *et al.*, 1997, Kass *et al.*, 1998).

In the present study we developed an improved isolated, left ventricular ejecting mouse heart model paying special attention to the impedance characteristics of the aortic outflow tract, and to perfusate composition and temperature. The stability of the murine heart in this model was investigated during prolonged normoxic perfusion (up to 100 min), using cardiac output, left ventricular developed pressure and the first maximal and minimal derivatives of left ventricular pressure as variables. Metabolic consequences of prolonged normoxic perfusion in this model were evaluated by measuring the tissue content of high energy phosphates and glycogen. Left ventricular function curves were obtained by stepwise variations of pre and afterload. The effect of perfusate calcium concentration on hemodynamic performance was addressed by varying preload,

using perfusion media containing either 1.5 or 2.5 mM nominally free Ca^{2+}.

Materials and methods

Chemicals

All chemicals used for the Krebs-Henseleit solution were of the highest grade available and purchased from Merck (Darmstadt, Germany) except for D(+)-glucose and pyruvate (Sigma Chemical Co., St. Louis, MO, USA).

Perfusion system

An assisted-mode perfusion system was used, which ensures proper coronary perfusion by an automatic switch from antegrade to retrograde perfusion when the heart is temporarily unable to generate its coronary perfusion in order to avoid unwanted underperfusion of the heart (Snoeckx *et al.*, 1986, Van Bilsen *et al.*, 1991). The perfusion system was placed in a temperature adjustable cage, the temperature of which was maintained at 38.5 ¡C. From a water-jacketed reservoir (38.5 ¡C) the perfusion fluid was pumped at a rate of 0.5 $l.min^{-1}$ through a Millipore filter (pore size 1.2 µm) into a 1.0 l bottle to suppress pressure pulsations generated by the pump. The perfusion solution passed through a film-oxygenator and was returned to the reservoir via an overflow (Figure 1).

In this way the total buffer solution was circulated, filtered, oxygenated and temperature controlled. The perfusion solution was periodically checked for pH, pO_2 and pCO_2.

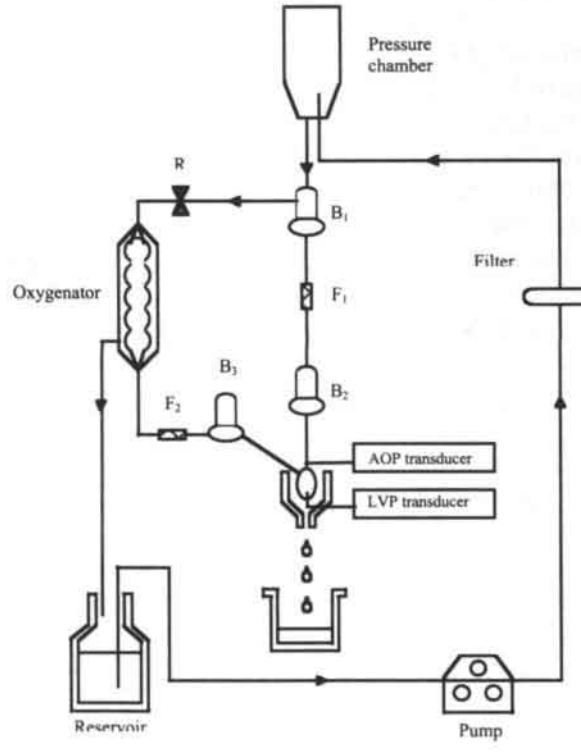


Figure 1

Schematic representation of the perfusion system for left ventricular ejecting mouse hearts. *Arrows* indicate the direction of the perfusate flow, *R*: variable resistance used to adjust perfusion pressure (retrograde perfusion) or afterload pressure (antegrade perfusion), B_1 bubble trap in aortic line, B_2 compliance chamber, B_3 bubble trap in venous return line, F_1 1N inline flow probe, F_2 2N inline flow probe, *AOP* aortic pressure transducer, *LVP* left ventricular pressure transducer.

pH values ranged between 7.40 and 7.45, while pO_2 exceeded 650 mm Hg.

Aortic pressure was regulated by the air compression in the bottle and by a variable hemodynamic resistance (R) placed between bubble trap B_1 and the film-oxygenator. An inline 1N flow probe (F_1; Transonic System Inc., Ithaca, NY, USA) was placed between bubble trap B_1 and the compliance chamber (B_2) to measure retrograde and antegrade aortic flow. Left atrial filling pressure was determined by the height of the overflow level in the film-oxygenator. Entry of air-bubbles, if any, was prevented by a bubble trap (B_3) in the left atrial filling line. An inline 2N flow probe (F_2; Transonic System Inc.) was placed before bubble trap B_3 to measure atrial filling flow (venous return).

Design of the aortic outflow tract

In the design of an artificial system for isolated ejecting hearts, the hemodynamic characteristics of the aortic conduit are often neglected. This may result in a too high aortic impedance, leading to an unphysiologically high pressure load to the left ventricle, and subsequently to poor performance of the isolated heart (Van Bilsen *et al.*, 1991). To avoid this situation, the aortic conduit was designed to match the hemodynamic characteristics of an isolated murine heart. The most critical part of the artificial aortic outflow tract is the short, rigid cannula between the heart and the compliance chamber distal to the aortic cannula. The major determinants of the pressure drop over the cannula are fluid mass acceleration (inertia term) and the loss of kinetic energy in the distal jet stream (Bernouilli term) (Van Bilsen *et al.*, 1991). The viscous losses in the Poisseuille term appear to be negligible. The pressure drop in the cannula should be kept below 4 kPa (31 mm Hg). Setting the pressure loss (P_{bern}) due to the Bernouilli term to this maximum leads to the following condition for the internal diameter d of the cannula:

$$d \geq \sqrt{AOF \sqrt{\frac{(\rho \cdot k_1)}{P_{bern}}}} \qquad (1)$$

where k_1 = a constant, AOF = mean aortic flow and ρ fluid density. Substitution of realistic values in equation (1), i.e., AOF = 0.25 $ml.s^{-1}$ (Barbee *et al.*, 1992), ρ = 1050 $kg.m^{-3}$ and $k_1 \approx 24$, results in $d \geq 0.79$ mm. To make the design of the cannula less critical, the internal cannula diameter should be chosen as large as possible. In our set-up we were able to set the diameter at $d = 0.92$ mm.

An important second design criterium is to keep the fluid inertia term of the pressure drop, P_{inert}, below about 2 kPa (15.5 mm Hg). This condition results in a maximum value for the length of the cannula l:

$$l \leq \frac{d^2 \cdot t_{cycl} \cdot P_{inert}}{\rho \cdot AOF \cdot k_2} \qquad (2)$$

Substituting for the diameter $d = 0.92$ mm, for the cardiac cycle duration t_{cycle} = 150

ms, for P_{inert} = 2 kPa (15.5 mm Hg) and for $k_2 \approx 237$, yields $l \leq 4.1$ mm. Based on these estimations an aortic cannula was made with a length of 4.0 mm and inner diameter of 0.92 mm. The outer diameter was 1.1 mm, which was just small enough for attachment of the murine aortic stump to the cannula (Hartley *et al.*, 1995). A schematic drawing of the cannula is presented in Figure 2.

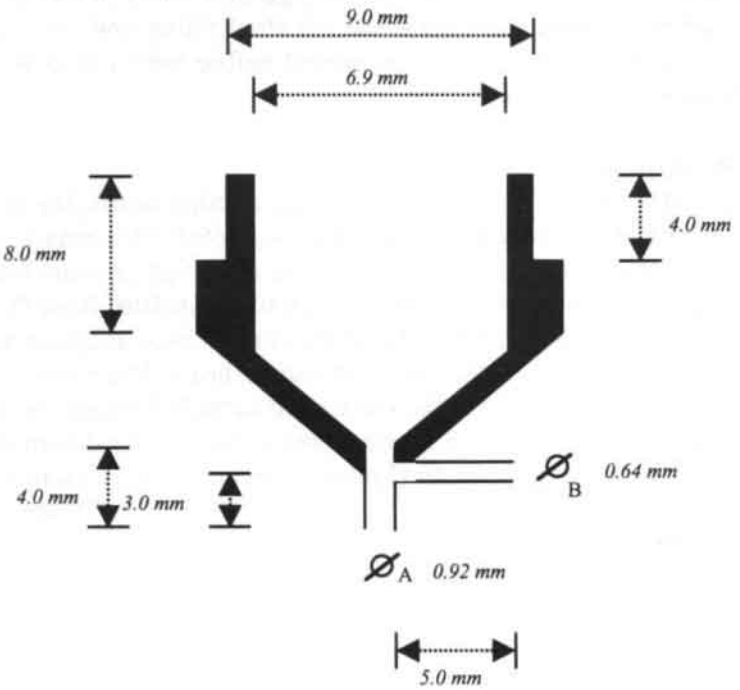


Figure 2
Detailed schematic representation of the dimensions of the outflow tract. *A* indicates the cannula to which the aorta of the heart is attached. The inner diameter ($\varnothing_A$) is 0.92 mm. The position of the aortic pressure outlet (*B*) is shown with an inner diameter ($\varnothing_B$) of 0.64 mm.

Animals and surgery

Adult female and male Swiss mice (Iffa Credo, Lyon, France), average body weights 33.7 – 3.1 g and 46.1 – 1.6 g, respectively, were kept under standard housing conditions with an artificial 12 h light cycle. The animals had free access to food (Diet SRM-A, Hope farms, Woerden, the Netherlands) and tap water. Experiments were approved by the Institutional Animal Care and Use Committee of the Maastricht University.

Before surgery, the mice underwent mild CO_2 anesthesia followed by a 50 $mg.kg^{-1}$ sodium pentobarbital injection i.p. (Nembutal, Sanofi Sante BV., Maassluis, the Netherlands). After thoracotomy the hearts (average weight of 197 – 29 and 266 – 26 mg, and heart weight/body weight ratio of 5.9 – 0.9 and 5.8 – 0.5 $mg.g^{-1}$ for female and male mice, respectively) were quickly excised and placed in ice-chilled perfusion buffer. After removal of remnant lung and fat tissue, the aorta was connected to the aortic cannula and retrograde perfusion was started immediately at a perfusion pressure of 50 mm Hg, after which the hearts started to beat spontaneously. The left atrium was cannulated with an atrial 20-gauge cannula (inner diameter 0.64 mm, outer diameter 0.90 mm, length 10.6 mm) through one of the lung veins.

Table I
Hemodynamic values measured in isolated, ejecting Swiss mouse hearts after 20 min of perfusion to allow the hearts to adapt to the working mode.

HW (mg)	197 ± 29
CO ($ml.min^{-1}$)	11.1 ± 1.7
AOF ($ml.min^{-1}$)	7.0 ± 2.1
CF ($ml.min^{-1}$)	4.1 ± 1.3
SV (µl)	29 ± 5
LVSP (mm Hg)	88 ± 5
LVEDP (mm Hg)	5 ± 3
LV dP/dt_{max} ($mm\ Hg.s^{-1}$)	6,283 ± 552
LV dP/dt_{min} ($mm\ Hg.s^{-1}$)	-3,374 ± 293

Data are expressed as means ± SD (n=8). Hearts were paced at 380 beats.min-1, pre- and afterload pressures were 10 and 50 mm Hg, respectively. HW, heart weight; CO, cardiac output; AOF, aortic flow; CF, coronary flow; SV, stroke volume; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; LV dP/dt_{max}, first maximal derivative of left ventricular pressure; LV dP/dt_{min}, first minimal derivative of left ventricular pressure.

Left ventricular pressure was measured with a PE-50 catheter, inserted into the left ventricular cavity through the apex, connected to a Baxter Pressure transducer (Baxter Healthcare Corp., Irvine, CA, USA). Aortic pressure was measured through a side branch of the aortic cannula, located 3 mm above its entrance (Figure 2).

The hearts were perfused with a modified Krebs-Henseleit solution, consisting of (in mM): NaCl (118), KCl (4.7), $CaCl_2$ (3.0), $MgSO_4$ (1.2), KH_2PO_4 (1.2), $NaHCO_3$ (25), Na-EDTA (0.5), D(+)-glucose (10) and Na-pyruvate (1.5), which was gassed with carbogen (95% O_2, 5% CO_2). Due to the presence of 0.5 mM EDTA, the nominally free calium concentration of this perfusion medium was 2.5 mM. Insulin (5 U/l) was added to the perfusate. Due to losses (sticking to the glass wall of the perfusion system) the final insulin concentration in the circulating

perfusion medium amounted to 0.15 U/l as determined by an insulin radioimmunoassay (Amersham, Buckinghamshire, UK).

During preliminary experiments the performance of the isolated perfused murine heart was found to be highly sensitive to temperature fluctuations. Therefore efforts were undertaken to avoid excessive cooling of the hearts due to, for instance, evaporation of moisture from the epicardial surface. Moreover, the perfusate entering the heart was consistently 38-39 ¡C, similar to core body temperatures in the mouse (Conroy *et al.*, 1980, Miller *et al.*, 1994).

Hemodynamic data

All hemodynamic variables were continuously recorded on a personal computer, using specialized software (Hemodynamic Data Acquisition System, Technical Department Maastricht University), allowing the on-line acquisition, calculation and presentation of aortic flow (AOF), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), diastolic aortic pressure (AOPD) and the first maximal and minimal derivatives of left ventricular pressure (LV dP/dt_{max} and LV dP/dt_{min}). Left ventricular developed pressure (LVDP) was defined as the difference between LVSP and LVEDP. Cardiac output (CO) was defined as the sum of aortic flow (AOF) and coronary flow (CF). CF was determined from the difference between aortic flow (AOF), as measured by flow probe F_1, and left atrial filling flow, as measured by the inline flow probe F_2 (Figure 1).

In a separate test, it was investigated whether LV dP/dt_{max} was limited by the frequency response of the pressure transducer (Baxter). In a closed container a pressure step was induced with a rise time of 2 ms as measured with a Millar pressure transducer. The rise time of the pressure measuring system used in the present study was 3 ms. The rise times of LVP as measured experimentally were found to be about five times higher, i.e., 15-20 ms. Therefore, it can be concluded that measured LV dP/dt_{max} was not limited by the frequency response of the pressure measurement system.

Experimental protocol

In the experiments to test the stability of the preparation, antegrade perfusion was started by opening the left atrial conduit after an initial 10 min retrograde stabilization period at 50 mm Hg. The hearts were perfused at a left atrial filling pressure (preload) of 10 mm Hg, while diastolic aortic pressure (afterload) was kept at 50 mm Hg. Five minutes after the onset of antegrade perfusion, the hearts were paced via platina electrodes attached to the right atrium at a rate of 380 beats.min^{-1} until the end of the experiment, i.e., 100 min after the onset of antegrade perfusion. After completion of the experiments the ventricles of the individual hearts were separated from the atria and immediately frozen between aluminum clamps, previously cooled in liquid nitrogen, and stored at -80¡C for further analysis. In a separate series of experiments, hearts were normoxically perfused for a 20 min stabilization period, after which the ventricles of the individual hearts were separated from the atria and immediately freeze clamped between aluminium clamps, previously cooled in liquid nitrogen, and stored at -80 °C for further biochemical analysis.

In another series of experiments left ventricular function curves were constructed by increasing left atrial filling pressure stepwise from 10 to 15, 15 to 20, and from 20 to 25 mm Hg at diastolic afterload pressures of 50, 75 and 100 mm Hg, respectively. At each preload pressure the paced hearts (380 beats. min^{-1}) were allowed to stabilize for 5 min during which hemodynamic data were recorded. After completion of data collection at the maximal preload pressure, hearts were allowed to recover in the antegrade mode for 10 min at baseline loading conditions (i.e., preload pressure of 10 mm Hg, afterload pressure of 50 mm Hg), after which the next function curve was performed.

In a third series of experiments the influence of different free Ca^{2+} concentrations in the perfusate (1.5 and 2.5 mM) on left ventricular function was investigated.

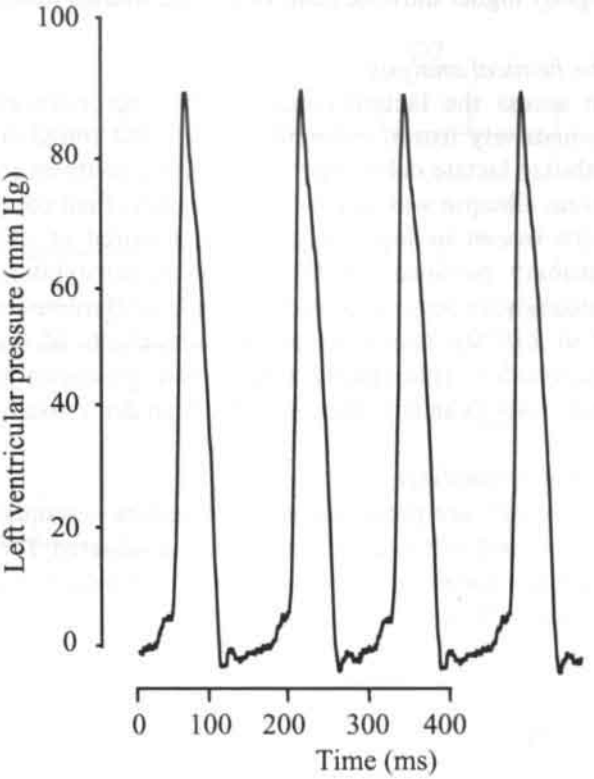


Figure 3
Typical recordings of left ventricular pressure curves of a normoxically perfused, isolated ejecting mouse heart. Depicted are left ventricular preessure tracings at a preload pressure of 10 mm Hg and an afterload pressure of 50 mm Hg.

To this end male murine hearts were perfused in an antegrade manner with Krebs-Henseleit perfusion medium containing 1.5 mM nominally free Ca^{2+} (2.0 mM $CaCl_2$ plus 0.5 mM Na-EDTA). Hearts were allowed to equilibrate for 20 min, after which left ventricular function curves were constructed by increasing preload pressure in a stepwise fashion from 10 to 15, 15 to 20, and 20 to 25 mm Hg at a constant afterload pressure of 50 mm Hg. At each preload pressure the hearts were allowed to stabilize for 5 min during which LVP, AOP, CO, AOF and CF were recorded. After data collection at the maximal preload pressure, the hearts were allowed to recover in the antegrade mode for 10 min at pre and afterload pressures of 10 and 50 mm Hg, respectively. Hereafter $CaCl_2$ was added to the perfusion medium to a nominally free Ca^{2+} concentration of 2.5 mM. Hearts were allowed to equilibrate for 10 min, whereafter the same experimental protocol was followed.

During the complete experiment hearts were paced at 400 beats.min^{-1} due to the slightly higher intrinsic heart rate of the murine hearts.

Biochemical analysis

To assess the lactate content in the coronary effluent, samples of 1 ml were immediately frozen in liquid nitrogen and stored at -80 ¡C for further analysis. To stabilize lactate dehydrogenase (LDH) activity in coronary effluent samples, bovine serum albumin was added to the samples (final concentration 3 %), after which they were frozen in liquid nitrogen and stored at -80 ¡C. Lactate and LDH in the coronary perfusate were assessed spectrophotometrically using a Cobas Bio autoanalyzer according to the method of Bergmeyer and Bernt ((1974) and Apstein *et al.* (1970), respectively. Tissue contents of high energy phosphates [adenine nucleotides, (phospho)creatine] and glycogen were determined as described previously (Van Bilsen *et al.*, 1991, Van der Vusse *et al.*, 1994).

Statistical analysis

The results are presented as mean values – standard deviations (SD). Differences within and between groups were evaluated for statistical significance using Student s t-test for paired and unpaired data, respectively. P values < 0.05 were considered to be statistically significant.

Results

Baseline hemodynamics and stability

The baseline values of the various hemodynamic variables as measured 20 min after the start of antegrade perfusion are presented in Table 1. Under the conditions applied (10 mm Hg preload pressure; 50 mm Hg afterload pressure; pacing at 380 beats.min^{-1}) AOF, CF and CO amounted to 7.0 – 2.1, 4.1 – 1.3 and 11.1 – 1.7 ml.min^{-1}, respectively. Values of 88 – 5, 5.0 – 3.2 and 83 – 5 mm Hg were found for left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP) and left ventricular developed pressure (LVDP), respectively. A representative tracing of a left ventricular pressure curve measured under basal conditions (pre and afterload pressures of 10 and 50 mm Hg, respectively) is shown in Figure 3.

To test the stability of the preparation, hearts were antegradely perfused for 100 min. During this period CO decreased by about 10 % (Figure 4). No significant changes were observed in LVEDP between the onset (6.0 – 3.2 mm Hg) and the end of antegrade perfusion (6.4 – 4.1 mm Hg). LVSP, and consequently LVDP, showed a decline of about 20 %. LV dP/dt_{max} decreased by about 25 %, whereas LV dP/dt_{min} did not change significantly (Figure 4). The changes in hemodynamic function were confined to the first 40 min of perfusion, whereafter cardiac function did not show a statistically significant change.

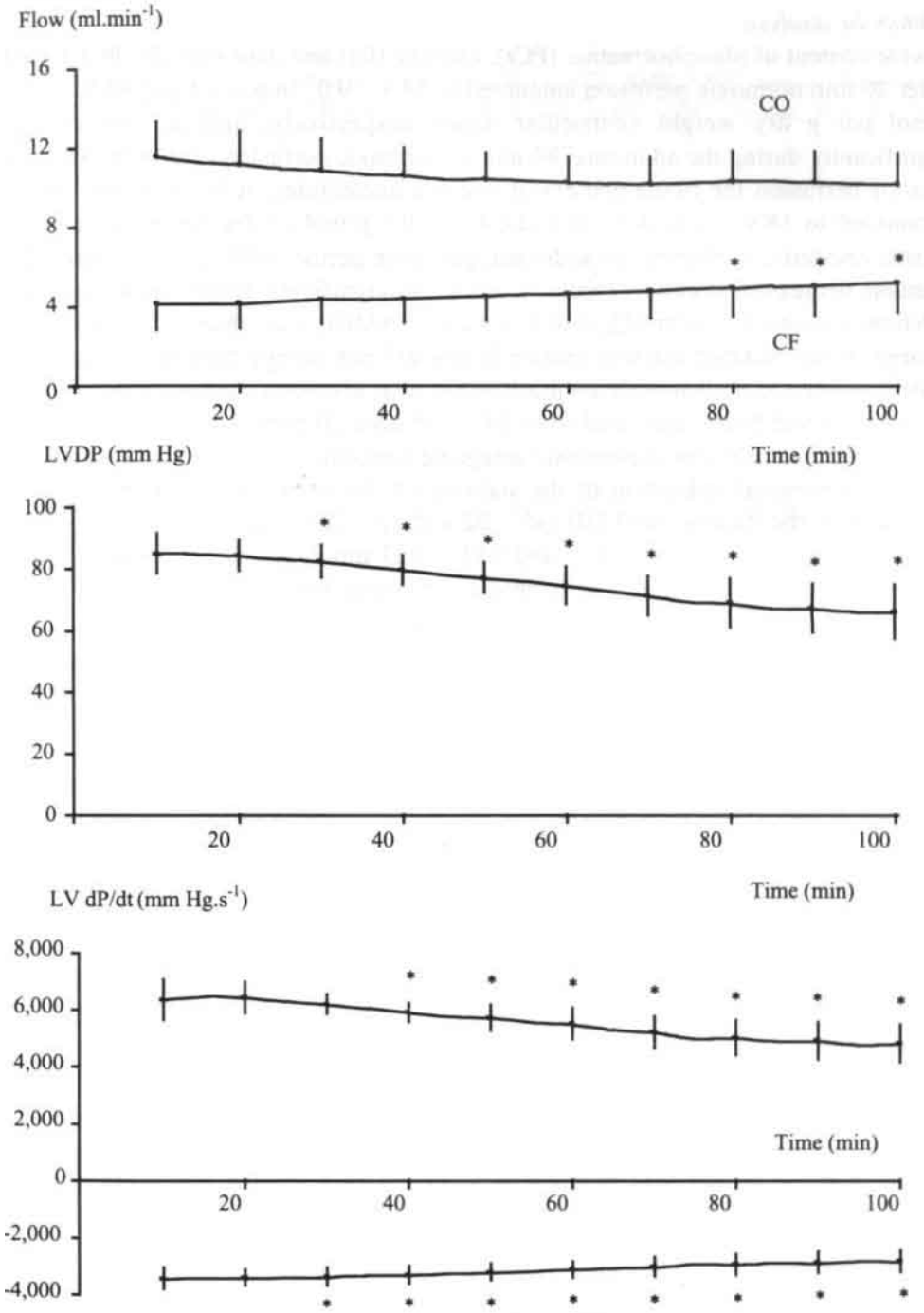


Figure 4
Cardiac output (CO) and coronary flow (CF; upper panel), left ventricular developed pressure (LVDP; middle panel), and in the lower panel the first maximal and minimal derivative of left ventricular pressure (LV P/dt_{max} upper trace and LV dP/dt_{min} lower trace, respectively) as a function of time in the normoxically perfused, isolated working mouse heart under basal conditions (10 and 50 mm Hg pre- and afterload, respectively). Data are expressed as means ± SD (n = 8). * $P < 0.05$ vs value after 20 min of normoxic perfusion.

Metabolic analysis

Tissue content of phosphocreatine (PCr), creatine (Cr) and their sum [Σ (PCr + Cr)] after 20 min normoxic perfusion amounted to 33.3 – 9.0, 36.6 – 6.4 and 69.9 – 12.0 µmol per g dry weight ventricular tissue, respectively, and did not change significantly during the additional 80 min of normoxic perfusion (Table 2). After 20 min of perfusion the tissue content of adenine nucleotides, ATP, ADP and AMP, amounted to 18.9 – 2.0, 4.3 – 0.5 and 0.6 – 0.1 $\mu mol.g^{-1}$ dry weight ventricular tissue, respectively. During the additional perfusion period of 80 min the tissue ATP content decreased slightly (Table 2), while no significant reduction in the total adenine nucleotide content [Σ (ATP + ADP + AMP)] was observed. The energy charge of the isolated ejecting murine hearts did not change between 20 and 100 min of normoxic perfusion. In contrast, ventricular glycogen content of the isolated ejecting murine hearts increased from 245 – 68 after 20 min to 409 – 100 $\mu mol.g^{-1}$ dry weight after 100 min of normoxic antegrade perfusion.

Additional indication of the stability of the working heart preparation is provided by the finding that LDH (45 – 22 and 22 – 20 $mU.min^{-1}.heart^{-1}$, onset vs. end; N.S.) and lactate (0.60 – 0.12 and 0.91 – 0.23 $\mu mol.min^{-1}.heart^{-1}$, onset vs. end; N.S.) release into the coronary effluent did not change between the onset (0-10 min) and the end of antegrade perfusion (90-100 min).

Left ventricular function curves

Left ventricular functions curves resembling Frank-Starling curves were constructed by varying both pre and afterload. At an afterload pressure of 50 mm Hg a marked rise in CO was observed in response to the stepwise elevation of preload pressure, increasing from 12.0 – 0.6 to 18.6 – 1.2 $ml.min^{-1}$ at preload values of 10 and 25 mm Hg, respectively (Figure 5, Panel A). As expected, the increases in CO were less pronounced at afterload pressures of 75 mm Hg and 100 mm Hg (Figure 5, Panel A). At all afterload pressures CF increased approximately 1.5 fold from the lowest to the highest preload pressure level tested, reaching maximal values of 7.8 – 2.3 $ml.min^{-1}$ at an afterload pressure of 100 mm Hg (Figure 5, Panel A). LVEDP substantially increased when preload pressure was enhanced (Figure 5, Panel B).

In general the left ventricular end-diastolic pressure (LVEDP) measured was lower than the preload pressure applied by the height of the oxygenator overflow level. Interestingly, at an afterload pressure of 50 mm Hg the preload dependent increase in CO observed (Figure 5, Panel A) was not associated with significant rises in LVSP and LV dP/dt_{max} (Figure 5, Panel B and C). In contrast, at afterload pressures of 75 and 100 mm Hg, LVSP and LV dP/dt_{max} displayed preload-dependent elevations (Figure 5, Panel B and C). LV dP/dt_{min} did not show a preload dependent change at any afterload tested, but showed a more negative value at 75 and 100 mm Hg, indicating faster relaxation, than at 50 mm Hg.

Table II

Tissue content of phosphocreatine (PCr), creatine (Cr), ATP, ADP, AMP, energy charge and glycogen of isolated left ventricular ejecting mouse hearts after 20 and 100 min of normoxic, antegrade perfusion.

Compound ($\mu mol.g^{-1}$ *dry weight)*	*Duration of perfusion (min)*	
	20	*100*
	33.3 ± 8.2	31.3 ± 4.7
PCr	36.6 ± 5.9	34.2 ± 6.2
Cr	69.9 ± 10.9	65.6 ± 5.4
Σ (PCr + Cr)		
	18.9 ± 1.8	15.3 ± 1.4 *
ATP	4.3 ± 0.4	4.6 ± 1.0
ADP	0.6 ± 0.1	0.7 ± 0.4
AMP	23.8 ± 2.0	20.6 ± 1.6
Σ (ATP + ADP + AMP)	0.89 ± 0.01	0.86 ± 0.04
Energy charge		
	245 ± 68	437 ± 114 *
Glycogen (glucose units)		

Values are presented as means ± SD (both groups n = 6). Energy charge is defined as (ATP + 0.5 ADP)/(ATP + ADP + AMP) and is unitless.

Effect of calcium on cardiac function

To test the effect of the perfusate calcium concentration on cardiac performance, left ventricular function curves were constructed at 1.5 mM and 2.5 mM free calcium. At both concentrations typical Frank-Starling curves were obtained by increasing the preload pressure in a stepwise manner from 10 to 25 mm Hg with intervals of 5 mm Hg at an afterload pressure of 50 mm Hg. In both the low and normal calcium group a significant rise in CO was observed in response to the stepwise elevation of preload pressure. In the low calcium group CO increased from

11.9 – 2.6 to 14.9 – 3.3 ml.min^{-1} ($P < 0.05$) when elevating preload pressure from 10 to 25 mm Hg (Figure 6). At the preload pressures tested CO values were significantly higher in the 2.5 mM Ca^{2+} group, increasing from 14.5 – 2.0 ml.min^{-1} to 21.8 – 2.6 ml.min^{-1}, when elevating preload pressure from 10 to 25 mm Hg (Figure 6). Similarly, AOF values were about 45 % lower in the low extracellular calcium group over the whole range of loading conditions tested (7.0 – 1.9 and 9.9 – 1.9 ml.min^{-1} at preload pressure of 10 and 25 mm Hg, respectively) as compared to those measured at a nominally free Ca^{2+} concentration of 2.5 mM (9.9 – 1.4 and 14.2 – 2.2 ml.min^{-1} at preload pressure of 10 and 25 mm Hg, respectively). In contrast, in both groups CF increased approximately 1.6 fold from the lowest to the highest preload pressure tested and did not demonstrate a significant difference between the two groups at corresponding loading conditions. LVEDP values demonstrated preload dependent elevations, but did not show significant differences between both groups at all corresponding preload pressures tested. In the low calcium group LVSP, LV dP/dt_{max} and LV dP/dt_{min} amounted to 80 – 16 mm Hg, 5100 – 1617 mm Hg.s^{-1} and -2885 – 476 mm Hg.s^{-1}, respectively, at baseline loading conditions. Elevation of the Ca^{2+}-concentration in the perfusion medium to 2.5 mM resulted in significantly higher values for LVSP, LV dP/dt_{max} and $LVdP/dt_{min}$ (109 – 17 mm Hg, 7480 – 1632 mm Hg.s^{-1} and -3537 – 705 mm Hg.s^{-1}, respectively) at similar pre and afterload pressures.

Discussion

In the present study an improved isolated working mouse heart model is described. In developing this model special attention was paid to the characteristics of the aortic outflow tract and the composition and temperature of the perfusion medium. The design of the aortic outflow tract is critical to avoid a too high aortic impedance and, hence, a too high load to the ejecting left ventricle (Van Bilsen *et al.*, 1991). A high impedance of the aortic outflow tract will lead to a high systolic left ventricular pressure and enhanced oxygen demand of the isolated heart (Neely *et al.*, 1967, Kobayashi *et al.*, 1983). The aortic cannula of the present study was designed using hemodynamic values as measured in the mouse *in vivo* (Barbee *et al.*, 1992, Hartley *et al.*, 1995) and the dimensions of the mouse ascending aorta (Hartley *et al.*, 1995). The proper design of the aortic outflow tract likely explains the physiological hemodynamic values observed during ejection (see below).

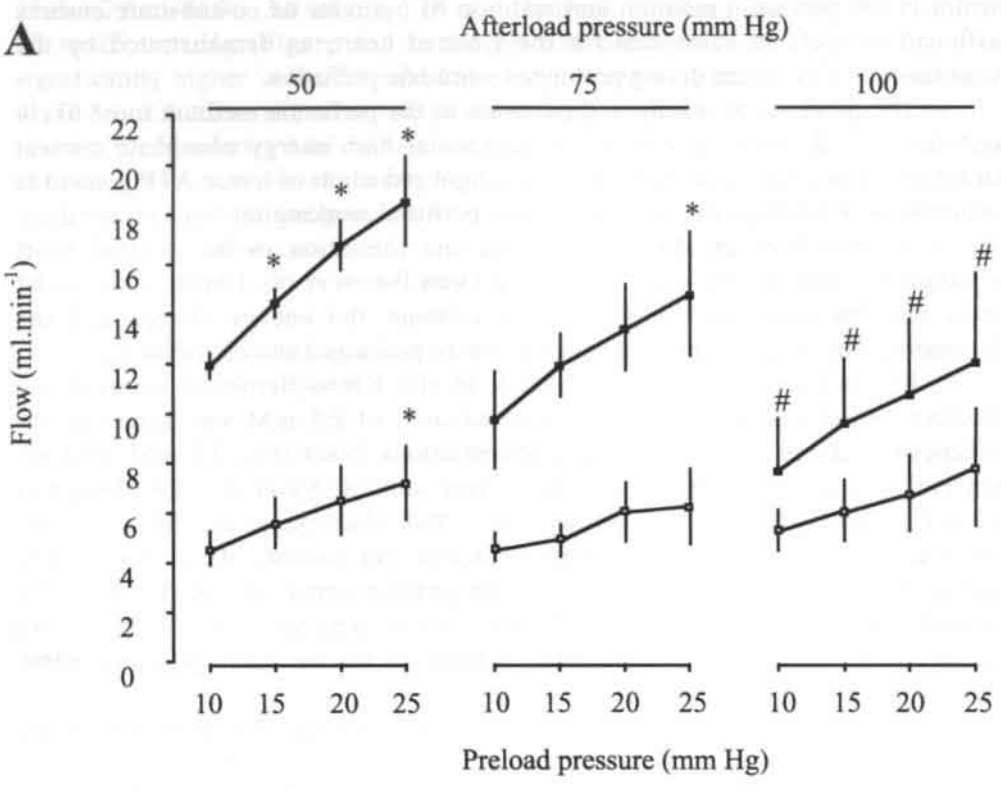
Another factor influencing hemodynamic performance of isolated, perfused mouse heart preparations is the substrate composition of the perfusion medium. Pyruvate was included as co-substrate to the perfusate solution, as its beneficial effects on hemodynamic and metabolic stability of isolated heart preparations have been well documented (Van Bilsen *et al.*, 1989 and 1991, De Groot *et al.*, 1993). A concentration of 1.5 mM pyruvate was chosen to substitute for all monocarboxylate substrates present in the physiological situations. Increasing the final concentration of pyruvate to 5 mM in the Krebs-Henseleit buffer, previously described for isolated rat heart preparations (Snoeckx *et al.*, 1986, Van Bilsen *et al.*, 1989 and 1991), did not improve hemodynamic stability (data not shown). The presence of

insulin in the perfusion medium and addition of pyruvate as co-substrate ensures sufficient extractable substrates for the isolated heart, as demonstrated by the accumulation of glycogen during prolonged normoxic perfusion.

The presence of insulin and pyruvate in the perfusion medium most likely contributed to the relative stability of ventricular high energy phosphate content during prolonged normoxic perfusion. The slight reduction of tissue ATP content is consistent with findings in the normoxically perfused working rat heart preparation. This imbalance between ATP degradation and formation in the isolated heart preparation is still incompletely understood (Van Bilsen *et al.*, 1989). It should be noted that the total adenine nucleotide content, the energy charge and the phosphocreatine content remained constant during prolonged aerobic perfusion.

By including 0.5 mM Na-EDTA in the Krebs-Henseleit solution, an effective concentration of nominally free calcium of 2.5 mM was achieved. As evidenced in the present study, Ca^{2+} concentrations lower than 2.5 mM, that are frequently being applied in isolated mouse heart studies (Ng *et al.*, 1991, Grupp *et al.*, 1993, Marber *et al.*, 1995, Yoshida *et al.*, 1996, Matherne *et al.*, 1997, Li *et al.*, 1997), clearly resulted in a lower cardiac output and contractility of the isolated ejecting mouse heart. The temperature of the perfusion medium and, hence, of the isolated hearts was set at 38- 39 ...C because this temperature corresponds well with the core body temperature of the intact laboratory mouse (Conroy *et al.*, 1980, Miller *et al.*, 1994).

It is shown that in the present experimental set-up, at loading conditions comparable to those reported by the groups of Bittner *et al.* (1996), Grupp *et al.* (1991 and 1993) and Gauthier *et al.* (1998a and 1998b) the hemodynamic function of the isolated working mouse heart is relatively stable for at least 100 min. Cardiac output decreased by less than 10 % over this period. More importantly, hemodynamic performance in terms of cardiac output and contractility compares favorable to the *in vivo* situation in contrast to the other studies. In the present study cardiac output increased from about 12 to 19 $ml.min^{-1}$ when preload was increased from 10 to 25 mm Hg at a fixed afterload pressure of 50 mm Hg. These cardiac output values are close to those (16 $ml.min^{-1}$) found in mice *in vivo* (Barbee *et al.*, 1992, Hartley *et al.*, 1995) and significantly higher than the cardiac output values of 1 - 4 $ml.min^{-1}$ reported by Bittner *et al* (Bitnner *et al.*, 1996) at comparable loading conditions as in the present study.


A
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Flow (ml.min-1)
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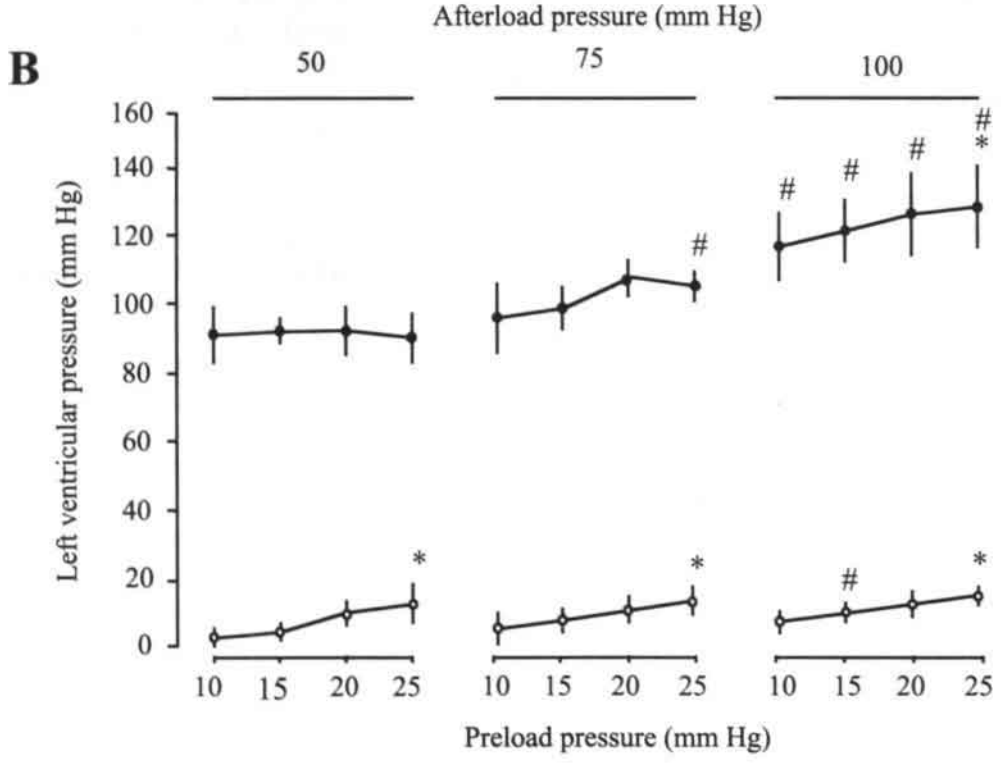
Preload pressure (mm Hg)


B
Afterload pressure (mm Hg)
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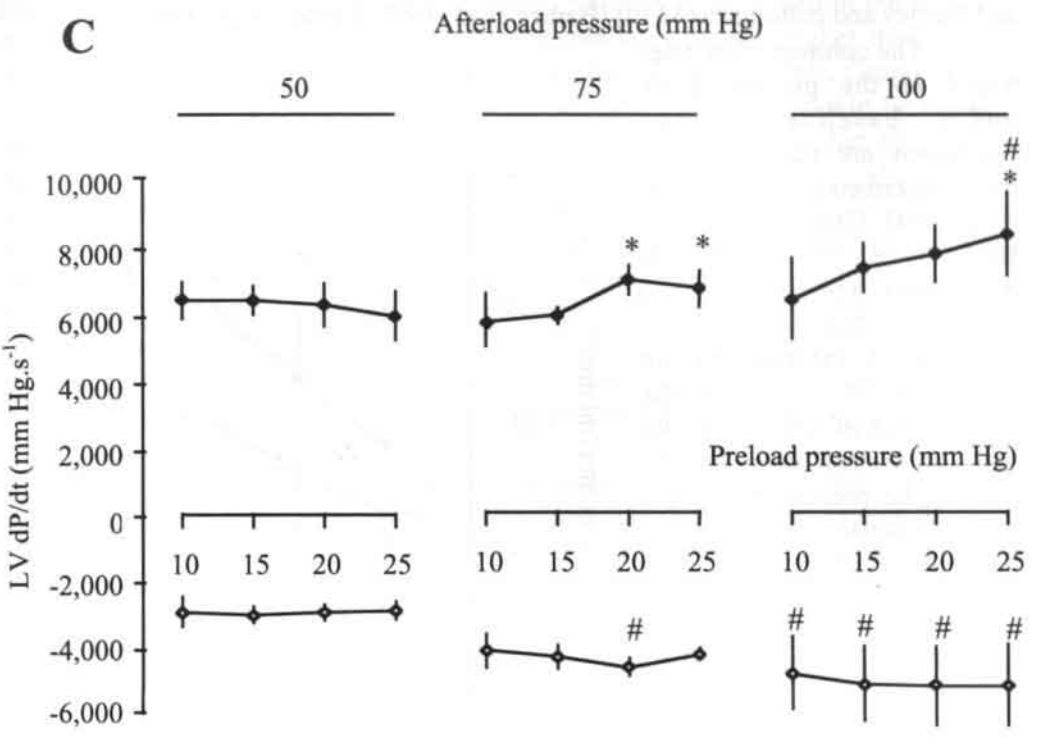

Figure 5
Left ventricular function curves of normoxically perfused, isolated working mouse hearts. Shown are the effects of varying preload and afterload pressures on **A** cardiac output (CO; closed squares) and coronary flow (CF; open squares); **B** left ventricular systolic pressure (closed circles) and left ventricular end-diastolic pressure (open squares); and **C** the first maximal derivative of left ventricular pressure (closed diamonds) and the first minimal derivative of left ventricular pressure (open diamonds) in isolated antegradely perfused, murine hearts. * indicates $P < 0.05$ vs lowest preload pressure (10 mm Hg) at comparable afterload pressure, # indicates $P < 0.05$ vs corresponding preload pressure.

A comparison with the functional characteristics reported by the group of Grupp (Ng *et al.*, 1991, Grupp *et al.*, 1993) is more difficult as these investigators standardly used a fixed left atrial inflow instead of a fixed filling pressure. Nevertheless, it is of interest to note that in their model at an afterload of 50 mm Hg approximately 90 % of cardiac output (4.6 of 5.1 $ml.min^{-1}$) is used for coronary flow (Grupp *et al.*, 1993). The model described in the present study compares favorably in this respect as less than 40 % of cardiac output is used to supply the coronary vasculature. Most likely, these differences in performance are attributable to differences in the impedance of the aortic outflow tract, since in other studies longer and narrower cannulas were used, illustrating the importance of proper design of this part of the perfusion system. The stroke volumes values of 29 – 5 µl

resemble those reported for the *in vivo* situation by Barbee and colleagues (26 µl) and Hartley and colleagues (33 µl) (Barbee *et al.*, 1992, Hartley *et al.*, 1995).

The coronary flow rates found in the present study under baseline loading conditions are comparable to those described previously (Ng *et al.*, 1991, Grupp *et al.*, 1993, Bittner *et al.*, 1996, Gauthier *et al.*, 1998a and 1998b). The observation that coronary flow rates could increase by an additional 50 % during the construction of left ventricular function curves, demonstrates that in the present model the murine coronary vascular bed is not fully dilated under basal conditions (10 and 50 mm Hg pre and afterload pressure, respectively). Additionally, the absence of and increase in LDH and lactate release during prolonged perfusion indicates that the mouse hearts are not compromised with regard to oxygen supply.

The finding that the effective preload pressure (LVEDP) was lower than the applied left atrial filling pressure is consistent with findings in other isolated working mouse heart models (Ng *et al.*, 1991, Grupp *et al.*, 1993, Gauthier *et al.*, 1998a and 1998b). It is of interest to note that this phenomenon was observed under all experimental conditions (varying pre and afterload). At present no explanation is at hand for this phenomenon.

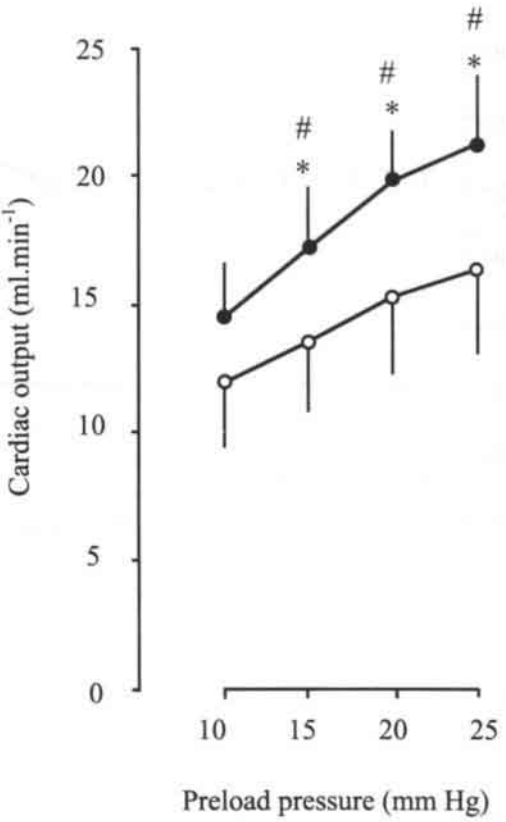

Figure 6: Left ventricular function curves of isolated working mouse hearts perfused with 1.5 mM calcium (open circles) and 2.5 mM calcium (closed circles). The curves were constructed by measuring cardiac output at varying preloads with the afterload kept constant at 50 mm Hg. Data are expressed as means ± SD (both groups n = 5). * indicates $P < 0.05$ vs lowest preload (10 mm Hg) at comparable Ca^{2+} concentration, # indicates $P < 0.05$ vs corresponding preload pressure.

Only a few reports give insight into the *in vivo* contractile state of the mouse heart. Lembo *et al.* (1996) and Milano *et al.* (1994) reported values for LV dP/dt_{max} in open-chest mice of 4000-4500 mm Hg.s^{-1}, while Lorenz *et al.* (1997)

found LV dP/dt_{max} values of 7830 – 670 mm $Hg.s^{-1}$ in anesthetized closed-chest mice. Again, the LV dP/dt_{max} values of 6000-7500 mm $Hg.s^{-1}$ found in the present study are comparable to the *in vivo* situation and considerable higher than those reported in other isolated ejecting mouse heart studies (Ng *et al.*, 1991, Grupp *et al.*, 1993, Bittner *et al.*, 1996, Gauthier *et al.*, 1998a and 1998b). It should be noted, however, that in the conscious mouse (heart rates typically higher than 550 $beats.min^{-1}$) LV dP/dt_{max} values of more than 14,000 mm $Hg.s^{-1}$ should be anticipated (Kass *et al.*, 1998). The observation that LV dP/dt_{max} does not change when increasing preload at an afterload of 50 mm Hg may be explained by the fact that the aortic valve opens before the physiological maximal value of the first derivative of left ventricular pressure has been reached, as earlier discussed by Reneman and Pollack (1971). This notion is supported by the finding that at higher afterloads LV dP/dt_{max} increases with enhanced preload pressure. Therefore, the use of this parameter of contractile performance has to be interpreted in the light of the actual pre and afterload pressures applied.

In conclusion, the model described in the present study allows detailed evaluation of the performance of isolated, antegradely perfused mouse hearts. Because the functional characteristics of the isolated heart model in general corresponds well with *in vivo* findings, the present model will be asset in the interpretation of the function of specific gene products investigated in transgenic and gene-targeting mouse models for heart diseases.

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Chapter 6

Functional recovery, energy metabolism and lipid homeostasis in the ischemic-reperfused mouse heart.

Submitted as full paper as:

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Parts of this study were published as:

LJ De Windt, J Willems, GJ Van der Vusse, RS Reneman, M Van Bilsen. Ischemia-reperfusion induced damage in isolated left ventricular ejecting mouse heart. *Faseb J* 1998;12:A75 (Abstract).

Abstract

In the murine heart the effects of ischemia and reperfusion on cardiac high energy metabolism and lipid metabolism were investigated and related to functional recovery and the degree of irreversible cell damage. To this end, isolated ejecting murine hearts were subjected to 10, 15 and 20 min of normothermic, no-flow ischemia, whether or not followed by 60 min of reperfusion. During ischemia cardiac ATP levels gradually declined, which was associated with a transient rise in ADP and a sharp increase in AMP content (from a pre-ischemic value of 0.6 ± 0.1 $\mu mol.g^{-1}$ dry weight to 6.3 ± 2.0 $\mu mol.g^{-1}$ dry weight after 20 min of ischemia). During reperfusion hemodynamic function almost completely recovered following 10 min of ischemia (83 ± 14 % recovery of cardiac output [CO]), but was severely depressed following 15 and 20 min of ischemia (40 ± 24 and 31 ± 24 % recovery of CO, respectively). Reperfusion following 20 min of ischemia was accompanied by disturbed reflow as evidenced by delayed wash-out of lactate dehydrogenase (LDH) and post-ischemic accumulation of small molecular weight degradation products such as lactate and (oxy)purines. The percentage necrotic cells as assessed by cumulative release of LDH during reperfusion was calculated to be 4.8, 8.4 and 10.0 % of total cardiac muscle cells following 10, 15 and 20 min of ischemia, respectively. Reperfusion *per se* was associated with an enhanced degradation of phospholipids as indicated by the accumulation of fatty acids (FA) in reperfused hearts (0.9 ± 0.1 $\mu mol.g^{-1}$ and 3.6 ± 1.3 $\mu mol.g^{-1}$ dry weight in pre-ischemic and reperfused hearts following 20 min of ischemia, respectively). In the reperfused heart a positive correlation was found between levels of FA ($r = 0.70$) and arachidonic acid ($r = 0.65$) on the one hand and cumulative release of LDH during reperfusion on the other. The latter observation strongly suggests that membrane phospholipid degradation and loss of cellular viability are closely related phenomena.

Introduction

Murine models are increasingly used to study the molecular mechanisms of cardiac dysfunction following ischemia and reperfusion due to the widespread availability of transgenic and gene-targeted models. The isolated heart preparation is particularly suitable to assess ischemia/reperfusion-induced functional and biochemical alterations independent of potentially compensatory extra-cardiac factors. So far, functional and structural alterations in the mouse heart following global ischemia and reperfusion have almost exclusively been investigated by means of retrogradely perfused heart preparations (Hampton *et al.*, 1998, Eberli *et al.*, 1998). In addition, Langendorff perfused hearts from transgenic mice have been used to document the protective effects of heat shock protein 72 (Marber *et al.*, 1995; Plumier *et al.*, 1995; Radford *et al.*, 1996; Trost *et al.*, 1998), catalase (Li *et al.*, 1997), A_1 adenosine receptor (Matherne *et al.*, 1997) and Cu-Zn superoxide dismutase (Wang *et al.*, 1997) on postischemic functional recovery. Furthermore, changes in high energy phosphate metabolism were subject of study in a number of genetically altered murine hearts (Headrick *et al.*, 1998; Spindler *et al.*, 1998; Saupe *et al.*, 1998), either or not in conjunction with ischemia/reperfusion.

An important limitation of the isolated Langendorff perfused heart model is that it does not perform external work. As a consequence, this empty-beating heart model has limited physiological resemblance and the biological significance of the functional parameters obtained is uncertain. These disadvantages can largely be overcome by using a left ventricular ejecting (working) heart preparation allowing, in addition to pump performance, the accurate assessment of left ventricular diastolic and systolic function. Using isolated rat hearts it has been shown that with the use of these parameters left ventricular dysfunction following an ischemic insult can be adequately described (Van Bilsen *et al.*, 1989a and 1989b, De Groot *et al.*, 1993a and 1993b, Cornelussen *et al.*, 1997).

In a previous study we developed and characterized a normoxically perfused isolated working murine heart model in terms of functional stability and its reponse to changes in workload (De Windt *et al.*, 1999). Up to now detailed information of the murine heart regarding the functional recovery and its relation to post-ischemic energy status and lipid metabolism are lacking. The first main objective of the present study was to delineate the vulnerability of the isolated murine heart towards normothermic ischemia followed by reperfusion. Therefore, we determined the degree of hemodynamic recovery and alterations in cardiac high energy phosphate levels and related compounds after ischemia, varying in duration from 10 to 20 min followed by reperfusion. Moreover, post-ischemic release of LDH was monitored to estimate the degree of irreversibly damaged cardiac cells. The second main objective was to assess whether and to which extent ischemia and/or reperfusion-induced damage of the murine heart is associated with disturbances in membrane phospholipid homeostasis reflected by accumulation of tissue fatty acids, including arachidonic acid.

Materials and methods

Chemicals
All chemicals used for the Krebs-Henseleit solution were of the highest grade available and purchased from Merck (Darmstadt, Germany), except for D(+)-glucose and pyruvate (Sigma Chemical Co., St. Louis, MO, USA). Insulin was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark).

Animals and surgery
For this study 3-4 month old female Swiss mice (Iffa Credo, Lyon, France) with an average body weight of 32.7 ± 2.4 g were used. The mice were kept under standard housing conditions with an artificial 12 h light cycle with free access to standard rodent food (Diet SRM-A, Hope farms, Woerden, the Netherlands) and tap water. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Maastricht University.

Isolated working mouse heart preparation
The isolated ejecting mouse heart preparation used in the present study has been described in detail before (De Windt *et al.*, 1999). Briefly, animals were anesthetized by 50 $mg.kg^{-1}$ sodium pentobarbital, i.p. (Nembutal, Sanofi Sante BV., Maassluis, the Netherlands). After thoracotomy the hearts were quickly excised and transferred to ice-chilled perfusion buffer (for composition see below). Remnant thymic and fatty tissue was carefully removed and the ascending aorta was cannulated with a recently described aortic cannula that was designed to match the hemodynamic aortic impedance characteristics of the isolated murine heart (De Windt *et al.*, 1999). Retrograde perfusion was started immediately at a perfusion pressure of 50 mm Hg, after which the hearts started to beat spontaneously. The left atrium was cannulated with an atrial cannula through one of the lung veins. The recirculating modified Krebs-Henseleit perfusion buffer was prefiltered by a microfilter (0.45 µm diameter; Millipore Corp.) and consisted of the following composition: 118 mM NaCl, 4.7 mM KCl, 3.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 0.5 mM Na-EDTA, 10 mM D(+)-glucose, 1.5 mM Na-pyruvate and 5 U/l insulin. The buffer was continously gassed with 95 % O_2 - 5 % CO_2. Care was taken to maintain the temperature of the perfusate, and thus the heart, at 38.5 ¡C. Left ventricular pressure was measured with a PE-50 catheter, inserted into the left ventricular cavity through the apex (De Windt *et al.*, 1999) and connected to a Baxter pressure transducer (Baxter Healthcare Corp., Irvine, CA, USA). Aortic pressure was measured through a side branch located 3 mm above the entrance of the aortic cannula using a pressure transducer (Baxter Healthcare Corp.).

Hemodynamic data
All hemodynamic variables were continuously recorded on a personal computer, using specialized software (Hemodynamic Data Acquisition System, Technical Department Maastricht University), allowing the on-line acquisition, presentation and calculation of left atrial filling flow, aortic flow (AOF), left ventricular systolic

pressure (LVSP), left ventricular end-diastolic pressure (LVED), diastolic aortic pressure (AODP) and the first maximal and minimal derivatives of left ventricular pressure (LV dP/dt_{max} and LV dP/dt_{min}). Left ventricular developed pressure (LVDP) was defined as the difference between LVSP and LVEDP. Cardiac output (CO) was defined as the sum of AOF and coronary flow (CF). CF was determined from the difference between AOF, as measured by a 1N inline aortic flow probe and left atrial filling flow as measured by a 2N inline flow probe placed in the left atrial inflow tract (De Windt *et al.*, 1999). Calculated CF data were periodically checked by timed collection of the coronary perfusate. CF data were used to calculate coronary resistance (CR), which was defined as AODP divided by CF normalized for individual heart weights.

Experimental protocol

During all experiments, antegrade perfusion was started by opening the left atrial conduit after an initial 10 min retrograde stabilization period at 50 mm Hg. Left atrial filling pressure was set at 10 mm Hg, while diastolic aortic pressure was kept at 50 mm Hg. Except for the ischemic period and during the first 5 min of reperfusion, the hearts were paced artificially throughout the whole experiment at a frequency of 380 beats.min^{-1}, which is slightly higher than the intrinsic rate of an isolated, perfused murine heart of this strain.

For ischemia/reperfusion experiments, the hearts were normoxically perfused for 20 min as described above (pre-ischemic period).

Table I

Pre-ischemic hemodynamic values measured in isolated, left ventricular ejecting murine hearts.

CO (ml.min^{-1})	10.8 ± 1.8
CF (ml.min^{-1})	3.8 ± 1.8
AOF (ml.min^{-1})	6.8 ± 1.8
SV (µl)	27 ± 6
LVDP (mm Hg)	77 ± 9
LVSP (mm Hg)	86 ± 9
LVPDia (mm Hg)	1 ± 3
LVEDP (mm Hg)	7 ± 4
LV dP/dt_{max} (mm Hg.s^{-1})	5,652 ± 831
LV dP/dt_{min} (mm Hg.s^{-1})	-3,054 ± 415

Data are expressed as means ± SD (n =13). Isolated hearts were perfused at a pre and afterlaod of 10 mm Hg, respectively. Hearts were paced at 380 beats.min^{-1}. CO, cardiac output; CF, coronary flow; AOF, aortic flow; SV, stroke volume; LVDP, left ventricular developed pressure; LVSP, left ventricular systolic pressure; LVPDia, left ventricular diastolic pressure; LVEDP, left ventricular end-diastolic pressure; LV dP/dt_{max}, first maximal derivative of left ventricular pressure; LV dP/dt_{min}, first minimal derivative of left ventricular pressure.

Just prior to the ischemic period the water-jacketed chamber was filled with warm (38.5 °C) perfusate solution until the heart was completely submersed, pacing was stopped, and the aortic and atrial lines were clamped for 10, 15 or 20 min to create normothermic, global ischemia. After the ischemic period, the water-jacketed chamber was emptied and hearts were reperfused retrogradely at a perfusion pressure of 50 mm Hg for 10 min. Subsequently, the left atrial conduit was re-opened and the hearts were allowed to work in the antegrade mode at a preload pressure of 10 mm Hg and a diastolic aortic pressure of 50 mm Hg for an additional 50 min.

Hearts were freeze-clamped either after the 20 min stabilization period (pre-ischemia), after 10, 15 or 20 min of no-flow normothermic ischemia (end-ischemia), or following ischemia and 60 min of reperfusion (ischemia/reperfusion). To assess the lactate content in the coronary effluent samples of 1 ml were immediately frozen in liquid nitrogen and stored at -80 ¡C for further analysis. For the determination of lactate dehydrogenase (LDH) activity in coronary effluent samples, bovine serum albumin was added to the samples (final concentration 3 %), after which they were frozen in liquid nitrogen and also stored at -80 ¡C.

Biochemical analysis

LDH and lactate content in the coronary perfusate were assessed spectrophotometrically using a Cobas Bio autoanalyzer as described earlier (Bergmeyer and Bernt *et al.*, 1974, Apstein *et al.*, 1970).

Tissue contents of adenine and guanine nucleotides, IMP, and (oxy)purines was determined by high-performance liquid chromatography (Varian Vista 5500 HPLC) according to a variation of the procedures of Wynants and Van Belle (1985) and as described previously in detail (Van Bilsen *et al.*, 1989a). Briefly, aliquots of deeply frozen tissue were extracted with perchloric acid and samples of the neutralized extract were applied to a precolumn (LiChroCART 4-4, Merck, Germany) and a reversed phase LiChrosorb RP-18 column (stainless steel, narrow bore, 250 × 4 mm ID, Merck). Stepwise gradient elution (flow speed 0.8 $ml.min^{-1}$), using two solvents, was applied to separate the compounds of interest. Solvent A [aqueous buffer of $NH_4(H_2PO_4)$ 150 mM, pH = 6.0] was prepared by mixing H_3PO_4 and NH_4OH. Solvent B consisted of a 1:1 (vol/vol) mixture of acetonitrile and methanol. Each run started with a 6 min elution period of 100 % solvent A. From 6 to 11 min solvent A was reduced to 95 % and solvent B increased to 5 %. From 11 to 27 min solvent A was further decreased to 85 %, after which solvent A was increased to 100 % within 2 min. Peaks were detected at 254 nm using an ultraviolet detector (Varian UV-200, flow cell 4.5 ml, path length 4 mm, spectral band width 5 nm). The sensitivity was set at 0.005 AUFS at a time constant of 0.5 s. Peaks were identified by comparing retention times with known standards.

The determination of cardiac fatty acids, phospholipids and triacylglycerols was performed as recently described in detail (Van der Vusse and Roemen, 1995). All fatty acyl values were expressed as moles per gram dry weight. Dry weight was determined in a subset of hearts by freeze drying overnight and amounted to 14.6 % of wet weight, which corresponds to a conversion factor of 6.85 for wet weight to dry weight murine ventricular tissue.

Table II
Total tissue content of individual purines and their sum in pre-ischemic, ischemic and ischemic-reperfused mouse hearts.

n	*6*	*6*	*10*	*6*	*9*	*5*	*11*
	Pre-I	*10 min I*	*10 min I/R*	*15 min I*	*15 min I/R*	*20 min I*	*20 min I/R*
A	0.07 ± 0.02	0.82 ± 0.35*	0.20 ± 0.20#	1.07 ± 0.57*	0.09 ± 0.07#	1.87 ± 0.45*	0.41 ± 0.20#*
I	ND	0.71 ± 0.26	0.03 ± 0.02#	1.27 ± 0.65	0.32 ± 0.45#	1.85 ± 0.38	1.20 ± 1.31
HX	0.04 ± 0.02	0.21 ± 0.04*	0.28 ± 0.31	0.34 ± 0.08*	ND	0.48 ± 0.14*	0.32 ± 0.08
X	ND	ND	0.81 ± 0.53	0.08 ± 0.07#	0.93 ± 0.72	0.21 ± 0.12	1.00 ± 0.70#
Total	0.08 ± 0.03	1.60 ± 0.60*	0.74 ± 0.73*	6.75 ± 1.65*	0.88 ± 1.21#*	4.13 ± 0.72*	2.28 ± 2.08*

Data are expressed as means – SD. Presented are individual purines in $\mu mol.g^{-1}$ dry weight ventricular tissue. n indicates number of hearts analyzed. Pre-I, Pre-ischemia; I, ischemia; R, reperfusion. Total refers to the sum of adenosine (A), inosine (I), hypoxanthine (HX) and xanthine (X) and is expressed as $\mu mol.g^{-1}$ dry weight. * indicates $P < 0.05$ vs pre-ischemic value, # indicates $P < 0.05$ vs corresponding end-ischemic value. ND, not detectable.

Statistical analysis

The results are presented as mean values – standard deviations (SD). All statistical analyses were performed using InStat 3.0 software (GraphPad Software Inc., San Diego, CA). Changes in functional variables in time were statistically analyzed by repeated measures ANOVA with Tukey s HSD post-hoc correction test for multiple comparisons. Differences between values of functional variables and biochemical parameters between experimental groups were analyzed using one-way ANOVA followed by the Tukey s test. Linear regression was performed with the least squares method and the Pearson rank correlation coefficient (r) was used to estimate the strength of the relation between two variables. In all tests significance was accepted at P values < 0.05.

Table III
Total tissue content of GTP, GDP, IMP, NADP and NAD in pre-ischemic, ischemic and ischemic-reperfused mouse hearts.

N	*6*	*6*	*10*	*6*	*9*	*5*	*11*
	Pre-I	*10 min I*	*10 min I/R*	*15 min I*	*15 min I/R*	*20 min I*	*20 min I/R*
GTP	1.3 ± 0.1	0.9 ± 0.2	0.9 ± 0.3	0.7 ± 0.2*	0.8 ± 0.2	0.5 ± 0.1*	0.7 ± 0.2*
GDP	0.2 ± 0.1	0.3 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1
GMP	ND	ND	ND	0.1 ± 0.1	ND	0.1 ± 0.1	0.1 ± 0.1
IMP	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0.4 ± 0.2	0.4 ± 0.2
NADP	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1
NAD	3.4 ± 0.3	3.2 ± 0.7	3.2 ± 0.7	3.4 ± 0.2	2.7 ± 0.5	3.0 ± 0.5	2.6 ± 0.7

Data are expressed as means – SD in $\mu mol.g^{-1}$ dry weight ventricular tissue. n indicates number of hearts analyzed. Pre-I, Pre-ischemia; I, ischemia; R, reperfusion; ND, not detectable. * indicates $P < 0.05$ vs pre-ischemic value.

Results

Functional recovery
Pre-ischemic values of the hemodynamic variables tested were not significantly different between the experimental groups and, hence, pooled data are presented in Table I. Following 10 min of global ischemia and 60 min of reperfusion functional recovery was almost complete. CO recovered to 83 ± 14 % of its pre-ischemic value ($P < 0.05$ vs pre-ischemia), while no significant difference was observed between pre-ischemic and post-ischemic CF (Figure 1A). Similarly, post-ischemic LVDP, LV dP/dt_{max} and LV dP/dt_{min} all recovered to near pre-ischemic values (Figure 1C).

Extending the ischemic duration to 15 min resulted in a depression of the recovery of CO and AOF (Figure 1A). Post-ischemic LVDP amounted to 62 ± 18 % of its pre-ischemic value ($P < 0.05$ vs pre-ischemia). Recovery of LV dP/dt_{max} and LV dP/dt_{min} was also more severely depressed after 15 min of ischemia (Figure 1C).

Hemodynamic recovery was severely impaired following 20 min of ischemia. CO recovered to only 31 ± 24 % of its pre-ischemic value ($P < 0.01$ vs pre-ischemia; Figure 1A), while in the majority of hearts virtually no recovery of AOF was observed. Additionally, CF was severely compromised and amounted to

2.6 ± 1.9 ml.min^{-1}, corresponding to a doubling of calculated coronary resistance (CR) from a pre-ischemic value of 15.8 ± 7.6 mm Hg.min.ml^{-1} to 30.0 ± 21.8 mm Hg.min.ml^{-1} ($P < 0.05$). Due to a marked rise in LVEDP and a reduction in systolic function LVDP recovered to only 28 ± 21 % of its pre-ischemic value ($P < 0.01$ vs. pre-ischemia; Figure 1B).

Enzyme and lactate release

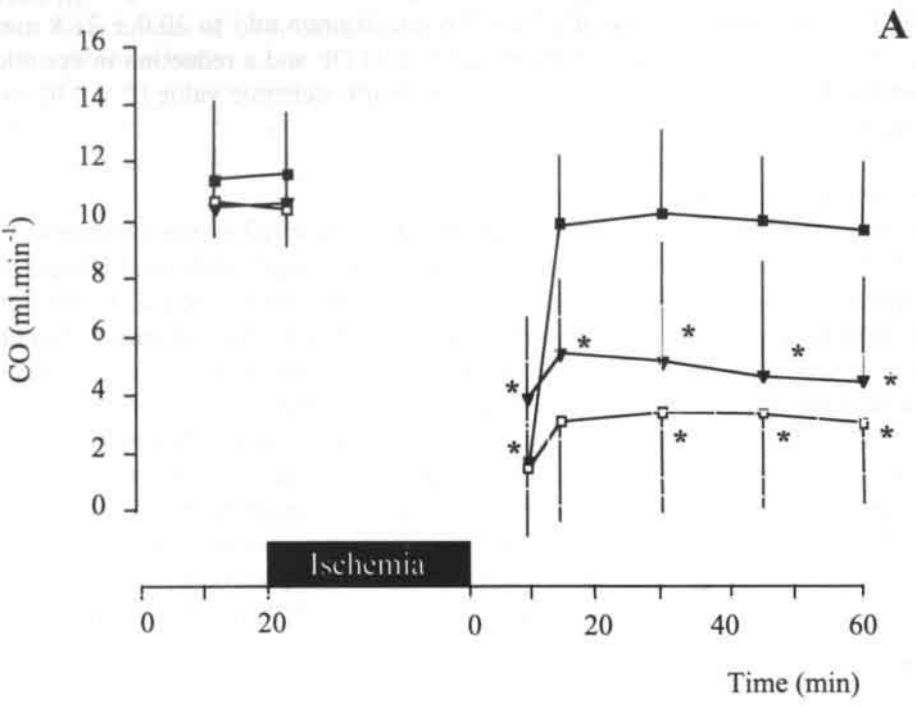
Pre-ischemic LDH release was low in all groups and amounted on the average to 37 ± 22 mU.min^{-1}. Following 10 min of global ischemia a small additional release of LDH was observed during the initial 20 min of the reperfusion phase. During 60 min of reperfusion, the cumulative release of LDH into the coronary effluent amounted to 2.9 – 0.8 U in this group. The cumulative release of LDH increased as a function of the duration of the preceding ischemic period and amounted to 5.1 – 1.7 U and 6.1 – 2.7 U following 15 and 20 min of ischemia, respectively. To estimate the percentage of irreversibly damaged cells, the cumulative release of LDH was normalized to the total LDH content of the murine heart (60.5 U/heart). Accordingly, the percentage of necrotic cardiac myocytes was calculated to be 4.8, 8.4 and 10.0 % following 10, 15 and 20 min of ischemia, respectively.

Prior to the onset of ischemia cardiac lactate release into the coronary effluent amounted to 0.8 – 0.2 µmol.min^{-1}. Following 10 and 15 min of ischemia a significantly higher lactate release rate was observed during the first 5 min of reperfusion (1.6 – 0.1 and 1.6 – 0.6 µmol. min^{-1}, respectively; $P < 0.05$ vs pre-ischemia), reflecting the wash-out of lactate accumulated during the preceding ischemic period. Thereafter, lactate release returned to pre-ischemic values. In contrast, following 20 min of ischemia no significant washout of lactate was observed in the initial reperfusion phase. Lactate release amounted to 0.9 – 0.3 µmol. min^{-1} and remained constant during the remainder of the reperfusion phase.

Tissue adenine nucleotides and degradation products

The pre-ischemic tissue content of ATP, ADP and AMP amounted to 18.9 – 2.0, 4.3 – 0.5 and 0.6 – 0.1 µmol.g^{-1} dry weight, respectively, corresponding to an adenylate energy charge (EC) of 0.89 ± 0.01 (Figure 2). The sum of adenine nucleotide degradation products, i.e., adenosine, inosine, hypoxanthine and xanthine, was very low in normoxically perfused pre-ischemic ventricular tissue (Table II).

During 10 and 15 min of ischemia tissue ATP content progressively decreased, while ADP and AMP levels increased (Figure 2). Extension of the ischemic duration to 20 min was associated with marked reductions in both ATP and ADP content. In contrast, tissue AMP levels increased dramatically. As a result, the tissue adenylate EC fell to 0.42 ± 0.16 following 20 min of ischemia (Figure 2). Adenine nucleotide degradation products substantially increased (Table II).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CO (ml.min-1)
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12
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8
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4
2
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Ischemia
0
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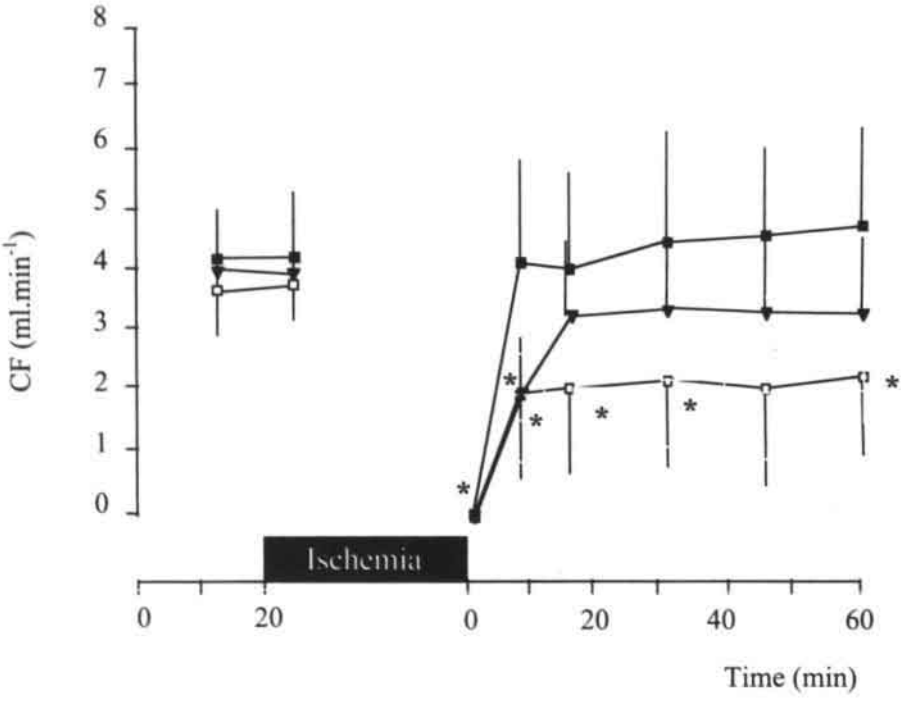
40
60
Time (min)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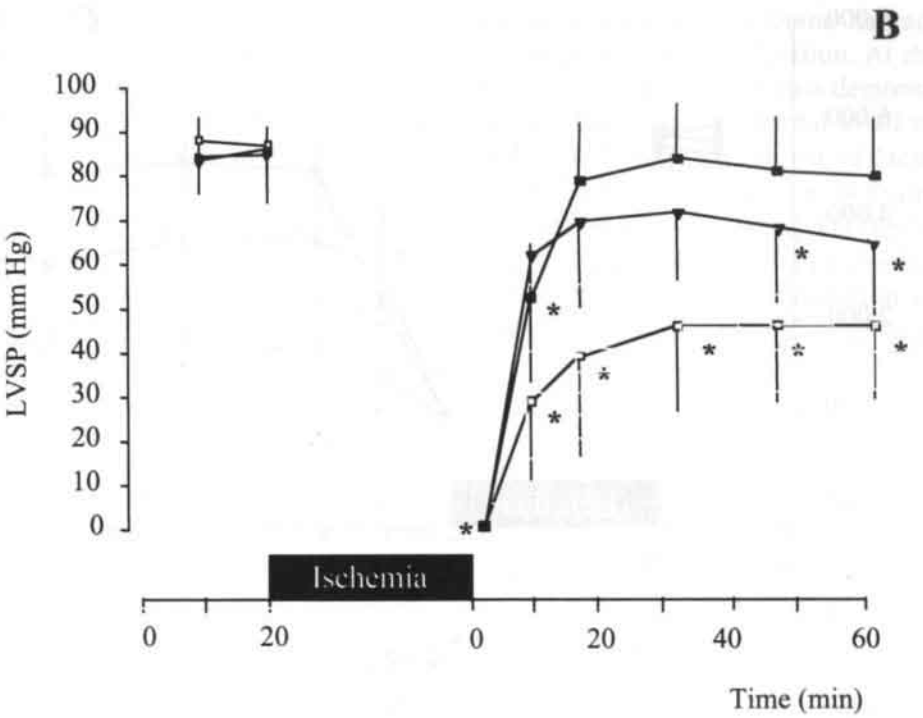
B
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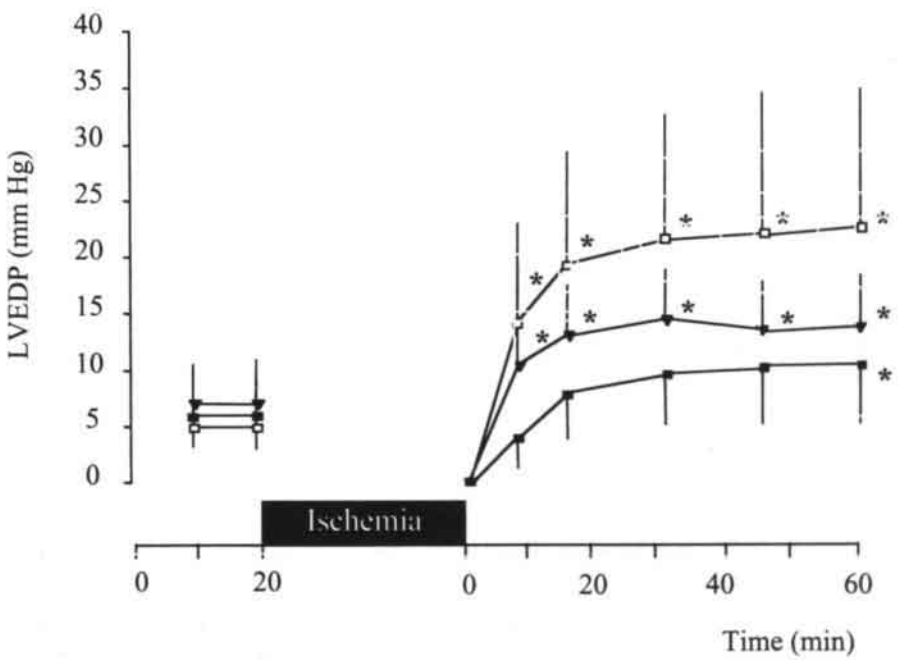
Time (min)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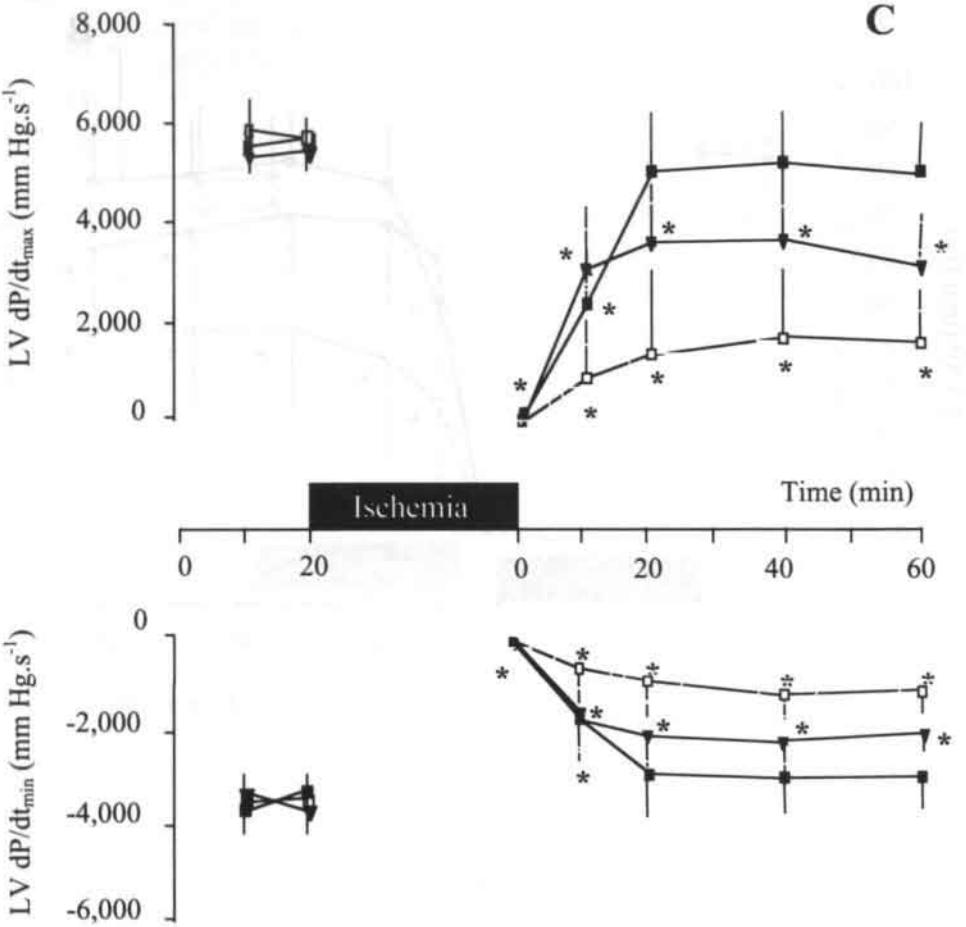

Figure 1
Post-ischemic recovery of cardiac output and coronary flow (CO and CF; Figure 1A), left ventricular systolic pressure and left ventricular end-diastolic pressure (LVSP and LVEDP; Figure 1B) and first maximal and minimal derivative of left ventricular pressure (LV dP/dt$_{max}$ and LV dP/dt$_{min}$; Figure 1C) following 10 (closed rectangles), 15 (closed triangles) and 20 min (open rectangles) of normothermic no-flow ischemia. Indicated on the x-axis is the 20 min pre-ischemic perfusion time in min, the global ischemic period and 60 min of reperfusion. Data are expressed as means – SD (n = 10, following 10 and 15 min of ischemia; n = 11, following 20 min of ischemia). indicates $P < 0.05$ vs postischemic recovery following 10 min of ischemia.

Restoration of flow for 60 min resulted in an almost complete recovery of tissue ATP levels following 10 min of ischemia. In contrast, following an ischemic insult of 15 or 20 min only partial restoration of tissue ATP content was observed (Figure 2). Tissue ADP and AMP levels returned to pre-ischemic values regardless of the duration of the preceding ischemic episode (Figure 2). EC recovered to pre-ischemic values following 10 and 15 min of ischemia, but only to 0.79 ± 0.09

following 20 min of ischemia and reperfusion ($P < 0.05$ vs pre-ischemia). Restoration of flow resulted in decreased tissue content of adenine degradation products relative to ischemic hearts regardless of the ischemic duration. At the end of reperfusion, however, hearts subjected to 10 or 15 min of ischemia demonstrated a significantly lower tissue (oxy)purine content than hearts subjected to 20 min of ischemia and 60 min of reperfusion (Table II). The tissue content of lactate in reperfused hearts was found to be 15.4 ± 11.3, 21.0 ± 9.6 and 41.1 ± 24.8 $\mu mol.g^{-1}$ dry weight following 10, 15 and 20 min of ischemia, respectively. The finding of markedly enhanced tissue levels of lactate and adenine degradation products in hearts subjected to 20 min of ischemia plus reperfusion, in combination with a substantially elevated postischemic coronary resistance, is indicative of impaired recovery of coronary flow in this group.

GTP, GDP, GMP, IMP and NAD(P)

Similar to ATP, guanine triphosphate (GTP) was gradually degraded in ischemic tissue following 20 min of ischemia ($P < 0.05$ vs pre-ischemia; Table III). This reduction, however, was not associated with significant changes in GDP and GMP levels. Restoration of flow during 60 min was not sufficient to restore tissue GTP content back to pre-ischemic values, regardless of the preceding ischemic duration. Tissue NAD and NADP contents remained unaffected during ischemia, or ischemia followed by 60 min of reperfusion (Table III).

Tissue fatty acids, triacylglycerol and phospholipids

The total pre-ischemic tissue content of fatty acids (FA), phospholipids (PL) and triacylglycerols (TG) in the murine hearts and their relative fatty acid composition is shown in Table IV. The fatty acyl composition of the PL and FA pool demonstrated high resemblances with a relative abundance of palmitic (16:0), stearic (18:0), linoleic (18:1) and docosahexaenoic acid (22:6), with each species accounting for 10-20 % of total fatty acids present (Table IV). The poly-unsaturated FA arachidonic acid (20:4) accounted for 6.7 ± 0.7 and 4.0 ± 0.4 % of total fatty acids in the PL and FA pool, respectively (Table IV). Cardiac TG s mainly comprised of 16:0, 18:1 and 18:2 moieties, each representing about 30 % of all TG fatty acyl moieties. In contrast to the FA and PL pool, 18:0, 20:4 and 22:6 accounted for only a small percentage of fatty acids esterified in the TG pool (Table IV).

Oxygen deprivation for 10, 15 or 20 min did not affect the tissue PL and TG content (Tables VI and VII). Total cardiac FA content declined significantly in hearts subjected to 10 or 15 min of ischemia ($P < 0.05$ vs pre-ischemia). Increasing the ischemic duration with an additional 5 min resulted in an elevation of cardiac FA content relative to the end-ischemic value at 15 min of ischemia (Figure 3 and Table V). The reduction in total FA content was associated with marked changes in the mol % of individual FA species (Table V). In general, the mol % of saturated FA species increased, whereas the mol % of poly-unsaturated FA s declined. In this respect the shift in the mol % of 16:0 and 22:6 were most outspoken. A small but significant increase of 22:6 in the PL pool and a decrease in the amount of 16:0 and a small increase in the amount of 18:2 esterified in the TG pool were observed.

Reperfusion itself resulted in a marked increase in tissue FA content following 10 and 15 min of ischemia, respectively ($P < 0.05$ vs pre-ischemia; Figure 3). The increase in tissue FA was found to be most pronounced in hearts previously deprived of oxygen for 20 min ($P < 0.01$ vs pre-ischemia). Irrespective of the duration of the preceding ischemic episode, reperfusion was accompanied by a substantial rise in the tissue content of 18:2, 20:4 and 22:6, in both absolute amounts (not shown) and in the percentage contribution to the total FA pool (Table V). Following reperfusion the amounts of the polyunsaturated fatty acyl species 20:4 and 22:6 in the PL pool tended to decrease compared to end-ischemic values, the decrease reaching statistical significance only for 22:6 following 10 min of ischemia and 60 min of reperfusion (Table VI). The relative fatty acyl composition of the cardiac TG pool showed a significant change only in the 18:2 content, which decreased during reperfusion following 20 min of ischemia (Table VII).

Discussion

Hemodynamic function in the ischemic-reperfused mouse heart

In the present study it is demonstrated that murine hearts tolerated 10 min of ischemia well, as evidenced by a near complete recovery of hemodynamic function and adenylate energy charge and the limited LDH release upon reperfusion. Nonetheless, reperfusion was associated with a marked rise in the tissue FA content, reflecting release of FA from endogenous lipid pools. Extending the ischemic duration to 15 or 20 min resulted in a diminished recovery of cardiac function, more pronounced decreases in the adenylate energy charge, enhanced tissue FA accumulation and release of cytosolic proteins.

The nearly complete recovery of hemodynamic function after 10 min of global ischemia is at variance with earlier observations of Bittner and colleagues, who reported that 6 min of global ischemia using their working mouse heart preparation was already sufficient to induce substantial impairment of postischemic cardiac output (Bittner *et al.*, 1996, Chen *et al.*, 1997). The apparent discrepancy between these studies and the present one are not clear. However, the pre-ischemic hemodynamic values of the quoted studies deviate to such an extent from the *in vivo* murine cardiac function and the present study, probably as a result of a higher aortic impedance and differences in perfusate composition, that comparisons are hard to make. This issue has been discussed earlier in more detail (De Windt *et al.*, 1999). In all other studies to date the so-called isolated Langendorff perfused heart model was typically used to study ischemia and reperfusion-induced cardiac dysfunction of isolated hearts, either or not derived from trangenic animals (Eberli *et al.*, 1998, Li *et al.*, 1997, Marber *et al.*, 1995, Headrick *et al.*, 1998, Matherne *et al.*, 1997, Plumier *et al.*, 1995, Radford *et al.*, 1996, Sumeray *et al.*, 1998, Trost *et al.*, 1998, Wang *et al.*, 1998, Yoshida *et al.*, 1996, Xi *et al.*, 1998). The minimal duration of flow-deprivation required to induce marked functional impairment on Langendorff perfused isolated mouse hearts in these studies ranges from 30 min (Yoshida *et al.*, 1996) to as long as 50 min (Li *et al.*, 1997). A number of factors may account for the large variation in ischemia tolerance of the murine hearts as reported by the various research groups.

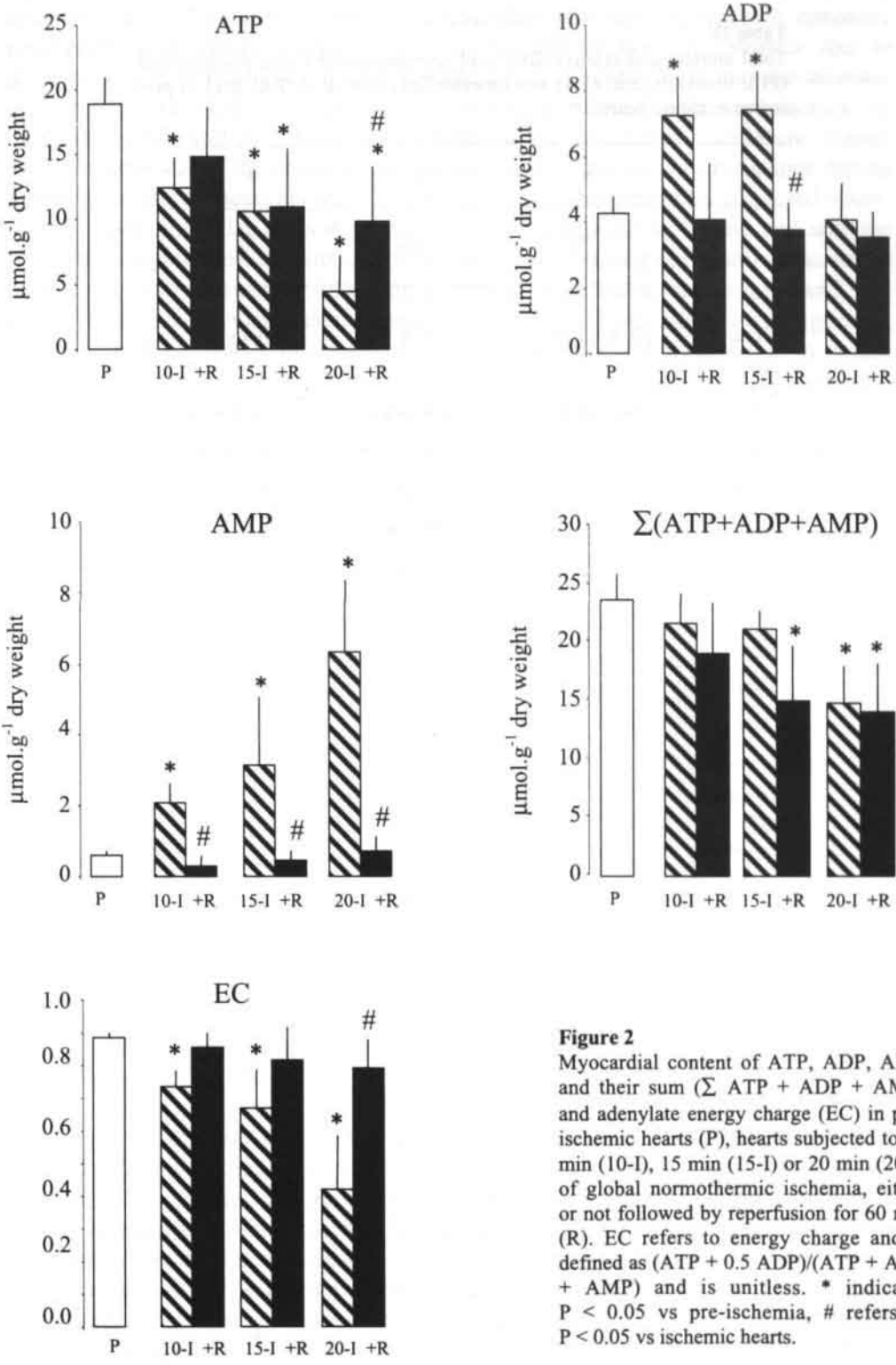

Figure 2
Myocardial content of ATP, ADP, AMP and their sum (Σ ATP + ADP + AMP) and adenylate energy charge (EC) in pre-ischemic hearts (P), hearts subjected to 10 min (10-I), 15 min (15-I) or 20 min (20-I) of global normothermic ischemia, either or not followed by reperfusion for 60 min (R). EC refers to energy charge and is defined as (ATP + 0.5 ADP)/(ATP + ADP + AMP) and is unitless. * indicates $P < 0.05$ vs pre-ischemia, # refers to $P < 0.05$ vs ischemic hearts.

Table IV
Total amount and relative fatty acid composition of tissue phospholipid (PL), triacylglycerol (TG) and (unesterified) fatty acid (FA) pool in pre-ischemic mouse hearts.

n	6	4	4
	PL	*TG*	*FA*
Total	198.4 ± 10.3	40.8 ± 5.3	0.9 ± 0.1
16:0	19.0 ± 0.5	30.9 ± 6.2	18.0 ± 1.6
16:1	0.5 ± 0.1	4.5 ± 1.2	0.7 ± 0.1
18:0	18.6 ± 0.8	5.4 ± 1.1	17.0 ± 2.5
18:1	11.4 ± 0.6	29.2 ± 4.0	13.2 ± 0.6
18:2	15.9 ± 0.5	27.3 ± 5.9	12.5 ± 0.8
20:0	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.1
20:4	6.7 ± 0.7	0.9 ± 0.1	4.0 ± 0.4
22:4	0.2 ± 0.0	0.3 ± 0.1	0.7 ± 0.1
22:6	22.7 ± 1.4	4.3 ± 1.0	28.7 ± 3.5

Data are expressed as means ± SD. Total refers to total (unesterified) fatty acid and is expressed as $mmol.g^{-1}$ dry weight. Individual fatty acids are presented as percentages of total tissue fatty acid.

Factors likely to play a role are variations in nominally free Ca^{2+} concentrations in the perfusate, choice of substrates, temperature of the isolated hearts during global ischemia and differences in workload (perfusion pressure, retrograde versus antegrade perfusion).

The findings in the present study are in line with the earlier expressed notion of a higher susceptibility of the mouse heart to ischemia and reperfusion-induced damage as compared to other species (Headrick *et al.*, 1998). Using also an assisted mode perfusion setup and comparable experimental conditions as in the present study, De Groot and colleagues found substantial cardiac dysfunction as measured by recovery of cardiac output, coronary resistance, post-ischemic LDH release and ventricular accumulation of arachidonic acid in isolated working rat hearts only following ischemic periods of 30 min or longer (De Groot *et al.*, 1993a). Reperfusion of mouse hearts following 20 min of ischemia was associated with a

significant increase in coronary resistance and, hence, a substantial decline in coronary flow. Although it cannot be excluded that this increase in coronary resistance results from a reduced oxygen demand of the left ventricle due to decreased work performance during reperfusion, it is more likely that the increase in coronary resistance is caused by ischemia-induced hypercontracture of cardiomyocytes and/or substantial damage to the coronary vasculature. Latter notion is stressed by the observation that the increase in coronary resistance during reperfusion and reduced myocardial perfusion is accompanied by impaired wash-out of small molecular mass degradation compounds such as lactate and adenine nucleotide degradation products. Indeed, a relatively strong correlation was found between coronary resistance following 60 min of reperfusion, on the one hand, and post-ischemic ventricular accumulation of lactate, on the other ($r = 0.87$; Figure 4). Although not the subject of the present study, it may be anticipated that in mouse hearts subjected to 20 min of global ischemia especially the inner layers of the left ventricle will be affected by flow impairment (De Groot *et al.*, 1993b).

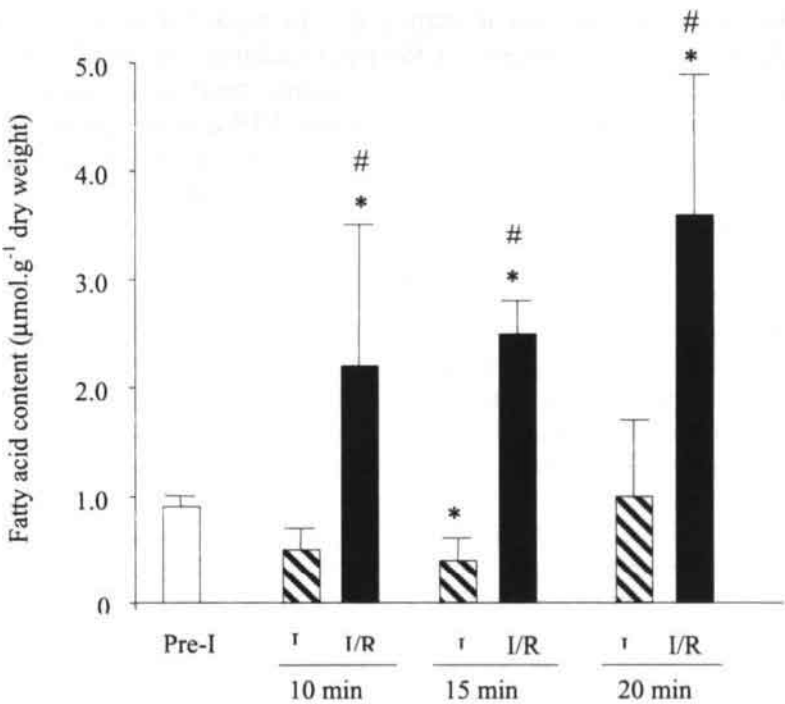

Figure 3.
Tissue content of (unesterified) fatty acids in the pre-ischemic myocardium and in mouse hearts subjected to 10, 15 and 20 min of ischemia, either or not followed by reperfusion. Data are expressed as means ± SD ($n = 4$ for pre-ischemic hearts, $n = 5$ for 10 min I/R, 20 min I and 20 min I/R, and $n = 6$ for the remaining groups). I, ischemia; R, reperfusion. * indicates $P < 0.05$ vs pre-ischemia, # indicates $P < 0.05$ vs corresponding end-ischemic period.

Energy metabolism in the ischemic-reperfused mouse heart

Prolonged ischemic duration resulted in a substantial reduction of ATP (to less of 25 % of pre-ischemia) and tissue accumulation of AMP and (oxy)purines, mainly adenosine and inosine. The accumulation of AMP suggests a limited activity of endo-5 -nucleotidase during ischemia or a confinement of AMP to a compartment distinct from the localization of this enzyme (Van Bilsen *et al.*, 1989a). Similarly, the accumulation of adenosine and inosine, combined with relatively low tissue levels of hypoxanthine and xanthine strongly suggests a constraint at the level of nucleoside phosphorylase in the ischemic murine heart. This might be caused by inhibition of the enzyme under ischemic conditions or a difference in localization of inosine production and nucleoside phosphorylase in the mouse heart. Similar findings were obtained in the isolated working rat heart subjected to varying periods of ischemia and reperfusion (Van Bilsen *et al.*, 1989a, De Groot *et al.*, 1993b)

The recovery of ATP and adenylate energy charge during reperfusion indicates that mitochondrial ATP production is not seriously hampered by the preceding ischemic insult or hampered by accumulating fatty acids. The data suggest that the reduced total adenine nucleotide pool (sum of ATP, ADP and AMP) at the end of reperfusion is mainly due to wash-out of its degradation products, which prevents resynthesis of the parent adenine nucleotides. Although post-ischemic hemodynamic function of the murine heart is severely impaired following 20 min of ischemia, the recovery of tissue ATP content and the adenylate energy charge still amounted to about 60 and 80 % of pre-ischemic levels, respectively, demonstrating a dissociation between hemodynamic recovery and post-ischemic energy metabolism.

Lipid homeostasis in the ischemic-reperfused mouse heart

The small but significant transient decrease in tissue fatty acid levels during initial ischemia is in accordance with previous findings in the ischemic rat heart, in which also a biphasic pattern in tissue fatty acid content was observed (Van Bilsen et al., 1989b). In these studies it was also found that substantial amounts of glycerol accumulated during early ischemia, which indicates increased triacylglycerol-fatty acid cycling (Van Bilsen et al., 1989b, De Groot et al., 1993a). The observation that the arachidonoyl content of the tissue (unesterified) fatty acid pool increased during prolonged ischemia is in favor of a switch from increased triacylglycerol cycling to enhanced deacylation and/or decreased reacylation of the phospholipid pool during more prolonged ischemia (Van der Vusse et al., 1992). Restoration of flow resulted in a substantial elevation of fatty acids, the level being dependent on the length of the preceding ischemic episode. The high contribution of poly-unsaturated fatty acids such as arachidonic acid and docosahexaenoic acid relative to saturated fatty acids is in agreement with an accelerated net degradation of the cardiac phospholipid pool during the reperfusion phase (Van der Vusse et al., 1992, Van der Vusse et al., 1998).

It is of interest to note that in the mouse hearts under investigation the phospholipid-plasmalogen content is substantially lower than earlier reported values in the rat heart (Van Bilsen et al., 1989), guinea pig heart (Arthur et al., 1985) and bovine heart (Shaikh et al., 1981). Ischemia and reperfusion did not affect the absolute

Table V

Relative fatty acid composition of the ventricular (unesterified) fatty acid content in pre-ischemic, ischemic and ischemic-reperfused mouse hearts.

n	4	6	5	6	6	6	5
	Pre-I	*10 min I*	*10 min I + 60 min R*	*15 min I*	*15 min I + 60 min R*	*20 min I*	*20 min I + 60 min R*
Total	0.9 ± 0.1	0.5 ± 0.2	2.2 ± 1.3 $^{\#*}$	0.4 ± 0.2 $^{\#}$	2.5 ± 0.3 $^{\#*}$	1.0 ± 0.7	3.6 ± 1.3 $^{\#*}$
16:0	18.0 ± 1.6	32.7 ± 5.7 $^{\#}$	21.8 ± 1.6 $^{\#*}$	31.9 ± 3.5 $^{\#}$	22.1 ± 2.7 $^{\#*}$	31.3 ± 6.1 $^{\#}$	22.2 ± 1.9 $^{\#*}$
16:1	0.7 ± 0.3	4.1 ± 1.1 $^{\#}$	0.4 ± 0.2 *	4.0 ± 1.9 $^{\#}$	0.5 ± 0.3 *	2.4 ± 1.4 $^{\#}$	1.4 ± 1.8
18:0	17.0 ± 2.5	22.3 ± 5.0	27.1 ± 3.9 $^{\#}$	27.0 ± 5.4 $^{\#}$	27.7 ± 3.5 $^{\#*}$	21.6 ± 3.4	21.5 ± 2.3 $^{\#}$
18:1	13.2 ± 0.6	14.0 ± 1.9	15.9 ± 1.4	12.5 ± 2.7	16.3 ± 2.3 $^{\#}$	13.5 ± 2.0	15.8 ± 1.5 $^{\#}$
18:2	12.5 ± 0.8	11.2 ±2.1	15.9 ± 1.4 $^{\#*}$	9.8 ± 3.2	12.1 ± 3.1	14.1 ± 2.3	14.9 ± 2.5
20:0	0.4 ± 0.1	1.8 ± 2.7	0.5 ± 0.2	0.4 ± 0.2	0.2 ± 0.1	0.5 ± 0.4	0.1 ± 0.1
20:4	4.0 ± 0.4	1.8 ± 1.7	2.9 ± 0.2 $^{\#}$	0.8 ± 0.9	3.0 ± 0.8 *	2.2 ± 1.4	4.4 ± 1.0 *
22:4	0.7 ± 0.1	N.D.	0.4 ± 0.1 $^{\#*}$	N.D.	0.5 ± 0.1 *	0.8 ± 0.2	0.5 ± 0.1
22:6	28.7 ± 3.5	7.2 ± 2.7	15.8 ± 2.2 $^{\#*}$	8.0 ± 3.2 $^{\#}$	15.0 ± 3.8 $^{\#*}$	9.3 ± 5.0 $^{\#}$	14.7 ± 1.5 $^{\#*}$

Data are expressed as means ± SD. Presented are percentages of total tissue (unesterified) fatty acids. *n* indicates number of hearts analyzed.

I: ischemia, *R*: reperfusion. *Total* refers to total ventricular (unesterified) fatty acid content and is expressed as μmol.g^{-1} dry weight. Values less than 0.1 % are indicated with N.D. (not detectable). * indicates $P < 0.05$ vs corresponding end-ischemic value. # indicates $P < 0.05$ vs pre-ischemic value.

Table VI

Total amount of ventricular phospholipids and relative fatty acyl composition in pre-ischemic, ischemic and ischemic-reperfused murine hearts.

n	4	6	5	6	6	5	5
	Pre-I	*10 min I*	*10 min I + 60 min R*	*15 min I*	*15 min I + 60 min R*	*20 min I*	*20 min I + 60 min R*
Total	198.4 ± 10.3	213.0 ± 9.5	194.0 ± 45.6	210.7 ± 20.0	202.7 ± 20.8	244.7 ± 21.9	200.0 ± 11.2
16:0	19.0 ± 0.5	19.4 ± 0.3	18.8 ± 0.4	19.4 ± 0.7	19.4 ± 0.6	19.4 ± 1.0	19.3 ± 0.1
16:0$_{PL}$	0.2 ± 0.0	0.4 ± 0.1	2.0 ± 1.2 *#	1.1 ± 1.1	2.3 ± 0.3 *	1.3 ± 1.1	2.5 ± 0.3 *
16:1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
18:0	18.6 ± 0.8	17.6 ± 0.4	18.0 ± 0.7	17.7 ± 0.5	18.3 ± 0.7	17.7 ± 0.5	18.2 ± 0.5
18:0$_{PL}$	0.5 ± 0.0	0.2 ± 0.0	0.8 ± 0.4	0.4 ± 0.3	0.9 ± 0.2	0.4 ± 0.3	0.9 ± 0.1
18:1	11.4 ± 0.6	11.0 ± 0.6	11.2 ± 0.8	11.0 ± 0.6	11.1 ± 0.7	10.9 ± 0.6	11.2 ± 0.4
18:1$_{PL}$	1.1 ± 0.3	0.1 ± 0.0	0.4 ± 0.1 *#	0.2 ± 0.1	0.4 ± 0.0 *#	0.2 ± 0.2	0.5 ± 0.1 *#
18:2	15.9 ± 0.5	17.0 ± 0.8	16.5 ± 0.5	16.3 ± 0.7	16.2 ± 0.9	17.3 ± 1.0	16.4 ± 0.9
20:0	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.2	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.0
20:4	6.7 ± 0.7	7.2 ± 0.5	6.9 ± 0.3	7.2 ± 0.6	6.6 ± 0.5	7.2 ± 0.6	7.0 ± 0.3
22:4	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.0
22:6	22.7 ± 1.4	25.6 ± 1.0 *	22.3 ± 0.4 #	24.8 ± 2.7 #	22.1 ± 0.7	23.5 ± 2.6	21.6 ± 0.5

Data are expressed as means ± SD. Presented are percentages of total tissue fatty acyl composition. *n* indicates number of hearts analyzed. I: ischemia, R: reperfusion, *Total* refers to total ventricular phospholipid content and is expressed as μmol.g^{-1} dry weight, PL refers to the dimethylacetal form of the corresponding fatty aldehyde.

* indicates $P < 0.05$ vs pre-ischemic value, # indicates $P < 0.05$ vs corresponding end-ischemic value.

Table VII

Total amount and relative fatty acyl composition of ventricular triacylglycerol in pre-ischemic, ischemic and ischemic-reperfused murine hearts.

n	4	6	5	6	6	6	5
	Pre-I	*10 min I*	*10 min I + 60 min R*	*15 min I*	*15 min I + 60 min R*	*20 min I*	*20 min I + 60 min R*
Total	40.8 ± 5.3	41.9 ± 8.9	31.1 ± 7.4	38.5 ± 7.4	27.8 ± 13.4	49.8 ± 18.5	34.0 ± 9.6
14:0	2.0 ± 0.2	1.9 ± 0.4	2.2 ± 0.2	1.9 ± 0.6	2.2 ± 0.6	1.9 ± 0.4	2.1 ± 0.4
16:0	30.9 ± 6.2	25.2 ± 3.8	25.0 ± 0.7	22.7 ± 2.9 *	24.5 ± 2.3	23.7 ± 2.8 *	24.0 ± 1.8
16:1	4.5 ± 1.2	3.4 ± 1.5	2.8 ± 0.3	2.7 ± 0.3	3.4 ± 1.7	3.1 ± 1.0	3.4 ± 1.2
18:0	5.4 ± 1.1	4.5 ± 0.2	4.8 ± 0.3	4.6 ± 0.3	4.6 ± 0.6	4.8 ± 0.6	4.6 ± 0.4
18:1	29.2 ± 4.0	29.2 ± 1.7	28.1 ± 0.8	29.2 ± 1.1	27.5 ± 1.8	27.5 ± 1.9	27.2 ± 1.8
18:2	27.3 ± 5.9	28.8 ± 2.4	28.2 ± 1.9	31.3 ± 2.8 *	28.0 ± 3.3	31.2 ± 2.8 *	27.2 ± 1.8 #
18:3	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
20:0	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
20:4	0.9 ± 0.1	1.1 ± 0.4	1.3 ± 0.2	1.3 ± 0.5	1.5 ± 0.4	1.3 ± 0.2	1.5 ± 0.4
22:4	0.3 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
22:6	4.3 ± 1.0	4.9 ± 2.5	5.7 ± 1.2	5.4 ± 2.0	6.3 ± 1.1	5.4 ± 2.1	7.2 ± 2.1

Data are expressed as means ± SD. Presented are percentages of total fatty acyl composition. *n* indicates number of hearts analyzed. I: ischemia, R: reperfusion. *Total* refers to total ventricular triacylglycerol content and is expressed as μmol.g^{-1} dry weight. * indicates $P < 0.05$ vs pre-ischemia, # indicates $P < 0.05$ vs corresponding end-ischemic value.

amount of plasmalogen, but caused a change in the nature of the aliphatic chain connected via an ether-bond to the glycerol backbone. The cause and significance of this alteration remains to be elucidated, especially in view of the potential importance of type VI plasmalogen-specific phospholipases A_2 in phospholipid hydrolysis in the ischemic myocardium (Hazen et al., 1991,).

Post-ischemic tissue fatty acid accumulation and irreversible cell damage

The positive correlation between the amount of LDH released during reperfusion, reflecting irreversible cardiac muscle cell injury, on the one hand, and ventricular total fatty acid or arachidonic acid accumulation during reperfusion, on the other (Figure 5), which can also be observed in the rat heart (Van Bilsen et al., 1989b), supports the notion of a causative relation between accelerated phospholipid degradation during ischemia and/or reperfusion, on the one hand, and irreversible cell damage, on the other (Van der Vusse et al., 1992, Van der Vusse et al., 1998). The data in the present study suggest that the basic mechanisms leading to disturbances in the lipid homeostasis during ischemia and reperfusion as documented and studied in ischemic-reperfused hearts derived from other species are also applicable to the murine heart. A distinct advantage of the availability of an isolated working mouse heart model is the current technological facilities to alter the expression level of one of the putative contributing factors, such as cardiac phospholipases A_2, to more definitively establish their roles in the sequela of events leading to ischemia and reperfusion-induced cardiac dysfunction.

In conclusion, the model described in the present study allows detailed evaluation of the performance of isolated, antegradely perfused mouse hearts during transient ischemia. The results of the present study are indicative of a high susceptibility of the mouse heart to ischemia and reperfusion-induced damage. This model will be asset in the interpretation of the function of specific genes investigated in transgenic and gene-targeted mouse models for heart diseases.

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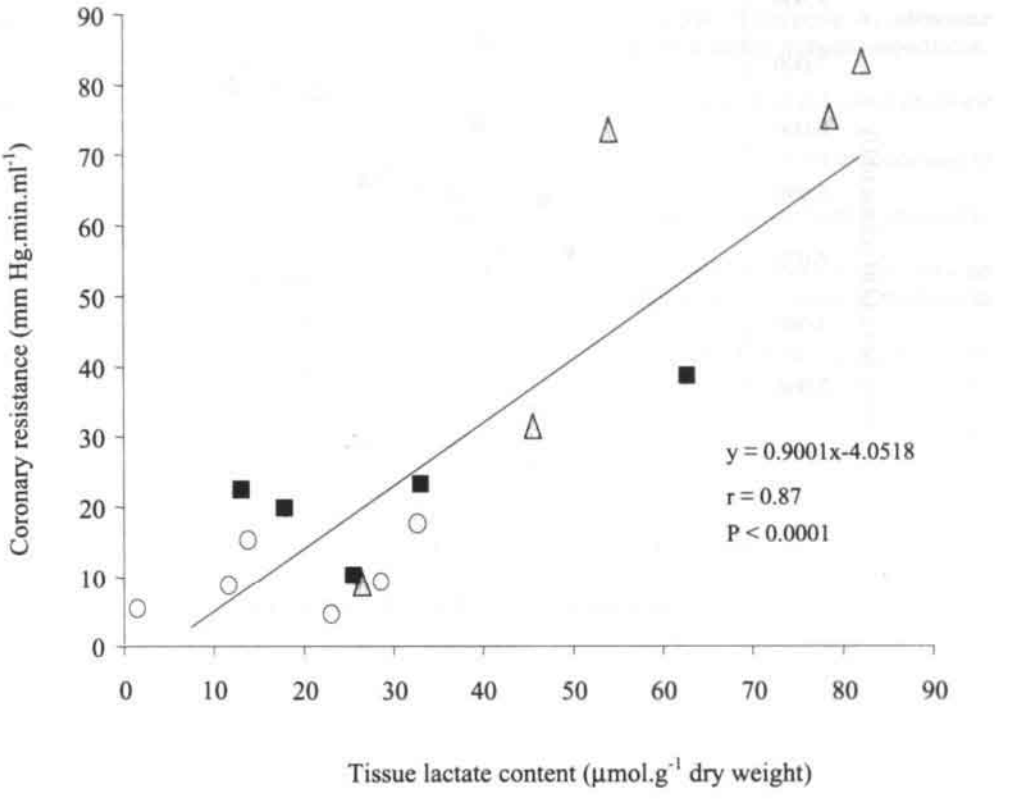

Figure 4
Relationship between post-ischemic coronary resistance and tissue content of lactate in reperfused ventricles of hearts subjected to 10 (○), 15 (■) and 20 min (Δ) of global, normothermic ischemia.

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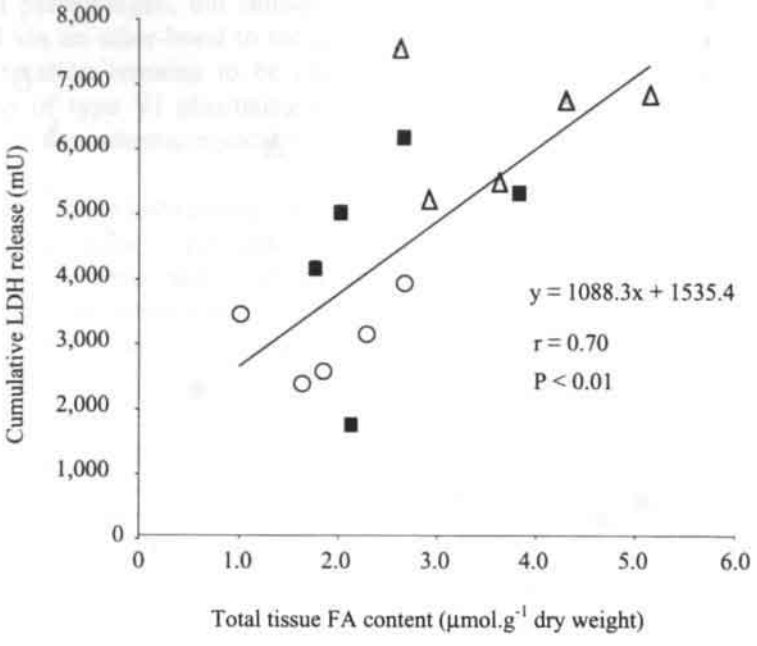

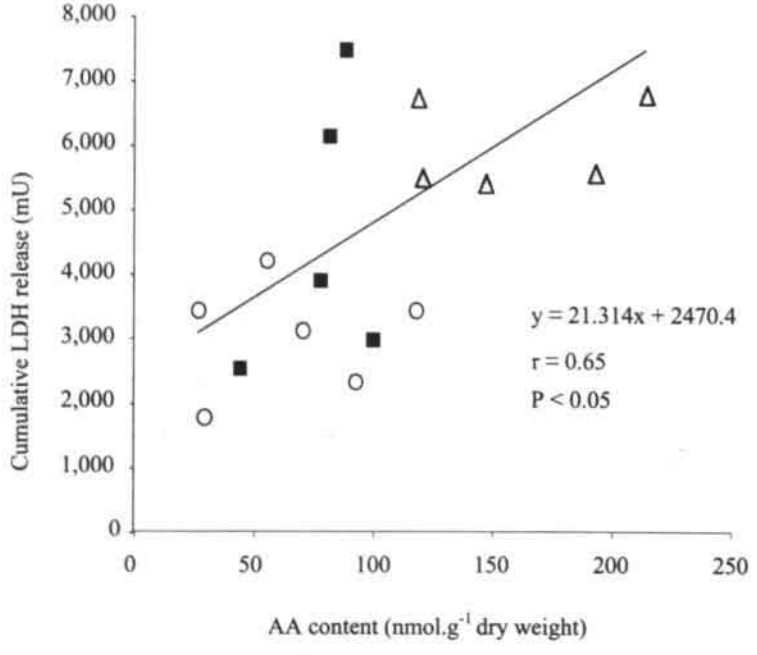

Figure 5
Relationship between cumulative LDH release into coronary effluent during 60 min of reperfusion and unesterified fatty acid content (top panel) or arachidonic acid content (bottom panel) in the reperfused heart subjected to 10 (○), 15 (■) or 20 min (Δ) of global, no-flow ischemia.

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Appendix

(Phospho)creatine content in murine heart

Routinely the ventricular phosphocreatine (PCr) and creatine (Cr) in pre-ischemic, ischemic and ischemic-reperfused murine ventricles were determined using the HPLC techniques. PCr and creatine (Cr) content in pre-ischemic hearts amounted to 33.3 ± 8.2 and 36.6 ± 5.4 $\mu mol.g^{-1}$ dry weight, respectively, as determined by HPLC. Following 10 min of ischemia the tissue PCr content tended to decrease to the expense of Cr, but this decrease did not reach the level of significance (Table VIII). However, extending the ischemic duration to 15 or 20 min of ischemia resulted in a substantial decrease of PCr content to 19.2 ± 1.6 and 18.7 ± 4.6 $\mu mol.g^{-1}$ dry weight, respectively, and significant increases in tissue Cr content, as determined by HPLC (Table VIII). Large fluctuations were observed in the sum of PCr and Cr following the different experimental procedures, reaching statistical significantly lower end-ischemic values compared to post-ischemic values. The PCr values in ischemic hearts were considered to be less reliable as close inspection of the chromatogram revealed additional peaks with almost identical RF values as the original PCr peak.

To explore this issue in more detail, a subset of hearts were subjected to 100 min of normoxic perfusion and PCr content was determined by both HPLC as well as fluorometrically. PCr content amounted to 22.0 ± 7.8 and 23.3 ± 3.8 $\mu mol.g^{-1}$ dry weight as determined by HPLC and fluorometry (n = 4), respectively. Subjecting the hearts to 20 min of ischemia resulted in a tissue PCr content of 26.7 ± 2.3 and 14.8 ± 2.0 $\mu mol.g^{-1}$ dry weight as determined by HPLC and fluorometry (n = 3), respectively. Finally, following 20 min of ischemia and 60 min of reperfusion, PCr content amounted to 11.4 ± 7.0 and 11.3 ± 6.8 $\mu mol.g^{-1}$ dry weight as determined by HPLC and fluorometry, respectively (Table VIII). The above findings indicate consistingly lower readings of tissue PCr content as determined by HPLC as compared to fluorometry, and, hence, PCr values obtained with HPLC techniques in ischemic hearts should be considered with caution.

Table VIII

Total tissue content phosphocreatine (PCr) and creatine (Cr) and their sum in pre-ischemic, ischemic and ischemic-reperfused mouse hearts.

n	*5*	*6*	*10*	*6*	*9*	*5*	*11*
	Pre-I	*10 min I*	*10 min I + 60 min R*	*15 min I*	*15 min I + 60 min R*	*20 min I*	*20 min I + 60 min R*
PCr	33.3 ± 8.2	23.1 ± 7.7	29.1 ± 7.6	19.2 ± 1.6 *	27.6 ± 6.1 #	18.7 ± 4.6 *	25.6 ± 8.1
Cr	36.6 ± 5.4	56.2 ± 10.2 *	34.7 ± 7.1 #	55.6 ± 5.0 *	28.5 ± 7.2 #	60.0 ± 11.6 *	31.3 ± 7.2 #
PCr + Cr	69.9 ± 10.9	79.3 ± 15.5	64.5 ± 9.5	74.9 ± 4.3	56.1 ± 6.2 #	78.7 ± 15.5	56.8 ± 9.4 #

Data are expressed as means ± SD. Data are presented in μmol g^{-1} dry weight ventricular tissue. *n* indicates number of hearts analyzed. *Pre-I*, pre-ischemia; *I*, ischemia; *R*, reperfusion. * indicates $P < 0.05$ vs pre-ischemic value, # indicates $P < 0.05$ vs corresponding end-ischemic value.

Chapter 7

Reduced ischemia tolerance of hearts from mice with targeted deletion of the insulin-like growth factor-1 gene.

Abstract

In the present study the ischemia tolerance of hearts derived from heterozygous, insulin-like growth factor-1 (IGF-1) knockout mice (IGF-1 +/-) was assessed in an isolated left ventricular ejecting mouse heart preparation. Pre-ischemic coronary flow (CF) was significantly lower and left ventricular end-diastolic pressure (LVEDP) was significantly higher in IGF-1 +/- hearts. During reperfusion following 15 min of ischemia functional recovery was more compromised in IGF-1 +/- hearts as compared to hearts from wildtype littermates, as evidenced by a reduced recovery of cardiac output (11 ± 5 vs 31 ± 15 % recovery; $P < 0.05$) and left ventricular developed pressure (26 ± 11 vs 57 ± 11 % recovery; $P < 0.05$). The percentage irreversibly damaged cardiac cells following 15 min of ischemia was higher in IGF-1 +/- than wildtype hearts, as indicated by the greater release of LDH into the coronary effluent during reperfusion (11.6 ± 4.1 and 5.1 ± 2.6 $U.g^{-1}$ heart weight per 60 min, respectively). In contrast, following 20 min of ischemia and 60 min of reperfusion hemodynamic recovery and the amount of LDH released from the heart was similar in IGF-1 +/- and wildtype hearts. Cardiac high-energy phosphate content was not significantly different in reperfused hearts from IGF +/- and wildtype mice following 15 as well as 20 min of ischemia. In contrast, tissue fatty acid levels were higher in the reperfused IGF-1 +/- heart following 15 min of ischemia due to substantial elevation of all major fatty acid species, including arachidonic acid (115 ± 32 and 41 ± 10 nmol. g^{-1} dry weight in IGF-1 +/- and wildtype hearts, respectively; $P < 0.05$), suggesting increased net phospholipid degradation in IGF +/- hearts. Collectively, these data indicate increased ischemic vulnerability of the IGF-1 +/- heart, suggesting that IGF-1 affords cardiac protection towards ischemia/reperfusion if the ischemic insult does not exceed 15 minutes.

Introduction

Insulin-like growth factor-1 (IGF-1) is part of the insulin family of peptides and acts as a pleiotropic growth factor in many tissues and cell types (for a review see Delafontaine, 1996). Recent studies indicate that IGF-1 may have beneficial effects in myocardial infarction and in the hypertrophied and failing heart (Reiss *et al.*, 1994, Jin *et al.*, 1995, Duerr *et al.*, 1996, Tanaka *et al.*, 1998). In transgenic mice with cardiac-specific overexpression of IGF-1 necrotic and apoptotic cell death following coronary artery ligation was found to be reduced and cardiac function was found to be better as compared to wildtype littermates (Li *et al.*, 1997, 1999).

In an earlier study, however, no differences in hemodynamic performance following myocardial infarction were found between heterozygous IGF-1 deficient mice and their wildtype littermates (Palmen *et al.*, 1999). Therefore, in the present study, it was tested whether partial deficiency of this growth factor would reduce the vulnerability of the heart towards global ischemia followed by reperfusion. To this end, hearts from heterozygous IGF-1 deficient mice, originally described by Powell-Braxton and colleagues (Powell-Braxton *et al.*, 1993), and their wildtype littermates were perfused *ex vivo* in the working ejecting mode and subjected to either 15 or 20 min of global ischemia followed by reperfusion for 60 min. The experiments were performed on isolated hearts to avoid differences in loading conditions and neurohumoral status which have been shown to be present in *in vivo* studies (Lembo *et al.*, 1996). The extent of tissue damage was assessed by measuring recovery of hemodynamic function and the release of lactate dehydrogenase (LDH) into the coronary effluent, a marker of irreversible cell damage, during reperfusion. In addition, tissue levels of high energy phosphates were monitored. Accumulation of fatty acids, if any, was used as a marker of disturbances in membrane phospholipid homeostasis in the ischemic/reperfused heart (Van der Vusse *et al.*, 1992).

Materials and Methods

Chemicals

All chemicals used for the Krebs-Henseleit solution were of the highest grade available and purchased from Merck (Darmstadt, Germany) except for D(+)-glucose and pyruvate (Sigma Chemical Co., St. Louis, MO, USA). Insulin was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark).

Animals

The IGF-1 deficient mice used in this study were originally generated by Powell-Braxton and colleagues (Powell-Braxton *et al.*, 1993) by disruption of the coding sequence in exon 3 of the murine *igf-1* locus in AB.1 embryonic stem cells, upon which recombinant clones were injected into C57BL/6J blastocysts. Resulting heterozygous (+/-) and homozygous (-/-) IGF-1 deficient mice were well characterized (Powell-Braxton *et al.*, 1993). More than 95 % of the homozygous

IGF-1 knockout mice die at birth and have < 60 % of the body weight of wildtype littermates. In contrast, heterozygous IGF-1 deficient mice are healthy and fertile in spite of a 30-40 % reduction of circulating IGF-1 levels. Heterozygous IGF-1 deficient mice are 10-20 % smaller than their wildtype littermates and no obvious abnormalities or differences can be detected upon histological examination (Powell-Braxton *et al.*, 1993). In the present study only male IGF-1 +/- mice (n = 10) and their wildtype littermates (n = 10) were included. The mice were kept under standard housing conditions with an artificial 12 h light cycle and had free access to standard rodent food (Diet SRM-A, Hope farms, Woerden, the Netherlands) and tap water. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Maastricht University.

Genotyping

Genotyping of the two mouse strains was performed by genomic PCR analysis to detect the presence of the targeted vector in the murine *igf-1* locus. Briefly, genomic DNA was isolated from tails using a Qiamp genomic DNA prep kit (Qiagen, Leusden, The Netherlands). PCR analysis was performed using primers directed against the neomycin cassette of the original targeting vector, which were designated L62 (5 -primer) [5 -TGCTCTGATGCCGC-CGTGTTCCGC-3] and L72 (3 -primer) [5 GGTCCGCCACACCCAGCCGGCCAC-3]. PCR reactions were cycled 40 times at 94 °C for 1 min, at 55 °C for 1 min and at 72°C for 2 min, with an additional amplification cycle of 10 min at 72°C using 1 µg of genomic DNA as template and 50 pmol of each primer. Detection of a specific product of about 600 bp was anticipated in the presence of the gene targeting vector and, hence, the targeted genomic *igf-1* allele in IGF-1 +/- mice.

Isolated working mouse heart preparation

The isolated ejecting mouse heart preparation used in the present study was previously described in detail (De Windt *et al.*, 1999). Briefly, hearts of anesthetized mice were mounted on the perfusion system and retrograde perfusion was started immediately at a perfusion pressure of 50 mm Hg. Subsequently, the left atrium was cannulated with an atrial cannula through one of the lung veins. The oxygenated (95 % O_2- 5 % CO_2) Krebs-Henseleit perfusion buffer consisted of: 118 mM NaCl, 4.7 mM KCl, 3.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 0.5 mM Na-EDTA, 10 mM D(+)-glucose, 1.5 mM Na-pyruvate and 100 mU/l insulin. Left ventricular pressure was measured with a PE-50 catheter, inserted into the left ventricular cavity through the apex, connected to a Baxter pressure transducer (Baxter Healthcare Corp., Irvine, CA, USA). Aortic pressure was measured through a side branch located 3 mm above the entrance of the aortic cannula by means of a pressure transducer (Baxter Healthcare Corp.).

Hemodynamic data

All hemodynamic variables were continuously recorded on a personal computer, using specialized software (Hemodynamic Data Acquisition System, Technical Department, Maastricht University), allowing the on-line acquisition, presentation and calculation of left atrial filling flow, aortic flow (AOF), left ventricular systolic

pressure (LVSP), left ventricular end-diastolic pressure (LVED), diastolic aortic pressure (AODP) and the first maximal and minimal derivatives of left ventricular pressure (LV dP/dt_{max} and LV dP/dt_{min}). Left ventricular developed pressure (LVDP) was defined as the difference between LVSP and LVEDP. Cardiac output (CO) was defined as the sum of AOF and coronary flow (CF). CF was determined from the difference between AOF, as measured by an 1N inline aortic flow probe, and left atrial filling flow, as measured by a 2N inline flow probe placed in the left atrial inflow tract. Calculated CF data were periodically checked by timed collection of the coronary perfusate.

Experimental protocol

Hearts were normoxically perfused in the antegrade mode for 20 min (pre-ischemic period), whereupon the hearts were subjected to normothermic (38.5 ...C) no-flow global ischemia for either 15 or 20 min (see Chapter 6 for details). Following this ischemic period the hearts were reperfused retrogradely (perfusion pressure 50 mm Hg). After 10 min the left atrial conduit was re-opened and the hearts were perfused in the antegrade mode (preload 10 mm Hg, afterload 50 mm Hg) for an additional 50 min. Except for the ischemic period and during the first 5 min of reperfusion, the hearts were paced throughout the experimental protocol at 450 $beats.min^{-1}$, a frequency slightly higher than the intrinsic denervated heart rate of this mouse strain. Coronary effluent was collected and samples were immediately frozen in liquid nitrogen and stored at -80 °C for further biochemical analysis. To stabilize lactate dehydrogenase (LDH) activity in coronary effluent samples, bovine serum albumin was included (final concentration 3%). After completion of the experimental protocol the ventricles of the individual hearts were separated from the atria and immediately frozen between aluminum clamps, previously cooled in liquid nitrogen, and stored at -80¡C for further analysis.

Biochemical analysis

LDH content in the coronary perfusate was assessed spectrophotometrically using a Cobas Bio autoanalyzer as described earlier (Bergmeyer and Bernt *et al.*, 1974). Tissue content of adenine nucleotides, IMP, nucleosides and (oxy)purines was determined by high-performance liquid chromatography (Varian Vista 5500 HPLC) as described in detail earlier (Chapter 6 and Van Bilsen *et al.*, 1989). The determination of cardiac fatty acids, phospholipids and triacylglycerols was performed as described in detail before (Chapter 6 and Van der Vusse and Roemen, 1995). All values were expressed as moles per gram dry weight of ventricular tissue.

Statistical analysis

The results are presented as mean values – standard deviations (M ± SD). All statistical analyses were performed using InStat 3.0 software (GraphPad Software Inc., San Diego, Ca). Differences between values of functional variables and biochemical parameters were statistically analyzed using one-way ANOVA followed by the Tukey s test. Linear regression was performed with the least squares method and the Pearson rank correlation coefficient (r) was used to estimate

the strength of the relation between two variables. In all tests significance was accepted at P values < 0.05.

Results

Genotyping

Heterozygous mice with the targeting vector, disrupting the *igf-1* gene, integrated into the genome, demonstrated a prominent PCR product of about 600 bp, whereas this product was absent in wildtype littermates (Figure 1). The IGF-1 +/- hearts used in the present study tended to be slightly smaller than wildtype hearts (215 ± 31 and 225 ± 30 mg wet weight, respectively; n = 10 for both groups), but the difference did not reach the level of significance. Similarly, heart weight-to-body weight ratios did not differ significantly between wildtype and IGF-1 +/- mice (6.3 ± 0.7 and 6.4 ± 0.4 mg/g, respectively).

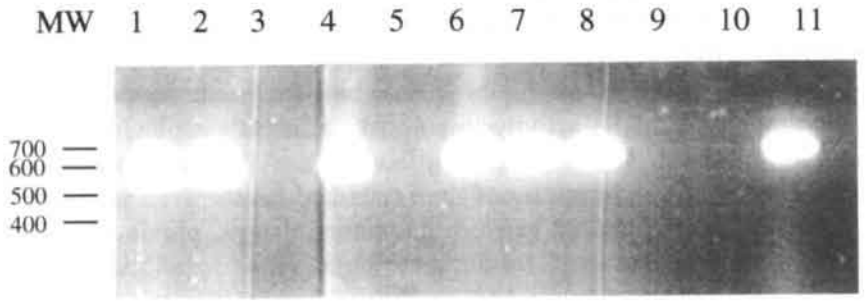


Figure 1. Example of IGF-1 PCR genotyping. Heterozygous IGF-1 deficient animals were identified by the presence of a prominent PCR product of about 600 bp following PCR on their genomic DNA as template, resulting from amplification of the integrated neomycine gene as part of the targeting vector (lanes 2, 3, 5 and 8; IGF-1 +/-), while in wildtype littermates this product was absent (lanes 1, 4, 6, 7; wt).

Functional recovery

Baseline hemodynamic performance, as measured during the pre-ischemic phase, of wildtype and IGF-1 +/- hearts is depicted in Table I. No significant differences in CO and AOF were observed between both groups. Coronary flow rate, however, was approximately 25 % lower ($P < 0.05$) in IGF-1 +/- hearts than in wildtype hearts. In addition, LVEDP was substantially higher in IGF-1 +/- hearts than in wildtype hearts. Baseline contractility as measured by LV dP/dt_{max} and LV dP/dt_{min} was similar in wildtype and IGF-1 +/- hearts (Table I).

Following 15 min of global ischemia and 60 min of reperfusion CO recovered to 31 ± 15 % and 11 ± 5 % of the corresponding pre-ischemic values in wildtype and IGF-1 +/- hearts, respectively ($P < 0.05$; Table II, Figure 2). LVDP

recovered to 57 ± 11% and 26 ± 11 % of their corresponding pre-ischemic values in wildtype and IGF-1 +/- hearts, respectively ($P < 0.05$; Table II and Figure 2). In both groups the decrease in post-ischemic LVDP could be attributed to both a rise in LVEDP and a decrease in LVSP. Post-ischemic LV dP/dt_{max} and LV dP/dt_{min} tended to be lower for IGF-1 +/- hearts, but the difference did not reach the level of significance (Table II and Figure 2).

When the ischemic period was extended to 20 min in IGF +/- hearts functional recovery during reperfusion was as poor as in the 15 min ischemia group (Table II). In contrast, in wildtype hearts further deterioration of cardiac function was observed (Table II). As a result, following 20 min of ischemia hemodynamic recovery was severely depressed in all hearts and comparable between the two groups. For instance, CO recovered to only 17 ± 20 % and 9 ± 11 % of their corresponding pre-ischemic value in wildtype and IGF-1 +/- hearts, respectively (N.S.; Figure 2).

Table I

Pre-ischemic values of functional parameters of isolated, left ventricular ejecting hearts from wildtype (wt) and heterozygous IGF-1 deficient mice [IGF-1(+/-)].

	wt	*IGF-1 (+/-)*
CO ($ml.min^{-1}.g^{-1}$)	55.3 ± 5.7	48.2 ± 11.4
CF ($ml.min^{-1}.g^{-1}$)	13.5 ± 3.6	10.2 ± 2.7 *
AOF ($ml.min^{-1}.g^{-1}$)	42.2 ± 7.1	37.2 ± 10.6
LVEDP (mm Hg)	8 ± 3	13 ± 2 **
LVSP (mm Hg)	87 ± 8	85 ± 10
LVDP (mm Hg)	77 ± 10	72 ± 7
LV dP/dt_{max} ($mm\ Hg.s^{-1}$)	6,393 ± 818	6,808 ± 1,102
LV dP/dt_{min} ($mm\ Hg.s^{-1}$)	-3,802 ± 865	-3,685 ± 514

Data are expressed as means ± SD (both groups n = 10). Isolated hearts were perfused in the antegrade mode for 20 min at a pre and afterload of 10 and 50 mm Hg, respectively, and paced at 450 $beats.min^{-1}$. CO, cardiac output; CF, coronary flow; AOF, aortic flow; LVDP, left ventricular developed pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; LV dP/dt_{max}, first maximal derivative of left ventricular pressure; LV dP/dt_{min}, first minimal derivative of left ventricular pressure. * indicates $P < 0.05$, ** indicates $P < 0.01$ between wildtype and IGF-1 (+/-) mice.

Enzyme release

Pre-ischemic LDH release was comparable in wildtype and IGF-1 +/- hearts and amounted to 0.2 ± 0.1 and 0.3 ± 0.1 $U.min^{-1}.g^{-1}$, respectively (N.S.; n=10 for both groups). In contrast, the post-ischemic cumulative LDH release was significantly higher in IGF-1 +/- hearts than in wildtype hearts following the shortest ischemic period tested and amounted to 11.6 ± 4.1 and 5.1 ± 2.6 $U.g^{-1}$ per 60 min, respectively ($P < 0.05$; $n = 5$ in both groups). Following 20 min of ischemia the cumulative release of LDH release amounted to 19.4 ± 5.7 and 14.7 ± 4.1 $U.g^{-1}$ for wildtype and IGF-1 +/- hearts, respectively, (N.S.; $n = 5$ in both groups).

Metabolic recovery

In reperfused hearts previously subjected to 15 min of ischemia the tissue levels of phosphocreatine (PCr) and creatine (Cr) were not significantly different between both groups (Table III). Also, tissue ATP, ADP and AMP levels and adenylate energy charge (EC) were similar between wildtype and IGF-1 +/- hearts (Table III). The tissue content of adenine nucleotide degradation products (i.e., sum of adenosine, inosine, hypoxanthine en xanthine), glycogen and lactate did not differ between both groups either (Table III).

Following 20 min of ischemia and 60 min of reperfusion no significant differences were observed in tissue PCr, ATP, ADP, AMP and EC between wildtype and IGF-1 +/- hearts (Table III). The same holds for the tissue content of (oxy)purines, glycogen and lactate (Table III). In reperfused hearts previously subjected to 20 min of ischemia in both groups the tissue content of (oxy)purines and lactate tended to be higher than after 15 min of ischemia. The tissue lactate content was significantly higher in IGF +/- than in wildtype hearts.

Fatty acids, phospholipids, triacylglycerols

No significant differences in either the cardiac phospholipid (PL) and triacylglycerol (TG) content (Figure 3) or the fatty acid composition of these lipid pools (data not shown) were observed between wildtype and IGF +/- hearts following either 15 or 20 min of ischemia and reperfusion. However, following 15 min of ischemia and reperfusion the tissue content of total (unesterified) fatty acids (FA) was significantly higher in IGF-1 +/- than in wildtype hearts (Figure 3). As demonstrated in Figure 4, tissue levels of all individual fatty acid species were 2 to 2.5-fold higher in reperfused IGF-1 +/- than in wildtype hearts. In contrast, in reperfused IGF +/- and wildtype hearts previously subjected to 20 min of ischemia total tissue FA levels (Figure 3) and the levels of individual FA species, including arachidonic acid (data not shown), showed no significant differences.

Discussion

The findings in the present study indicate that IGF-1 may have a protective effect in myocardial ischemia of short duration, bacause the myocardium of heterozygous IGF-1 deficient mice was more sensitive to ischemia/reperfusion than that of the wildtype littermates following 15 min of ischemia. After 20 min of ischemia,

however, the differences between the strains had disappeared. These results support the notion that the trophic effects of IGF-1 delay cardiac ischemia/reperfusion-induced dysfunction (Ross *et al.*, 1996). The findings are also in line with observations of protective effects on hemodynamic function of IGF-1 following myocardial infarction in transgenic mice overexpressing IGF-1 specifically in the mouse heart (Li *et al.*, 1997).

Table II

Hemodynamic function of reperfused wildtype (wt) and IGF-1 (+/-) hearts following 15 and 20 min of global ischemia and 60 min of reperfusion.

	15 min Ischemia		*20 min Ischemia*	
	wt	*IGF-1 (+/-)*	*wt*	*IGF-1 (+/-)*
CO ($ml.min^{-1}.g^{-1}$)	31.0 ±15.1	11.3 ± 4.9 *	4.7 ± 5.9 #	9.1 ± 11.1
AOF ($ml.min^{-1}.g^{-1}$)	11.3 ± 17.1	0.2 ± 8.0	-5.1 ± 0.8	-1.0 ± 8.7
CF ($ml.min^{-1}.g^{-1}$)	19.7 ± 9.7	12.9 ± 3.7	9.8 ± 6.5	10.1 ± 9.0
LVEDP (mm Hg)	10 ± 3	18 ± 4 *	16 ± 5 #	18 ± 3
LVSP (mm Hg)	68 ± 6	43 ± 28	26 ± 25 #	46 ± 13
LVDP (mmHg)	57 ± 11	26 ± 11 *	17 ± 13	22 ± 16
LV dP/dt_{max} ($mm\ Hg.s^{-1}$)	4,133 ± 669	3,005 ± 2,235	1,138 ± 77 #	2,067 ± 1,949
LV dP/dt_{max} ($mm\ Hg.s^{-1}$)	-2,676 ± 450	-1,672 ± 1,062	-992 ± 641 #	-1,300 ± 867

Data are expressed as means ± SD (n = 5 for wt and IGF-1 (+/-) following each ischemic period). Isolated hearts were perfused in the antegrade mode at a pre and afterload of 10 and 50 mm Hg, respectively, and paced at 450 $beats.min^{-1}$. CO, cardiac output; CF, coronary flow; AOF, aortic flow; LVDP, left ventricular developed pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; LV dP/dt_{max}, first maximal derivative of left ventricular pressure; LV dP/dt_{min}, first minimal derivative of left ventricular pressure. * indicates $P < 0.05$ between wt and IGF-1 (+/-) following identical ischemic period, # indicates $P < 0.05$ vs group with identical genotype subjected to 15 min of ischemia

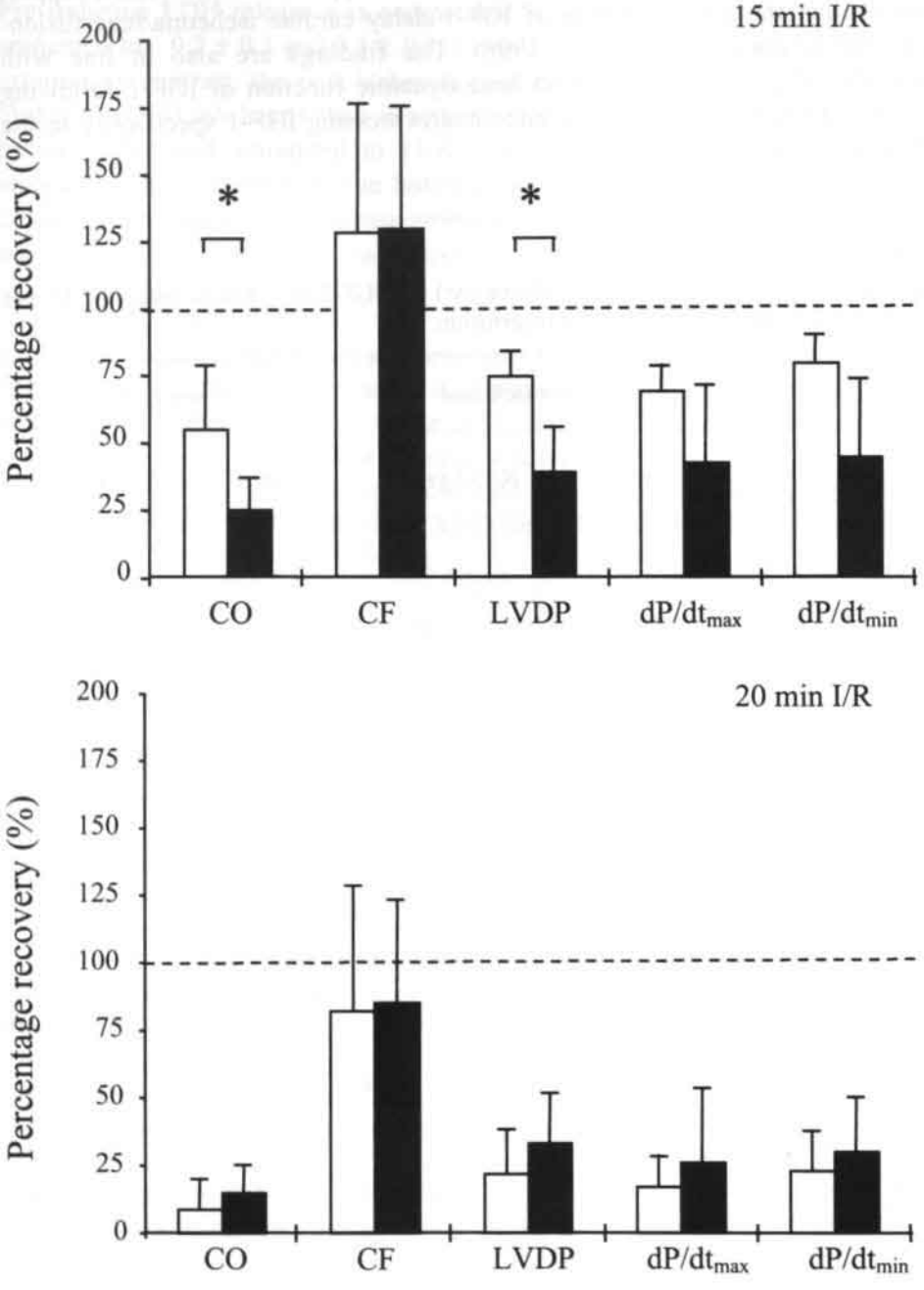


Figure 2. Post-ischemic percentage recovery of hemodynamic variables in isolated wildtype hearts (wt; open bars) and IGF-1 deficient (IGF-1 +/-; closed bars) following 15 (upper panel) or 20 min of global ischemia (lower panel). Data are presented as means ± SD. CO, cardiac output; CF, coronary flow; LVDP, left ventricular developed pressure; LVSP, left ventricular systolic pressure; LV dP/dt_{max}, first maximal derivative of left ventricular pressure; LV dP/dt_{min}, first minimal derivative of left ventricular pressure. * indicates $P < 0.05$ between wildtype and IGF +/-.

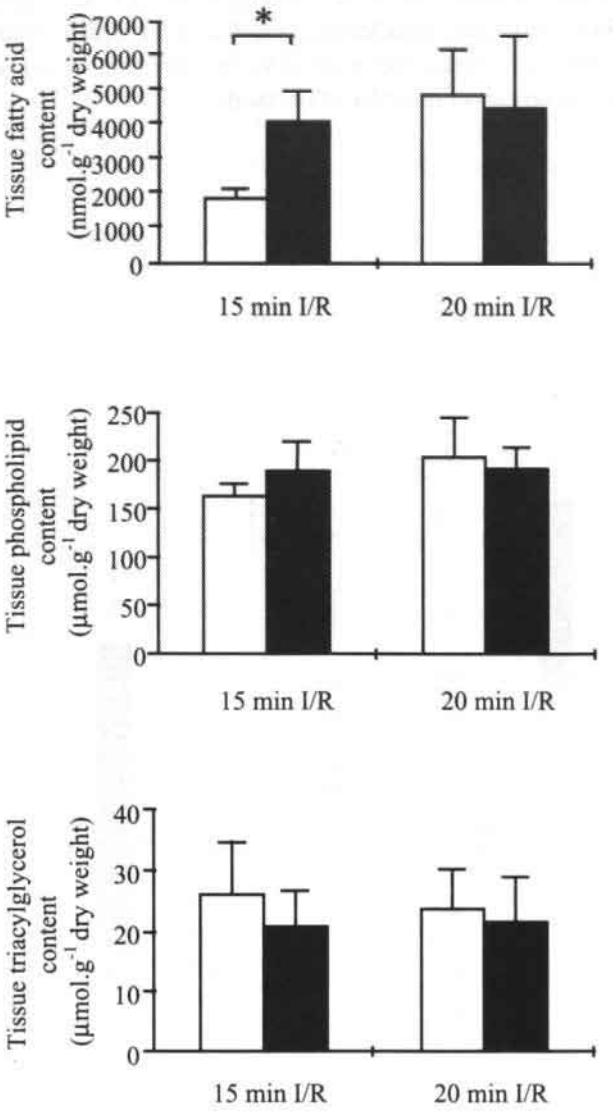
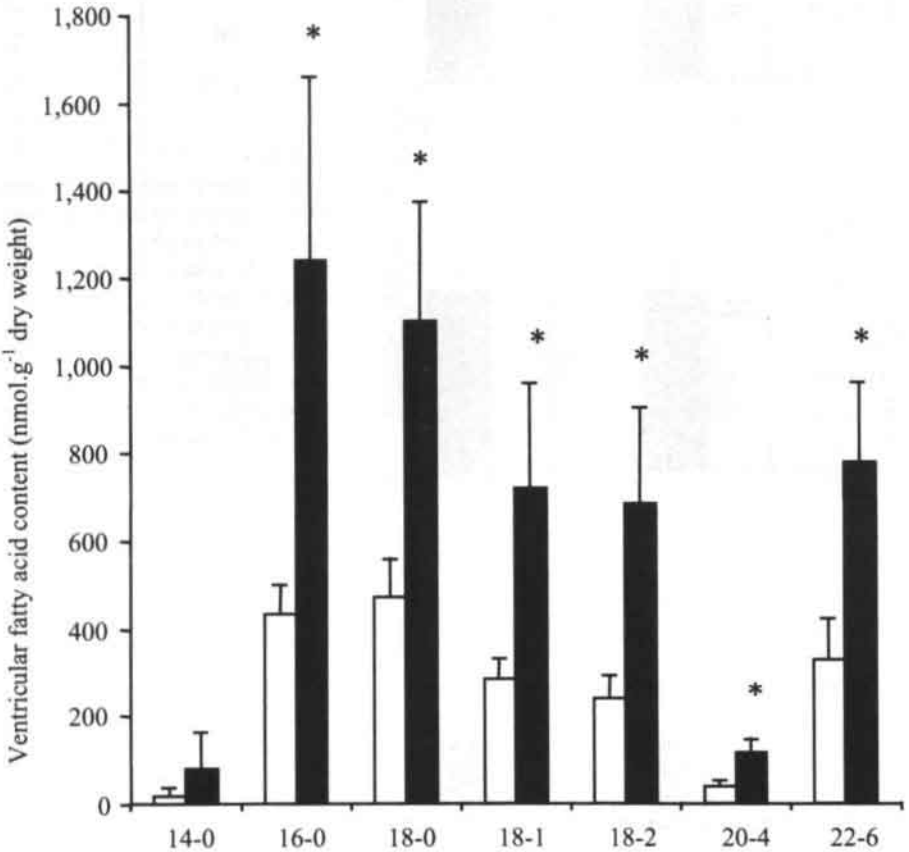


Figure 3
Post-ischemic ventricular content of the total (unesterified) fatty acid, phospholipid and triacylglycerol content in IGF-1 deficient (IGF-1 +/-; closed bars) and wildtype hearts (wt; open bars) following 15 and 20 min of ischemia. Data are presented as means ± SD. * indicates $P < 0.05$ between IGF-1 +/- and wt.

Following 15 min of global ischemia a significantly greater extent of irreversibly damaged cardiac muscle cells were found in the IGF-1 +/- mouse heart. The present findings corroborate with observed protective effects of IGF-1 towards necrotic and apoptotic cell death following myocardial infarction in transgenic mice with cardiac-specific overexpression of IGF-1 (Li *et al.*, 1997) and following non-occlusive coronary artery constriction in the mouse (Li *et al.*, 1999). Similar beneficial effects of IGF-1 on cardiac performance of regional infarction have been documented in experimental rat heart models where IGF-1 was administered exogenously (Reiss *et al.*, 1994, Jin *et al.*, 1995, Duerr *et al.*, 1996). The exact mechanism through which IGF-1 exerts its protective effect is incompletely understood. Previous studies provide indications that IGF-1 has potent anti-apoptotic effects on the cardiomyocyte both *in vivo* and *in vitro* (Reiss *et al.*, 1994,

Li *et al.*, 1997, Parrizas *et al.*, 1997, Wang *et al.*, 1998a, Wang *et al.*, 1998b, Leri *et al.*, 1999, Palmen *et al.*, 1999). Although accelerated cardiac cell death in the absence of IGF-1 may provide an attractive explanation for the observed phenomena, other possible explanations should not be dismissed.



Post-ischemic ventricular content of the major individual fatty acid species in wildtype hearts (wt; open bars) and IGF-1 deficient (IGF-1 +/-; closed bars) folowing 15 min of ischemia. Individual fatty acid species are indicated by their chemical notation. Data are presented as means ± SD. * indicates $P < 0.05$ between IGF-1 +/- and wt.

In this respect the observed differences in baseline normoxic hemodynamic performance between IGF-1 deficient and wildtype hearts deserve attention. Of note are the observed differences in end-diastolic left ventricular pressure and coronary flow. The significantly higher left ventricular end-diastolic pressure indicates diastolic dysfunction and filling abnormalities in the heterozygous IGF-1 deficient (IGF-1 +/-) heart under normoxic conditions. Another IGF-1 targeted mouse model, the so-called IGF-1 midi mouse in which plasma IGF-1 levels are approximately 30-40 % of normal, also display alterations in cardiac performance in

terms of fluid ejection and left ventricular pressure development as measured *in vivo* (Lembo *et al.*, 1996). The significantly lower baseline coronary flow rate in IGF-1 +/- hearts than in wildtype hearts is in line with a recent observation of reduced capillary density in the hearts of the same IGF-1 +/- mice (Palmen *et al.*, 1999) and the reported requirement of IGF-1 for the proper development of the microvasculature in other tissues (Sonntag *et al.*, 1997, Tonshoff *et al.*, 1998). Reduced capillary density and, hence, diminished tissue perfusion may contribute to the reduced ischemia tolerance under conditions of chronic IGF-1 shortage.

The present data demonstrate enhanced accumulation of (unesterified) fatty acids, including arachidonic acid, indicative of increased net phospholipid degradation, in IGF +/- reperfused hearts subjected to 15 min of ischemia. The present findings, however, do not allow to conclude that the increased net phospholipid degradation is directly related to enhanced phospholipase activity in the hearts of IGF +/- mice. Nonetheless, relatively strong correlations were observed between the extent of arachidonic acid accumulation in reperfused hearts, on the one hand, and the cumulative release of LDH or recovery of cardiac output during the reperfusion phase, on the other (Figure 5).

A number of studies have provided evidence for an inhibitory effect of IGF-1 on phospholipase activity via trancriptional as well as post-transcriptional mechanisms in various cell systems (Berenbaum *et al.*, 1994, Jacques *et al.*, 1997;Pruzanski *et al.*, 1998). It is tempting to speculate that *mutatis mutandis* reduced IGF-1 levels are associated with a stimulatory effect on cardiac phospholipase activity. Collectively, these observations suggest that phospholipase A_2-mediated net degradation of membrane phospholipids might play a role in the events associated with the increased ischemia vulnerability of the IGF-1 deficient heart.

In conclusion, using an isolated working murine heart model, it was found that hearts from mice with chronically reduced IGF-1 levels are more susceptible to cardiac dysfunction following no-flow ischemia of short duration, as demonstrated by reduced recovery of cardiac output, increased irreversible cell damage, and higher tissue fatty acid levels following 15 min of ischemia and reperfusion. These findings lend additional support to the notion that IGF-1 exerts salutatory effects on the ischemic/reperfused myocardium. The present findings also support the notion that the *ex vivo* ejecting mouse heart model as characterized in *Chapter 5* and *Chapter 6* allows the detection of both subtle differences in hemodynamic function under baseline conditions and small differences in susceptibility towards global ischemia and reperfusion between hearts derived from gene-targeted or transgenic mouse models and their wildtype littermates.

References

1. Berenbaum F, Thomas G, Poiraudeau S, Bereziat G, Corvol MT, Masliah J. Insulin-like growth factors counteract the effect of interleukin-1 beta on type II phospholipase A_2 expression and arachidonic acid release by rabbit articular chondrocytes. *FEBS Lett* 1994;340:51-55.

Table III
Tissue high energy phosphate, glycogen and lactate content of ischemic-reperfused wildtype (wt) and IGF-1 (+/-) hearts.

	15 min Ischemia		*20 min Ischemia*	
	wt	*IGF-1 (+/-)*	*wt*	*IGF-1 (+/-)*
PCr	18.1 ± 5.2	15.0 ± 6.6	15.4 ± 6.9	17.2 ± 7.7
Cr	31.8 ± 7.0	29.1 ± 6.9	31.9 ± 9.7	38.6 ± 5.8
ATP	11.6 ± 2.4	8.4 ± 3.3	7.8 ± 3.7	6.6 ± 2.0
ADP	3.5 ± 0.5	3.0 ± 0.9	3.0 ± 0.5	2.7 ± 0.3
AMP	0.6 ± 0.2	0.7 ± 0.3	1.0 ± 0.5	1.2 ± 0.5
EC	0.84 ± 0.03	0.81 ± 0.04	0.77 ± 0.10	0.75 ± 0.06
(Oxy)purines	0.3 ± 0.6	0.8 ± 1.2	1.7 ± 1.8	3.5 ± 2.7
Glycogen	200 ± 111	144 ± 84	125 ± 25	167 ± 61
Lactate	5.7 ± 6.4	11.9 ± 3.8	18.3 ± 21.3	44.0 ± 12.5

Data are expressed as means ± SD in µmol.g-1 dry weight from ventricular tissue freeze clamped following 60 min of reperfusion (n = 5 for both wt and IGF-1 (+/-) hearts). EC, energy charge is defined as (ATP + 0.5 ADP)/(ATP + ADP + AMP) and is unitless; PCr, phosphocreatine; Cr, creatine; (oxy)purines are defined as the sum of adenosine, inosine, hypoxanthine and xanthine. indicates $P < 0.05$ vs group with identical genotype subjected to 15 min of ischemia.

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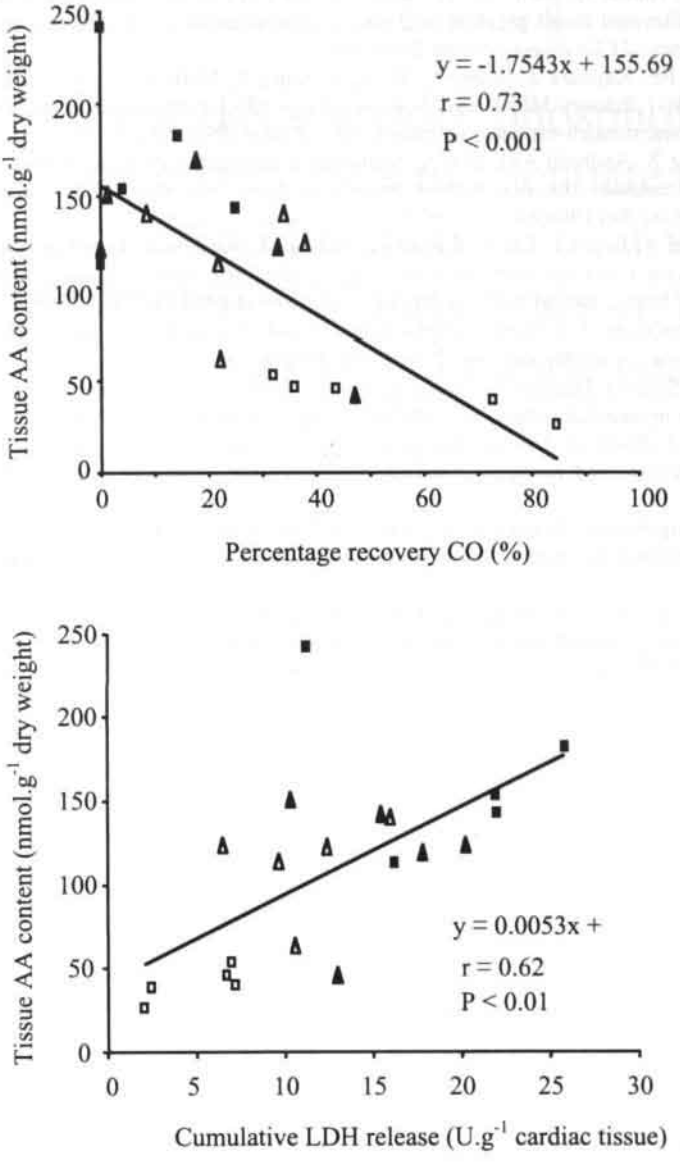

Figure 5.
Correlation between percentage recovery of cardiac output (CO) and post-ischemic tissue arachidonic acid content (upper panel) and cumulative LDH release during reperfusion and post-ischemic tissue arachidonic acid content (lower panel) in IGF-1 deficient (open and closed triangles) and wildtype hearts (open and closed rectangles) following 15 or 20 min min of ischemia and 60 min of reperfusion, respectively.

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Chapter 8

Type IIA secretory phospholipase A_2-deficiency fails to attenuate decline in mechanical function, cellular viability and accumulation of fatty acids in the ischemic-reperfused mouse heart.

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Abstract

In the present study the role of type IIA secretory phospholipase A_2 (type IIA $sPLA_2$) in cardiac ischemia/reperfusion-induced fatty acid accumulation, cellular damage and loss of function was investigated by subjecting isolated ejecting hearts derived from wildtype (C57BL/Ks) and type IIA $sPLA_2$-deficient mice (C57BL/6) to 17.5 min of global ischemia followed by 60 min of reperfusion. Pre-ischemic cardiac output (CO), aortic flow (AOF) and coronary flow (CF) normalized for heart weight was comparable between the two groups. Post-ischemic functional and metabolic recovery between type IIA $sPLA_2$ wildtype and type IIA $sPLA_2$ deficient hearts did not demonstrate significant differences (37 ± 23 and 39 ± 25 % recovery of CO, respectively; recovery of energy charge to 0.79 ± 0.04 and 0.83 ± 0.05, respectively). The post-ischemic tissue content of total unesterified fatty acids (FA) and arachidonic acid amounted to 2671 ± 1151 and 70 ± 32 $nmol.g^{-1}$ dry weight for wildtype and 2683 ± 911 and 107 ± 87 $nmol.g^{-1}$ dry weight for type IIA $sPLA_2$-deficient hearts, respectively. Cumulative lactate dehydrogenase release into the coronary effluent during reperfusion, reflecting irreversible cell damage, amounted to 10526 ± 3610 and 11306 ± 3545 $mU.g^{-1}$ wet weight for C57BL/Ks and C57BL/6 hearts, respectively. In addition, no differences were observed in either total phospholipid and triacylglycerol content or their respective fatty acyl composition between the two strains. Based on these results it is doubtful whether cardiac type IIA $sPLA_2$ plays a substantial role, if any, in acute ischemia/reperfusion-induced injury of cardiac structures.

Introduction

Type IIA secretory phospholipase A_2 (type IIA sPLA$_2$) catalyzes the hydrolysis of *sn-2* fatty acyl ester bonds of phospholipids, yielding lysophospolipids and unesterified fatty acids (FA), including arachidonic acid (AA). Type IIA sPLA$_2$ has been detected in a wide variety of cell types where it plays a role in AA-dependent cell signaling, in the physiological turnover of membrane phospholipids and in inflammatory processes such as rheumatoid arthritis and septic shock (Vadas *et al.*, 1993, Lin *et al.*, 1996, Murakami *et al.*, 1995). Likewise, type IIA sPLA$_2$ transgenic mice display more severe inflammatory responses to proinflammatory stimuli (Grass *et al.*, 1996, Nevelainen *et al.*, 1997, Fox *et al.*, 1996). Additionally, its activity has been associated with intestinal tumorigenesis (Cornier *et al.*, 1997), and brain and intestinal ischemia-reperfusion injury (Otamiri *et al.*, 1987, Lauritzen *et al.*, 1994).

In contrast to other tissues (Balsinde *et al.*, 1996, Reddy *et al.*, 1997, Murakami *et al.*, 1998), the physiological role of phospholipase A_2 (PLA$_2$) and its potential involvement in ischemia and reperfusion-induced cellular damage has not been well defined in cardiac tissue. Cardiac PLA$_2$s have been implicated in the deacylation cycle of the physiological membrane phospholipid turnover (Van der Vusse *et al.*, 1992), in mediating the rate-limiting step in the AA cascade (Van Bilsen *et al.*, 1995), and in playing a role in myocardial ischemia and reperfusion-induced phospholipid degradation (Van der Vusse *et al.*, 1982, Chien *et al.*, 1984, Otani *et al.*, 1989, Van Bilsen *et al.*, 1989b). Accelerated hydrolysis of cardiac membrane phospholipids during ischemia and reperfusion is thought to contribute to membrane destabilization and concomitant generation of biologically active lipid second messengers, which ultimately precipitate to electrophysiological dysfunction and cellular injury (Gross *et al.*, 1992, Corr *et al.*, 1995, Van der Vusse *et al.*, 1992). The assessment whether PLA$_2$ activity indeed is crucial in the transition from reversible to irreversible myocardial ischemia and reperfusion-induced injury and identification of the dominant cardiac PLA$_2$(s) responsible in this process could therefore be of therapeutic importance.

It should, however, be noted that until now at least four different types of PLA$_2$ enzymes have been detected in myocardial tissue; the high molecular mass (85-100 kDa) arachidonoyl-specific type IV cytosolic PLA$_2$ (Sharp *et al.*, 1993), the calcium-independent, plasmalogen-specific type VI PLA$_2$ (Hazen *et al.*, 1993), and the functionally closely related type IIA and type V secretory PLA$_2$s (Chen *et al.*, 1994, De Windt *et al.*, 1997). Which type of PLA$_2$ likely plays a dominant role in mediating cardiac I/R-induced membrane phospholipid-degradation remains a matter of continuing speculation (Gross *et al.*, 1992, Van Bilsen *et al.*, 1995, Van der Vusse *et al.*, 1997, De Windt *et al.*, 1998). In this light, the recent finding of a number of inbred mouse strains which display a frameshift mutation in the *pla2ga* gene, resulting in the absence of a functional type IIA sPLA$_2$ enzyme, is of interest (Kennedy *et al.*, 1995, MacPhee *et al.*, 1995).

In an attempt to establish the role of type IIA sPLA$_2$ in mediating cardiac ischemia and reperfusion-induced membrane phospholipid degradation, isolated ejecting hearts from the wildtype C57BL/Ks and the type IIA sPLA$_2$-deficient

C57BL/6 strain were subjected to 17.5 min of global, normothermic ischemia followed by 60 min of reperfusion. Loss of function was monitored by hemodynamic variables as cardiac output (CO), left ventricular developed pressure (LVDP) and the first maximal and minimal derivative of left ventricular pressure (LV dP/dt_{max} and LV dP/dt_{min}). The percentage irreversibly damaged cardiomyocytes was determined by the cumulative LDH release into the coronary effluent during the reperfusion phase. Post-ischemic high energy phosphate and glycogen content was determined to compare the metabolic state of the hearts. Post-ischemic cardiac AA content was assessed to specifically monitor potential differences in PLA_2 activity.

Materials and Methods

Chemicals

All chemicals used for the Krebs-Henseleit solution were of the highest grade available and purchased from Merck (Darmstadt, Germany) except for D(+)-glucose and pyruvate (Sigma Chemical Co., St. Louis, MO, USA). Insulin was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark).

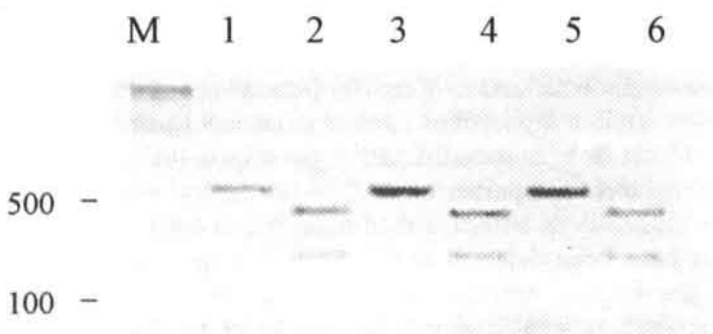

Figure 1. Genomic PCR analysis of *pla2ga* gene. Analysis by PCR amplification of exon 3 of the *pla2ga* gene of genomic DNA isolated from C57BL/Ks (lanes 2, 4, 6) and C57BL/6 mice (lanes 1, 3, 5). After amplification as described under Materials and Methods PCR product were digested by BamHI. As demonstrated, *pla2ga* exon 3 derived from C57BL/Ks mice was readily digested by BamHI resulting in two fragments of about 200 and 300 bp (lane 2, 4, 6). In contrast, T insertional mutation disrupted the *pla2ga* exon 3 BamHI site in C57BL/6 mice (lane 1, 3, 7).

Animals

Adult male inbred C57BL/Ks (wildtype *pla2ga* gene) and inbred C57BL/6 mice (mutated *pla2ga* gene) were purchased from M & B, Ry, Denmark and B & K, Hull, United Kingdom, respectively. The animals were allowed to adjust to the new housing conditions for two weeks before admission to experimental use.

Table I

Characteristics of the C57BL/Ks mice [$sPLA_2$ (+/+)] and C57BL/6 mice [$sPLA_2$ (-/-)] used in the present study.

	$sPLA_2$ (+/+)	*$sPLA_2$ (-/-)*
Age (weeks)	15 ± 2	18 ± 2 *
BW (g)	23.7 ± 2.2	30.2 ± 1.6 *
HW (mg)	204 ± 8	249 ± 17 *
HW/BW ($mg.g^{-1}$)	8.6 ± 0.3	8.3 ± 0.5

Data are expressed as means ± SD (n = 11 and 10 for $sPLA_2$ (+/+) and $sPLA_2$ (-/-), respectively). BW, body weight; HW, heart weight. * indicates $P < 0.05$ between $sPLA_2$ (+/+) and $sPLA_2$ (-/-).

The mice were kept under standard housing conditions with an artificial 12 h light cycle. Standard rodent food (Diet SRM-A, Hope farms, Woerden, the Netherlands) and tap water were freely accessible. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Maastricht University.

PCR genotyping

Genotyping of the two mouse strains to detect functional type IIA $sPLA_2$ deficiency was performed as described previously (Kennedy *et al.*, 1995). Briefly, genomic DNA was isolated from tails using a Qiamp genomic DNA prep kit (Qiagen, Leusden, The Netherlands). PCR analysis of type IIA $sPLA_2$ exon 3 was performed using the following primers: 5 -primer (5 -CTGGCTTTCCTTCCTGTCAGCCTGG CC-3); 3 -primer (5 -GGAAACCACTGGGACACTGAGGTAGTG-3). PCR reactions were cycled 35 times at 94 °C for 30 s, 60 °C for 30 s and 72°C for 30 s, with an additional amplification cycle of 10 min at 72°C using 100 ng of genomic DNA as template and 50 pmol of each primer. Since in type IIA $sPLA_2$-deficient mouse strains a thymidine insertion disrupts a BamHI site in exon 3 of the *Pla2ga* gene, PCR products were digested with BamHI prior to subjection to gel electrophoresis to confirm disruption of the BamHI restriction site and, hence, occurrence of the mutation.

Isolated working mouse heart preparation

The isolated ejecting mouse heart preparation used in the present study was previously described in detail (De Windt *et al.*, 1999). Briefly, animals were anesthetized by a 50 $mg.kg^{-1}$ sodium pentobarbital injection i.p. (Nembutal, Sanofi Sante BV., Maassluis, the Netherlands). After thoracotomy the hearts were quickly excised and transferred to ice-chilled perfusion modified Krebs-Henseleit buffer

(for composition see below). Remnant thymic and adipose tissue was carefully removed and the ascending aorta was cannulated with a recently described aortic cannula designed to match the hemodynamic aortic impedance characteristics of the isolated murine heart (De Windt *et al.*, 1999). Retrograde perfusion was started immediately at a perfusion pressure of 50 mm Hg, after which the hearts started to beat spontaneously. The left atrium was cannulated with an atrial cannula through one of the lung veins. The recirculating modified Krebs-Henseleit perfusion buffer was prefiltered by a microfilter (0.45 µm diameter; Millipore Corp.) and consisted of the following composition: 118 mM NaCl, 4.7 mM KCl, 3.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 0.5 mM Na-EDTA, 10 mM D(+)-glucose, 1.5 mM Na-pyruvate and 100 mU/l insulin. The buffer was continously gassed with 95 % O_2- 5 % CO_2. Cardiac output (CO) was defined as the sum of aortic flow (AOF) and coronary flow (CF). CF was determined from the difference between aortic flow (AOF), as measured by an 1N inline flow probe (Transonic System Inc., Ithaca, NY, USA) and left atrial filling flow as measured by an 2N inline flow probe (Transonic System Inc.) placed in the left atrial filling line. Aortic pressure was measured through a side branch located 3 mm above the entrance of the aortic cannula using a pressure transducer (Baxter Healthcare Corp.). Left ventricular pressure was measured with a PE-50 catheter, inserted into the left ventricular cavity through the apex (De Windt *et al.*, 1999), connected to a Baxter pressure transducer (Baxter Healthcare Corp., Irvine, CA). Left ventricular developed pressure (LVDP) was defined as the difference between left ventricular systolic (LVSP) and end-diastolic pressure (LVEDP).

Experimental protocol

Hearts were perfused under normoxic conditions for 20 min in the antegrade mode following an initial 10 min retrograde perfusion period to adjust to the perfusion apparatus (pre-ischemic period). Just prior to the ischemic period the water-jacketed chamber was filled with perfusate solution until the heart was completely submersed, pacing was stopped, and the aortic and atrial lines were clamped to create a normothermic (38.5 °C) global ischemia. During prelimenary experiments a limited number of wildtype C57BL/Ks hearts were subjected to either 15, 17.5 or 20 min of ischemia and their post-ischemic functional recovery and LDH release was monitored. It was found that 15 min of ischemia resulted in a near complete post-ischemic functional recovery and minimal LDH release. In contrast, 20 min of ischemia resulted in a substantial loss of function (less than 10 % recovery of CO) and loss of about 10 % of total cardiac LDH content. To this end, it was decided to subject all hearts in the present study to 17.5 min of ischemia to obtain a loss of function and LDH release in C57BL/Ks hearts which still allows measurable potential improvement or deterioration of these parameters in the absence of type IIA $sPLA_2$. After the ischemic period, the water-jacketed chamber was emptied and hearts were reperfused in a retrograde manner at

Table II
Pre-ischemic hemodynamic values measured in isolated, left ventricular ejecting murine hearts from the C57BL/Ks [$sPLA_2$(+/+)] and C57BL/6 [$sPLA_2$ (-/-)] strain.

	$sPLA_2$ (+/+)	*$sPLA_2$ (-/-)*
CO ($ml.min^{-1}.g^{-1}$)	49.0 ± 8.9	52.3 ± 11.0
AOF ($ml.min^{-1}.g^{-1}$)	37.9 ± 7.1	44.3 ± 7.8
CF ($ml.min^{-1}.g^{-1}$)	7.4 ± 1.3	8.0 ± 1.6
LVDP (mm Hg)	68 ± 9	86 ± 14 *
LVSP (mm Hg)	85 ± 9	97 ± 15 *
LVEDP (mm Hg)	7 ± 4	5 ± 3
LV dP/dt_{max} ($mm\ Hg.s^{-1}$)	6,461 ± 1,398	8,578 ± 1,734 *
LV dP/dt_{min} ($mm\ Hg.s^{-1}$)	-3,435 ± 434	-4,102 ± 545 *

Data are expressed as means ± SD (n =9 and 8 for $sPLA_2$ (+/+) and $sPLA_2$ (-/-), respectively). Isolated hearts were perfused in the antegrade mode at a pre and afterload of 10 mm Hg, respectively, and paced at 380 $beats.min^{-1}$. CO, cardiac output; CF, coronary flow; AOF, aortic flow; LVDP, left ventricular developed pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; LV dP/dt_{max}, first maximal derivative of left ventricular pressure; LV dP/dt_{min}, first minimal derivative of left ventricular pressure. * indicates $P < 0.05$ between $sPLA_2$ (+/+) and $sPLA_2$ (-/-).

a perfusion pressure of 50 mm Hg for 10 min. The left atrial conduit was re-opened and the hearts were allowed to work in the antegrade mode at a preload pressure of 10 mm Hg and a diastolic aortic pressure of 50 mm Hg for an additional 50 min. Except for the ischemic period and during the first 5 min of retrograde perfusion in the reperfusion phase, the hearts were paced throughout the whole experiment at a frequency of 380 $beats.min^{-1}$ that was slightly higher than their intrinsic denervated heart rate. Coronary perfusate was collected and samples were immediately frozen in liquid nitrogen and stored at -80 °C for further biochemical analysis. To stabilize lactate dehydrogenase (LDH) activity in coronary effluent samples, bovine serum

albumin was included (final concentration 3%). After completion of the experimental protocol the ventricles of the individual hearts were separated from the atria and immediately frozen between aluminum clamps, previously cooled in liquid nitrogen, and stored at -80¡C for further analysis.

Biochemical analysis

LDH content in the coronary perfusate were assessed spectrophotometrically using a Cobas Bio autoanalyzer as described earlier (Bergmeyer and Bernt *et al.*, 1974).

Tissue content of adenine and guanine nucleotides, IMP, nucleosides, (oxy)purines and (phospho)creatine was determined by high-performance liquid chromatography (Varian Vista 5500 HPLC) as described previously in detail (Chapter 6 and Van Bilsen *et al.*, 1989a).

The determination of cardiac fatty acids, phospholipids and triacylglycerols was performed by thin-layer chromatography followed by gas chromatography as recently described in detail (Van der Vusse and Roemen, 1995).

All values were expressed as moles per gram dry weight tissue. Dry weight was determined in a subset of hearts by freeze drying overnight and amounted to 14.6 % of wet weight, which corresponds to a conversion factor of 6.85 for wet weight to dry weight murine ventricular tissue.

Statistical analysis

The results are presented as mean values – standard deviations (SD). All statistical analyses were performed using InStat 3.0 software (GraphPad Software Inc., San Diego, Ca). Changes in functional variables were statistically analyzed by repeated measures ANOVA with Tukey s HSD post-hoc correction test for multiple comparisons. Differences between values of functional variables between experimental groups were analyzed using one-way ANOVA followed by the Tukey s test. Differences between values of biochemical parameters were tested using Student s t-test. In all tests significance was accepted at P values < 0.05.

Results

Genotyping

To confirm thymidine insertional mutation in exon 3 of the *pla2ga* gene in the inbred C57BL/6 strain and its absence in the inbred C57BL/Ks strain, exon 3 was amplified from a number of individual mice from both strains and digested with BamHI. Full length genomic PCR products from both strains were of the expected length of 500 bp. The C57BL/6 exon 3 PCR fragments could not be digested by BamHI, indicating disruption of the wildtype BamHI site, while digestion of the C57BL/Ks PCR products resulted in two cleavage signals of 300 and 200 bp, respectively (Figure 1). In the remainder of this chapter C57BL/Ks and C57BL/6 mice are referred to as $sPLA_2$ (+/+) and $sPLA_2$ (-/-) mice, respectively. Table I presents some morphometric characteristics (age, body weight, heart weight) of the mice used in the present study. It should be noted that the $sPLA_2$ (+/+) mice used in the present study had substantially lower heart and body weights as the $sPLA_2$ (-/-)

mice, which partially may be related to small differences in age and/or vendors of the inbred mice (Table I).

Functional recovery

Pre-ischemic values of the hemodynamic variables tested of both experimental groups are presented in Table II. No significant differences were found in absolute pre-ischemic CF and LVEDP values between the two groups tested. Absolute values for CO, AOF, LVSP, LV dP/dt_{max} and LV dP/dt_{min} were significantly lower for hearts from $sPLA_2$ (+/+) than for $sPLA_2$ (-/-) hearts. When corrected for heart weight, however, CO and AOF were comparable between both strains and amounted to 49.0 ± 8.9 and 37.9 ± 7.1 $ml.min^{-1}.g^{-1}$ for $sPLA_2$ (+/+) hearts and 52.3 ± 11.0 and 44.3 ± 7.8 $ml.min^{-1}.g^{-1}$ for $sPLA_2$ (-/-) hearts, respectively (N.S.). Pre-ischemic CF normalized to heart weight amounted to 7.4 ± 1.3 and 8.0 ± 1.6 $ml.min^{-1}.g^{-1}$ for $sPLA_2$ (+/+) and $sPLA_2$ (-/-) hearts, respectively ($P < 0.05$; Table II)).

Table III

Percentage recovery of hemodynamic parameters of $sPLA_2$ (+/+) and $sPLA_2$ (-/-) hearts following 17.5 min of ischemia and 60 min of reperfusion.

	$sPLA_2$ (+/+)	*$sPLA_2$ (-/-)*
CO	37 ± 23	39 ± 25
AOF	15 ± 19	21 ± 34
CF	127 ± 46	104 ± 58
LVDP	69 ± 21	57 ± 27
LVSP	76 ± 15	68 ± 25
LVEDP	253 ± 15	231 ± 105
LV dP/dt_{max}	65 ± 18	70 ± 32
LV dP/dt_{min}	67 ± 24	55 ± 29

Data are expressed as means ± SD (n =9 and 8 for $sPLA_2$ (+/+) and $sPLA_2$ (-/-), respectively). Isolated hearts were perfused in the antegrade mode at a pre and afterlaod of 10 mm Hg, respectively, and paced at 380 $beats.min^{-1}$. CO, cardiac output; CF, coronary flow; AOF, aortic flow; LVDP, left ventricular developed pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; LV dP/dt_{max}, first maximal derivative of left ventricular pressure; LV dP/dt_{min}, first minimal derivative of left ventricular pressure. * indicates $P < 0.05$ between $sPLA_2$ (+/+) and $sPLA_2$ (-/-).

Following 17.5 min of global, normothermic ischemia and 60 min of reperfusion CO recovered to 19.6 ± 16.8 and 20.4 ± 13.9 $ml.min^{-1}.g^{-1}$, representing 37 ± 23 and 39 ± 25 % of the corresponding pre-ischemic values in $sPLA_2$ (+/+) and $sPLA_2$ (-/-) hearts, respectively (N.S.). LVDP values recovered to 46 ± 13 and 47 ± 19 mm Hg at the end of the reperfusion period in wildtype and type IIA $sPLA_2$ deficient hearts, respectively (N.S.). The decrease in postischemic LVDP was caused by both a rise in LVEDP and a reduction of LVSP, which decreased to 64 ± 14 and 64 ± 25 mm Hg in $sPLA_2$ (+/+) and $sPLA_2$ (-/-) hearts following 60 min of reperfusion ($P < 0.05$ vs pre-ischemia; Table III). In both groups, post-ischemic LV dP/dt_{max} and LV dP/dt_{min} were substantially reduced in the acute reperfusion phase as compared to their pre-ischemic values and amounted to 4240 ± 1507 and —2306 ±841 mm $Hg.s^{-1}$ for $sPLA_2$ (+/+) and 4527 ± 2085 and —2748 ±1058 mm $Hg.s^{-1}$ for $sPLA_2$ (-/-) hearts, respectively (Table III).

Enzyme release

To measure the extent of irreversibly damaged cardiac muscle cells following ischemia and reperfusion, the release of LDH into the coronary effluent was continuously monitored throughout the experimental protocol. Pre-ischemic LDH release was low in both the wildtype and type IIA $sPLA_2$-deficient hearts and amounted to 162 ± 113 and 148 ± 51 $mU.min^{-1}.g^{-1}$, respectively (N.S.). Cumulative LDH release collected during 60 min of reperfusion was not significantly different between the two strains (10526 ± 3610 and 11306 ± 3545 $mU.g^{-1}$ wet heart weight for $sPLA_2$ (+/+) and $sPLA_2$ (-/-) hearts, respectively (N.S.).

Metabolic recovery

Post-ischemic values of ventricular ATP content in $sPLA_2$ (+/+) and $sPLA_2$ (-/-) amounted to 8.2 ± 2.3 and 10.3 ± 3.5 $\mu mol.g^{-1}$ dry weight tissue, respectively. ADP and AMP values were low and comparable in both strains (Table IV). The adenylate energy charge (EC) amounted to 0.79 ± 0.04 and 0.83 ± 0.05 for $sPLA_2$ (+/+) and $sPLA_2$ (-/-) hearts, respectively (N.S.). No significant differences were observed either between the two groups for total tissue glycogen content at the end of reperfusion. Tissue content of small molecular weight degradation products such as (oxy)purines and lactate were low and comparable in both groups (Table IV).

Fatty acids, phospholipids, triacylglycerols

Pre-ischemic total fatty acid (FA) levels were low and comparable in pre-ischemic $sPLA_2$ (+/+) and $sPLA_2$ (-/-) hearts (198 ± 80 and 334 ± 127 $nmol.g^{-1}$ dry weight, respectively; N.S.). Similarly, no significant differences were either found in pre-ischemic arachidonic acid levels in the pre-ischemic myocardium of both strains (Table V). Total fatty acid levels significantly increased following ischemia and reperfusion in both groups and amounted to 2671 ± 1151 and 2683 ± 911 $nmol.g^{-1}$ dry weight for $sPLA_2$ (+/+) and $sPLA_2$ (-/-) hearts, respectively (Table V). Among the most abundant saturated FA species to accumulate were palmitic (C16:0) and stearic acid (C18:0), each representing about 20 % of total accumulated FA (Table V). Of the individual poly-unsaturated FA species accumulating in the post-ischemic heart the majority was comprised of linoleic acid (C18:2) and

docosahexaenoic acid (C22:6); about 20 and 13 %, respectively in both groups (Table IV). No significant differences with respect to the arachidonic acid (AA; C20:4) levels, a sensitive marker reflecting membrane phospholipid degradadion, was found between the two mouse strains. The tissue content of AA at the end of the reperfusion period amounted to 70 ± 32 and 107 ± 87 nmol.g^{-1} dry weight for sPLA$_2$ (+/+) and sPLA$_2$ (-/-) hearts, respectively, representing 2.6 ± 0.3 and 3.6 ± 1.7 % of the total post-ischemic tissue FA pool, respectively (Table V).

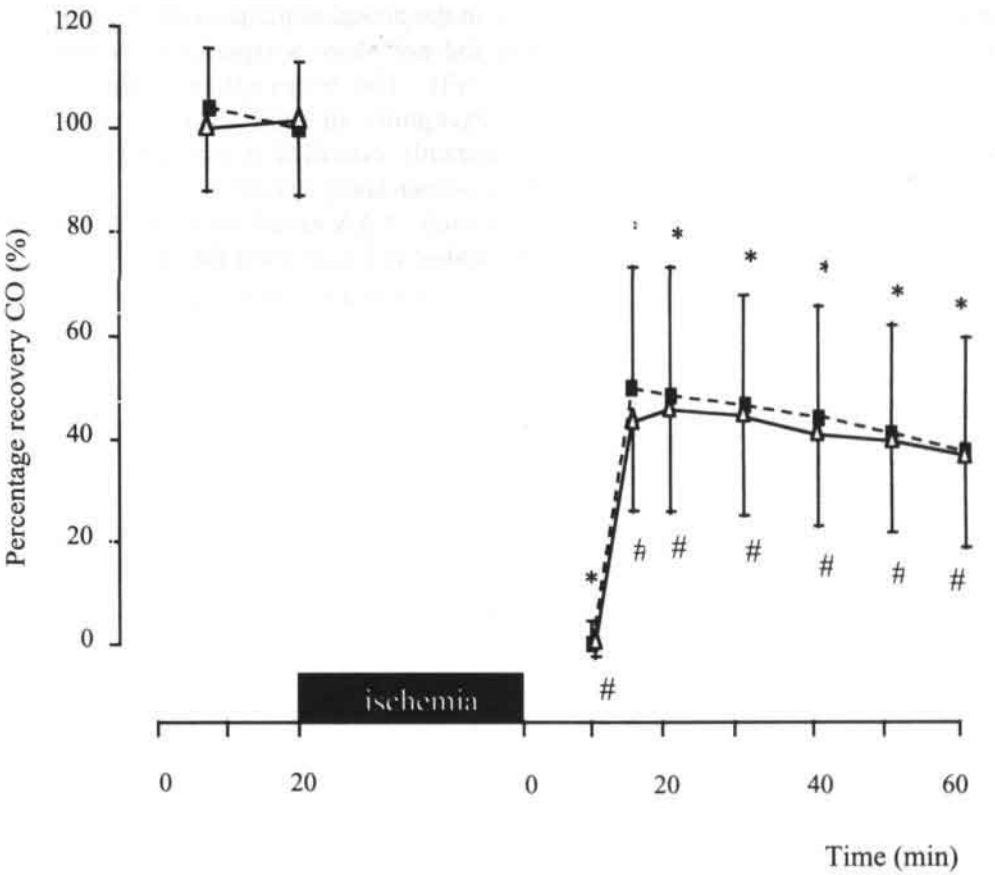

Figure 2
Post-ischemic recovery of cardiac output (CO) of isolated ejecting sPLA$_2$ (+/+) (closed rectangles) and sPLA$_2$ (-/-) hearts (open triangles) following 17.5 min of normothermic no-flow ischemia. Data are expressed as means – SD (n = 10 and 11 for sPLA$_2$ (+/+) and sPLA$_2$ (-/-) hearts, respectively). * indicates $P < 0.05$ vs respective pre-ischemic value of sPLA$_2$ (+/+) hearts, # indicates $P < 0.05$ vs respective pre-ischemic value of sPLA$_2$ (-/-) hearts.

Pre-ischemic total cardiac phospholipid (PL) and triacylglycerol (TG) levels were comparable between both strains (Table VI and VII). Total post-ischemic phospholipid and triacylglycerol content did not differ between the two groups and amounted to 170 ± 22 and 174 ± 9 μmol.g^{-1} dry weight for total PL content and 34 ± 11 and 38 ± 10 μmol.g^{-1} dry weight for total TG content in $sPLA_2$ (+/+) and $sPLA_2$ (-/-) hearts, respectively. The fatty acyl composition of both esterified lipid pools did not differ between hearts from both strains (Table VI). The total amount of fatty acids esterified at the glycerol backbone via an ether bond was low in both strains and amounted to 4.0 ± 3.2 and 4.5 ± 3.6 % of FA present in the PL pool of $sPLA_2$ (+/+) and $sPLA_2$ (-/-) hearts, respectively. Among the most abundant FA species esterified in the PL pool were palmitic, stearic, linoleic and docosahexaenoic acid, each representing about 20 % of the total amount of esterified FA. AA accounted for 6.3 ± 0.7 and 6.5 ± 0.8 % of FA present in the phospholipid pool of $sPLA_2$ (+/+) and $sPLA_2$ (-/-) hearts, respectively, and did not show a significant difference between the two mouse strains (Table VI). The triacylglycerol fatty acyl composition was different from the PL pool, in that stearic acid and docosahexaenoic acid were much less abundantly esterified in this lipid pool. In general poly-unsaturated FA species were less abundantly present in the TG pool in both mouse strains (Table VI). The total amount of AA esterified in the ischemic-reperfused TG pool was very low in both strains and accounted for only 1.6 ± 0.5 and 1.7 ± 0.3 % of total FA species in $sPLA_2$ (+/+) and $sPLA_2$ (-/-) hearts (N.S.).

Table IV

Ventricular high energy phosphate, (oxy)purine, glycogen and lactate content of reperfused $sPLA_2$ (+/+) and $sPLA_2$ (-/-) hearts.

	$sPLA_2$ (+/+)	*$sPLA_2$ (-/-)*
ATP	8.2 ± 2.3	10.3 ± 3.5
ADP	3.4 ± 0.4	3.1 ± 0.5
AMP	0.8 ± 0.3	1.0 ± 1.1
EC	0.79 ± 0.04	0.83 ± 0.05
(oxy)purines	0.08 ± 0.10	0.03 ± 0.04
glycogen	232 ± 50	259 ± 99
lactate	19.5 ± 9.3	23.6 ± 19.8

Data are expressed as means ± SD in μmol.g^{-1} dry weight from ventricular tissue freeze clamped following 17.5 min of ischemia and 60 min of reperfusion (n = 9 and n = 8 for $sPLA_2$ (+/+) and $sPLA_2$ (-/-) hearts, respectively). EC, energy charge and is defined as (ATP + 0.5 ADP)/(ATP + ADP + AMP) and is unitless; (oxy)purines are defined as the sum of adenosine, inosine, hypoxanthine and xanthine.

Discussion

In the present study the role of type IIA secretory phospholipase A_2 (type IIA sPLA$_2$) in cardiac ischemia/reperfusion-induced loss of function and phospholipid degradation was explored by using isolated, left ventricular-ejecting mouse hearts derived from the inbred C57BL/Ks and C57BL/6 mouse strains, the latter mouse strain being functionally deficient for this sPLA$_2$ (Kennedy *et al.*, 1995, MacPhee *et al.*, 1995).

Table V

Total tissue content and fatty acyl composition of the (unesterified) fatty acid pool of pre-ischemic and ischemic-reperfused sPLA$_2$ (+/+) and sPLA$_2$ (-/-) hearts.

	sPLA$_2$ (+/+)		*sPLA$_2$ (-/-)*	
	Pre	*I/R*	*Pre*	*I/R*
Total	198 ± 80	2,671 ± 1,151 *	334 ± 127	2,683 ± 911 *
16:0	35 ± 25	594 ± 240 *	59 ± 24	586 ± 206 *
16:1	-	29 ± 16	-	32 ± 15
18:0	43 ± 16	533 ± 213 *	59 ± 22	518 ± 121 *
18:1	35 ± 12	459 ± 226 *	42 ± 21	518 ± 121 *
18:2	30 ± 11	554 ± 315 *	35 ± 11	520 ± 231 *
20:0	-	11 ± 10	-	31 ± 40
20:4	3 ± 5	70 ± 32 *	9 ± 14	107 ± 87 *
22:4	-	3 ± 6	-	3 ± 5
22:6	47 ± 29	345 ± 160 *	74 ± 26	337 ± 93 *

Data are expressed as means ± SD in nmol.g^{-1} dry weight ventricular tissue (n = 5 and n = 6 for pre-ischemic sPLA$_2$ (+/+) and sPLA$_2$ (-/-) hearts, respectively; n = 9 and n = 8 for reperfused sPLA$_2$ (+/+) and sPLA$_2$ (-/-) hearts, respectively). Pre: pre-ischemic, I/R: ischemic-reperfused. Individual fatty acids are denoted by their chemical notation. —not-detectable. * indicates $P < 0.05$ vs corresponding pre-ischemic value.

Although pre-ischemic values of a number of functional parameters in C57BL/6 hearts were slightly but significantly higher as compared to those in C57BL/Ks hearts, post-ischemic percentual functional recovery was comparable between the two strains. Similarly, the amount of irreversibly damaged cardiac muscle cells, as assessed by the extent of LDH release into the coronary effluent, was comparable between the two strains. In addition, no significant differences were observed in metabolic recovery, tissue arachidonic acid (AA) content, total fatty acid (FA) accumulation, or in the fatty acyl composition of cardiac phospholipids and triacylglycerols between hearts from both strains. The data in the present study argue against a major role of type IIA $sPLA_2$ in acute ischemia and reperfusion-induced AA accumulation, cellular injury and loss of function.Aim of the present study was to determine whether type IIA $sPLA_2$ plays a significant role in cardiac membrane phospholipid degradation as reflected by AA accumulation during experimental ischemia and reperfusion at the intact organ level. Potential experimental caveats of the present study might be caused by the fact that some differences in age and pre-ischemic hemodynamic values were observed between the experimental groups, the latter difference not being easily explainable by the age factor. However, in the present study we preferred randomization of the perfusion experiments with the $sPLA_2$ (+/+) and $sPLA_2$ (-/-) mice over age-matching of the two experimental groups. Although the two mouse strains are genetically identical for 86 % (Naggert *et al.*, 1995, Lueders, 1995, Kennedy *et al.*, 1997), it can not be excluded that the remaining unidentical 14 % of the genome might account for undetermined phenotypical differences which might precipitate in differences in cardiac ischemia tolerance, if any, between the two strains. A more laborious approach to avoid this potential caveat would involve back-crossing of the mutation into the wildtype genotype or the creation of a targeted disruption of the *pla2ga* gene in a *pla2ga* genetic wildtype background (Kennedy *et al.*, 1997). Nevertheless, the availability of a type IIA $sPLA_2$-deficient experimental animal model has enabled a number of groups to demonstrate the specific contribution of this enzyme in cellular prostaglandin generation (Reddy *et al.*, 1997, Murakami *et al.*, 1997), intestinal tumorigenesis (Cormier *et al.*, 1997) and the gastric epithelial response to *Helicobacter* infection (Wang *et al.*, 1998). We used a similar experimental approach and demonstrated that complete absence of functional type IIA $sPLA_2$ failed to attenuate ischemia and reperfusion-induced AA accumulation, irreversible cell damage and functional recovery. These findings therefore present for the first time evidence against a significant role for type IIA $sPLA_2$ in cardiac membrane phospholipid degradation as observed during ischemia and reperfusion at the whole organ level.

Table VI

Total tissue content and fatty acyl composition of the tissue phospholipid pool in pre-ischemic and ischemic-reperfused sPLA$_2$ (+/+) and sPLA$_2$ (-/-) hearts.

	sPLA$_2$ (+/+)		*sPLA$_2$ (-/-)*	
	Pre	*I/R*	*Pre*	*I/R*
Total	180 ± 21	170 ± 22	171 ± 23	174 ± 9
14:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
16:0	17.5 ± 0.3	17.8 ± 0.6	17.7 ± 0.3	17.7 ± 0.7
16:0$_{PL}$	1.7 ± 0.3	1.2 ± 1.3	2.1 ± 0.3	1.3 ± 1.2
16:1	0.5 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	0.4 ± 0.1
18:0	17.7 ± 0.4	19.5 ± 2.6	17.7 ± 0.2	19.2 ± 2.4
18:0$_{PL}$	1.0 ± 0.3	0.6 ± 0.6	1.3 ± 0.2	0.7 ± 0.6
18:1	8.9 ± 0.4	9.1 ± 0.5	9.5 ± 0.4	9.6 ± 0.6
18:1$_{PL}$	0.4 ± 0.1	0.2 ± 0.2	0.5 ± 0.1	0.3 ± 0.3
18:2	17.0 ± 0.1	18.6 ± 0.6 *	16.3 ± 0.3 #	18.1 ± 0.7 *
20:4	6.4 ± 0.3	6.3 ± 0.7	6.5 ± 0.3	6.5 ± 0.8
22:6	26.4 ± 0.9	24.1 ± 0.8	25.4 ± 1.2	24.1 ± 0.9

Total amounts are given in μmol.g^{-1} dry weight ventricular tissue and fatty acyl composition is given as relative composition (in %). Data are expressed as means ± SD (n = 5 and n = 6 for pre-ischemic values for sPLA$_2$ (+/+) and sPLA$_2$ (-/-) hearts, respectively; n = 8 for both ischemic-reperfused sPLA$_2$ (+/+) and sPLA$_2$ (-/-) hearts). *PL* indicates the plasmalogen form of the particular fatty acid. Phospholipids are expressed as fatty acyl equivalents. * indicates $P < 0.05$ vs corresponding pre-ischemic value, # indicates $P < 0.05$ corresponding lipid pool of different genotype.

Type IIA $sPLA_2$ has been implicated as a candidate for cardiac ischemia/reperfusion induced membrane phopholipid degradation because of its Ca^{2+}-dependency and non-specific hydrolytic action towards phopholipids (Van Bilsen *et al.*, 1995, De Windt *et al.*, 1998). In accordance with this notion it was found that in the post-ischemic rat or mouse heart, accumulation of individual FA species is not confined to AA only, but demonstrates a more general elevation of all major FA species present in the phospholipid pool (Van Bilsen *et al.*, 1989b, Chapter 6). Moreover, earlier studies have demonstrated that antibodies raised against type IIA $sPLA_2$ and chemical inhibition resulted in a reduction of both ischemia and reperfusion-induced membrane degradation and cellular damage of the rat heart (Das *et al.*, 1986, Prasad *et al.*, 1991).

Table VII

Total tissue content and fatty acyl composition of the tissue triacylglycerol pool in pre-ischemic and ischemic-reperfused $sPLA_2$ (+/+) and $sPLA_2$ (-/-) hearts.

	$sPLA_2$ (+/+)		*$sPLA_2$ (-/-)*	
	Pre	*I/R*	*Pre*	*I/R*
Total	31 ± 7	34 ± 11	36 ± 10	38 ± 10
14:0	1.4 ± 0.4	1.2 ± 0.1	1.2 ± 0.2	1.3 ± 0.2
16:0	20.1 ± 2.1	18.2 ± 2.2	18.7 ± 0.7	18.2 ± 2.9
16:1	2.0 ± 0.7	0.8 ± 0.2 *	1.4 ± 0.3	0.9 ± 0.1 *
18:0	5.0 ± 0.6	4.0 ± 0.4 *	4.6 ± 0.1	4.0 ± 0.3 *
18:1	29.5 ± 2.1	24.3 ± 2.9	27.2 ± 1.5	25.5 ± 2.2
18:2	28.8 ± 1.8	34.5 ± 2.1 *	28.6 ± 1.7	36.2 ± 1.2 *
20:4	1.1 ± 0.3	1.6 ± 0.5	1.1 ± 0.2	1.7 ± 0.3
22:6	8.4 ± 3.0	10.9 ± 4.6	12.7 ± 1.9	8.4 ± 2.4

Total amounts are given in $\mu mol.g^{-1}$ dry weight ventricular tissue and fatty acyl composition is given as relative composition (in %). Data are expressed as means ± SD (n = 5 and n = 6 for pre-ischemic values for $sPLA_2$ (+/+) and $sPLA_2$ (-/-) hearts, respectively; n = 8 for both ischemic-reperfused $sPLA_2$ (+/+) and $sPLA_2$ (-/-) hearts). triacylglycerols are expressed as fatty acyl equivalents. * indicates $P < 0.05$ vs corresponding pre-ischemic value.

Furthermore, since reperfusion is associated with an elevation of intracellular Ca^{2+}, the involvement of this Ca^{2+}-dependent, nonspecific hydrolytic enzyme was anticipated. The present study clearly demonstrates, however, that a complete functional absence of type IIA sPLA$_2$ neither results in a reduction of membrane phospholipid degradation as measured by the tissue AA content, nor in a reduction in the degree of cellular injury or post-ischemic functional recovery.Among the other candidate cardiac PLA$_2$ enzymes responsible for ischemia/reperfusion-induced phospholipid hydrolysis are type V sPLA$_2$, type IV cPLA$_2$ and type VI iPLA$_2$, since all other major mammalian PLA$_2$ species (type IB sPLA$_2$, type IIC sPLA$_2$ and type X sPLA$_2$) remain to be detected in cardiac tissue (Tischfield *et al.*, 1997). In light of the present study, the identification, cloning and characterization of the other low molecular weight, Ca^{2+}-dependent secretory phospholipase A$_2$ (type V sPLA$_2$) abundantly present in the heart is of interest (Chen *et al.*, 1994, Chen *et al.*, 1998). Recent characterization of the recombinant human enzyme indicated a limited fatty acid preference of linoleoyl > palmitoyl > arachidonoyl acyl moieties at the *sn-2* position of PE and PC (Chen *et al.*, 1998). More importantly, it was demonstrated that both sPLA$_2$s are functionally redundant to each other in terms of AA liberation (Murakami *et al.*, 1998). Therefore, It is feasible that type IIA and type V sPLA$_2$ fulfill similar redundant functions in the heart as far as ischemia and reperfusion-induced AA or FA accumulation, lysophospholipid accumulation and concomitant cellular damage are concerned. The individual relevance and relationship between both cardiac sPLA$_2$ enzymes remains subject to future experimental study.

Interestingly, targeted disruption of the intracellularly located type IV cPLA$_2$ resulted in a significant reduction of post-ischemic brain infarct size in the mouse (Bonventre *et al.*, 1997). Because of its intracellular localization and specificity towards arachidonoyl acyl moieties in diacyl phospholipid species, a role of this particular enzyme in cardiac ischemia and reperfusion-induced AA liberation and cellular injury has as yet not been anticipated (Van der Vusse *et al.*, 1997, De Windt *et al.*, 1998). Regarding the promising results obtained in the ischemic brain (Bonventre *et al.*, 1997), however, a more thorough investigation of the role of this enzyme in the ischemic-reperfused heart seems justified and the focus of future studies.

Finally, Hazen and coworkers have demonstrated that the activity of type VI iPLA$_2$ is greatly enhanced soon after the onset of cardiac ischemia (Hazen *et al.*, 1991), making this plasmalogen-specific enzyme another candidate for ischemia and reperfusion-induced membrane anomalies in the heart. The significance of this particular PLA$_2$ in ischemia and reperfusion-associated phospholipid degradation and cardiac muscle cell death is subject of debate, however, since a number of studies demonstrated that the diacyl form rather than the plasmalogen form of phospholipids is subject to hydrolysis, while Davis and coworkers demonstrated that the levels of lysoplasmenylcholine and lysoplasmenylethanolamine did not increase in the ischemic rat heart (Davies *et al.*, 1992). Additionally, the time course of arachidonic acid accumulation during reperfusion does not coincide with that of type VI iPLA$_2$, since the activity of this enzyme quickly goes down to normal levels after ischemia, whereas AA continues to accumulate (Van der Vusse *et al.*, 1982, Van Bilsen *et al.*, 1989, De Windt *et al.*, 1999). Furthermore, Vesterqvist and

colleagues reported a marked decrease in type VI iPLA$_2$ activity in isolated rabbit hearts subjected to a prolonged period of global ischemia, using intact isolated sarcolemmal membranes as substrate to measure enzyme activity, while Hazen and colleagues used exogenously added phospholipid substrates (Hazen *et al.*, 1991, Vesterqvist *et al.*, 1996). The conflicting results might, however, be explained in the light of the different experimental models used. In this way, choline plasmalogen content in the rat heart represents < 2 % of choline phospholipids compared to 57 % in canine and 36 % in human myocardium (Davies *et al.*, 1992). Together with the findings that over 95 % of phospholipase A$_2$ activity in the human end-staged failed heart was identified as type VI iPLA$_2$ (Hazen *et al.*, 1992) and the potent arrhythmogenic properties and slow clearance of plasmalogen-derived lysophospholipids (Gross *et al.*, 1992, Caldwell *et al.*, 1998), a more throrough investigation of the role of type VI iPLA$_2$ in the pathophysiological cardiac phospholipid metabolism in different experimental models is certainly required.

Clearly, the individual role of each cardiac PLA$_2$ in phospholipid hydrolysis as observed during ischemia and reperfusion will remain subject of future investigation. In this light, loss-of-function experimental approaches are particularly efficient in delineating the role of each PLA$_2$ isozyme but does not exclude whether (PLA$_2$-mediated) membrane phospholipid degradation *per se* might play a pivotal role in the transition from reversible to irreversible injury or whether it merely reflects autolysis of irreversibly damaged cardiac muscle cells (Van der Vusse *et al.*, 1994). Gain-of-function experiments with a pre-existing increased type IIA sPLA$_2$ activity as generated previously (Fox *et al.*, 1996, Grass *et al.*, 1996, Nevelainen *et al.*, 1997) might therefore provide a more straightforward approach to study the relevance of membrane phospholipid degradation in the ischemic-reperfused heart (De Windt *et al.*, 1998).

In conlusion, the findings in the present study provide evidence against a decisive role of type IIA sPLA$_2$ in ischemia/reperfusion-induced arachidonic acid accumulation, cellular injury and cardiac metabolic and functional dysfunction. Future studies using mouse models with either a cardiac-specific overexpression of PLA$_2$ activity or a targeted disruption of other cardiac candidate PLA$_2$ enzymes might delineate whether PLA$_2$-mediated phospholipid degradation contributes to the development of ischemia/reperfusion-induced cellular death *in vivo* and/or which type of PLA$_2$ is involved in this pathophysiological process.

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Chapter 9

Prelimenary results on generating transgenic mice with a cardiac-specific overexpression of type IIA secretory phospholipase A_2.

Abstract

In the present study transgenic mice were generated to selectively overexpress type IIA $sPLA_2$ in the myocardium. To this end, the rat type IIA $sPLA_2$ cDNA was cloned downstream of the 250 bp ventricular myosin light chain-2 ($MLC\text{-}2_v$) promoter. Following multiple rounds of oocyte micro-injections one founder was obtained and germline transmission was confirmed by PCR. Additionally, a dot blot procedure was developed which allowed for determination of the copy number of transgene integration, amounted to 9 copies per haploid genome, and identification of heterozygous and homozygous transgenic animals. However, Northern blot and RT-PCR analysis of myocardial RNA demonstrated that homozygous transgenic $MLC\text{-}2_v$-$sPLA_2$ mice did not overexpress type IIA $sPLA_2$ in the heart. Subsequently, the cDNA of rat type IIA $sPLA_2$ was cloned downstream of the α-myosin heavy chain promoter (α-MHC). Following one round of micro-injections, multiple founder animals were identified by using a specific PCR analysis of the genome. The existence of potential α-MHC-$sPLA_2$ founder candidates was confirmed by Southern blot analysis. Interestingly, among these candidates the founder animal with the highest number of transgene copies as determined by Southern blot analysis became ill soon after birth and died a few weeks later. This finding coincidences with the activation of the α-MHC promoter and might indicate that very high levels of type IIA $sPLA_2$ in the heart are unreunitable with normal cardiac function in the mouse.

Introduction

The current technological ability to genetically alter the mouse in combination with miniaturized physiological *in vivo* and *ex vivo* experimental setups offers the opportunity to study the (patho)physiological role of single genes (Chien *et al.*, 1996). To more specifically target a given gene to the myocardium in a gain-in-function approach, independent laboratories have performed efforts to use promoter constructs to specifically and exclusively drive transgene expression in the heart *in vivo* (Figure 1). The majority of these promoter constructs were from genes encoding cardiac proteins expressed abundantly and exclusively in the myocardium. In this way promoter constructs for α-myosin heavy chain (α-MyHC), ventricular myosin light chain-2 (MLC-2_v) and cardiac troponin I have been used to drive transgene expression to the ventricular compartment of the myocardium *in vivo* (Subramaniam *et al.*, 1991, Lee *et al.*, 1992, De Lisi *et al.*, 1998), while promoters of the atrial natriuretic peptide (ANF) and atrial myosin light chain-2 (MLC-2_A) drive heterologous expression to the atria (Field *et al.*, 1988, Doevendans, 1998).

A number of reports provided circumstantial evidence that phospholipase A_2 (PLA_2) mediated membrane phospholipid degradation might play a pivotal role in the transition from reversible to irreversible cellular injury during cardiac ischemia and reperfusion (Van der Vusse *et al.*, 1982, Chien *et al.*, 1984, Das *et al.*, 1986, Otani *et al.*, 1989, Van Bilsen *et al.*, 1989, Van der Vusse *et al.*, 1989, Prasad *et al.*, 1991,Van der Vusse *et al.*, 1997). To study the significance of ischemia/reperfusion-mediated cardiac membrane phospholipid degradation in the transition of reversible to irreversible cellular injury and, consequently, the extent of functional recovery, experiments were initiated to generate mice with a cardiac-specific overexpression of type IIA phospholipase A_2 *in vivo*. Subjecting the hearts from these transgenic animals to ischemia/reperfusion in a recently developed isolated ejecting mouse heart preparation (De Windt *et al.*, 1999) will help to understand the role of this particular type of PLA_2 in the development of irreversible cell damage and loss of cardiac function during ischemia and reperfusion. To this end, the recently cloned and characterized cDNA coding for type IIA PLA_2 present in the rat heart (De Windt *et al.*, 1997) was cloned downstream of a 250 bp fragment of the rat MLC-2_v promoter and downstream of the mouse α-MyHC promoter to specifically target type IIA $sPLA_2$ to the cardiac ventricles.

Materials and Methods

Animals

The MLC-2_v-$sPLA_2$ transgenic mouse colony was maintained and bred under standard housing conditions with an artificial 12 h light cycle and free access to tap water and standard rodent food (Diet SRM-A, Hope farms, Woerden, the Netherlands). In all experiments involving surgical procedures, animals were

anesthetized with a 50 $mg.kg^{-1}$ sodium pentobarbital injection i.p. (Nembutal, Sanofi Sante BV, Maassluis, the Netherlands) prior to surgery. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Maastricht University.

PCR genotyping

To detect the presence of the transgene in individual MLC-2_v-$sPLA_2$ and α-MHC-$sPLA_2$ mice the genomic DNA was isolated from tail clips using a Qiamp genomic DNA prep kit (Qiagen, Leusden, the Netherlands) and subjected to PCR genotyping. In both PCR analyses a 3 -primer designated PLA-R3 [5 -GAATCATTTGCCCAAACTC-3], directed against bp 73 — 91 of the cDNA coding for rat heart type IIA $sPLA_2$ (De Windt *et al.*, 1997), was used. For the MLC-2_v-$sPLA_2$ transgenic line a 5 -primer designated MLC-F1 [5 -TCTGCCTACCTACAACTGCC-3] specific for the rat MLC-2_v promoter was used, while in the case of α-MyHC-$sPLA_2$ transgenic mice a 5 -primer designated MHC-F1 [5 -GGAAACCACTGGGACAC-3], specific for the mouse α-MHC promoter was used. PCR reactions were cycled 35 times at 94 °C for 30 s, at 60 °C for 60 s and at 72°C for 90 s, with an additional amplification cycle of 10 min at 72°C using 100 ng of genomic DNA, 50 pmol of each primer and 1.5 U Taq polymerase (Gibco BRL). In both MLC-2_v-$sPLA_2$ as well as α-MHC-$sPLA_2$ PCR genotyping a specific PCR product of about 300 bp is anticipated if the transgene is present in the genome.

In a subset of FVB/N mice (MLC-2_v-$sPLA_2$ mouse strain) the genomic DNA was used as template in a PCR reaction for genotype the mice in order to detect the genomic type IIA $sPLA_2$ mutation as described previously (Chapter 8). The genomic DNA of all the mice tested this way were found to consistent with the wildtype genome, suggesting that the type IIA $sPLA_2$ mutation is absent in the genetic background of the transgenic MLC-2_v-$sPLA_2$ mice.

Dot blot determination of copy number

Genomic dot blot analysis was used to determine the copy number of the transgene in the MLC-2_v-$sPLA_2$ transgenic line using a modification of a method described earlier (Hogan *et al.*, 1986, Subramaniam *et al.*, 1991). In short, genomic DNA was isolated from tail clips using the Qiamp genomic DNA isolation kit according to the manufacturer s directions (Qiagen) and quantitated spectrophotometrically in triplo. Subsequently, 5 µg of genomic DNA from each individual mouse was digested overnight with excess EcoRI (5 U/µg). The digested genomic DNA was resuspended in denaturating buffer (10 mM Tris, 0.2 M NaOH, 1 mM EDTA, 100 mM NaCl), heated to 70 °C for 5 min and spotted in dots in duplo onto nylon filter (Amersham) in serial 2-fold dilutions using denaturing buffer starting with 1 µg down to 62.5 ng. As a standard a set of blots was generated using Hind III linearized pMLCSVOA-$sPLA_2$ (see Figure 2) in serial 2-fold dilutions from 100 pg down to 6.25 pg. After UV crosslinking and baking for 30 min at 80°C, the blots were prehybridized for 2 h in 6 × SSC, 5 × Denhardt s, 0.5 % sodium dodecyl sulfate and 100 µg/ml salmon sperm DNA at 58 °C. Subsequently, the blots were

probed overnight with 1.10^6 cpm.ml^{-1} α-^{32}P-labeled PLA probe (De Windt *et al.*, 1997) in the same solution. The probe used was a Xho I/Pst I fragment of part of the cDNA coding for rat heart type II secretory PLA$_2$ in a pBluescript vector (De Windt *et al.*, 1997). Following two washes with 2 × SSC, 0.1 % SDS, the blots were exposed to storage phosphor screens for 24 h and scanned and quantified using a PhosphorImager and the ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

Northern blot analysis

Total RNA from MLC-2$_v$-sPLA$_2$ transgenic hearts (both ventricles and atria), from the hearts of their corresponding wildtype littermates and from rat ileum was isolated with the use of TRIzol Reagent (Gibco BRL). The RNA (10 µg and 5 µg for cardiac tissue and ileum, respectively) was size-fractionated on a 1% agarose, 18% formaldehyde gel according to the method described in Sambrook *et al.* (1989). The RNA was transferred to a nylon filter (Hybond-N, Amersham) by capillary transfer overnight. After UV crosslinking and baking at 80 °C for 2 h, the filters were prehybridized for 2 h in 6 × SSC, 5 × Denhardt s solution, 0.5% SDS, 100 µg/ml salmon sperm DNA at 58 °C and hybridized in the same solution with 10^6 cpm/ml of ^{32}P-labeled PLA- probe (Xho I/Pst I fragment of rat heart type IIA sPLA$_2$ cDNA). Final washing conditions were 1 × SSC with 0.1% SDS at 50 °C for 30 min. To correct for possible differences in loading and transfer, the immobilized RNA on the filters was visualized by methylene blue staining prior to hybridization. The Northern blots were exposed to storage phosphor screens for 90 h and subsequently scanned and quantified using a PhosphorImager and the ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

RT-PCR

Total RNA from MLC-2$_v$-sPLA$_2$ mouse hearts and their corresponding nontransgenic littermates was prepared with the use of TRIzol Reagent (Gibco BRL). Reverse transcription (RT) was performed with Superscript™-II RNAse H$^-$ reverse transcriptase (Gibco BRL) according to the instructions of the supplier. Polymerase chain reaction (PCR) was performed on a DNA thermal cycler (Perkin Elmer Co., Norwark, USA) using Thermus Aquaticus (Taq) polymerase (Gibco BRL). After an initial incubation of 5 min at 95 °C, the reactions were cycled 15, 20 or 25 times at 94 °C, at 55 °C, and at 72 °C, all for 1 min. An additional extension reaction at 72 °C for 10 min was included after cycle 25. Samples were size-fractionated by agarose gel electrophoresis and visualized after staining with ethidium bromide. RT-PCR was performed using a 5 primer designated PLA-F1 [5'-GAGTTTGGGCAAATGATTC-3'] and a 3 primer designated PLA-R1 [5'-CAGCTTTATCGCACTGGCA-3']. PLA-F1 and PLA-R1 correspond to bp 73-91 and bp 328-346 of the sequence of type IIA sPLA$_2$ present in the heart (De Windt *et al.*, 1997).

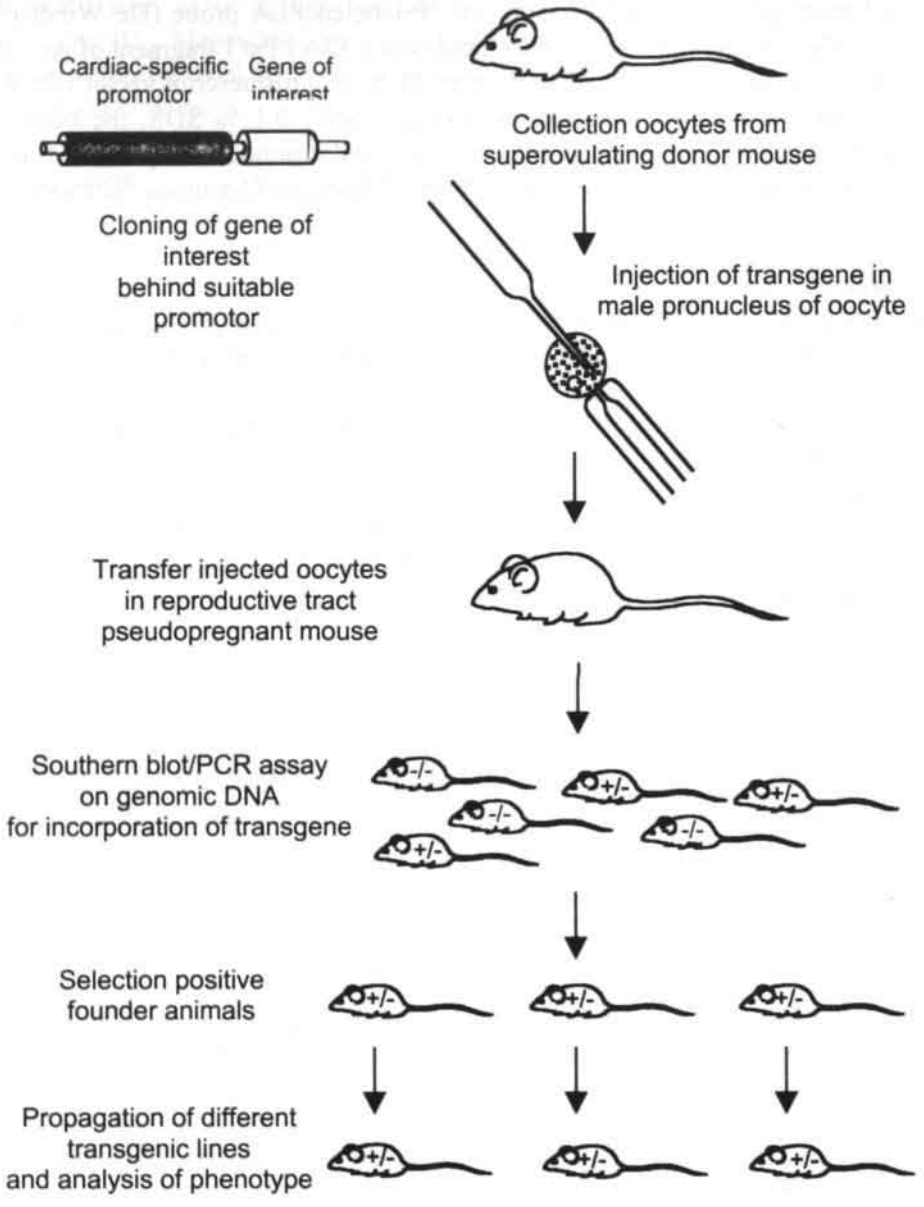

Figure 1. Schematic overview of the generation of transgenic mice. The gene of interest is placed under control of a promoter sequence (e.g. a cardiac-specific promoter such as the α-MyHC promoter) to target the expression of the gene to a specific organ, in this case the heart. The transgene is micro-injected into the male pronucleus of uncleaved fertilized oocytes. The oocytes are subsequently transferred into the reproductive tract of a pseudopregnant foster mouse, where the oocytes come to term. Genomic DNA is tested by Southern blot or PCR procedures to determine whether the transgene has been integrated into the mouse genome. In the different transgenic lines different numbers of transgenes at different integration sites are usually incorporated. After confirmation of overexpression of the transgene at the RNA and protein level and documentation of the copy number of the transgene integration, the phenotype of the transgenic mouse lines is investigated.
(Figure reprinted with permission from De Windt *et al.*, 1998).

Results

Cloning of MLC-2$_v$-sPLA$_2$ construct

To obtain a MLC-2$_v$-sPLA$_2$ expression vector to drive type IIA sPLA$_2$ specifically to the heart of mice, a number of subcloning strategies were applied to clone full length cardiac type IIA sPLA$_2$ cDNA downstream of the 250 bp fragment of the rat MLC-2$_v$ gene promoter. To this end, type IIA sPLA$_2$ was excised from a parental pBluescript vector (De Windt *et al.*, 1997) via an EcoR I/Not I double digest and inserted into a EcoR I/Not I linearized PCRII vector. Resulting PCRII-sPLA$_2$ was digested with Spe I to release a ~ 900 bp fragment containing the full length type IIA sPLA$_2$ cDNA clone (Figure 2). Finally, this Spe I fragment was ligated into the multiple cloning site of pMLCSVOA, a vector containing the 250 bp trunctated rat MLC-2$_v$ promoter upstream and a luciferase poly adenylation (poly A) signal downstream of the multiple cloning site, respectively. Resulting transgenic vector (pMLCSVOA-sPLA$_2$) was subjected to extensive restriction digest and sequencing analysis to confirm correct orientation and integrity of the open reading frame of sPLA$_2$.The transgenic vector was digested with Hind III and Kpn I, which cut immediately 5 of the promoter and 3 of the polyadenylation site, respectively (Figure 2). In this manner, no contaminating plasmid vector sequences were included into the microinjected DNA. Subsequent DNA purification and transgene animal generation was performed as described previously using standard techniques at the Department of Medicine, University of California - San Diego, CA USA (Hogan *et al.*, 1986, Lee *et al.*, 1992). Following multiple rounds of micro-injections only one male founder was obtained and subsequently shipped to the Maastricht University, The Netherlands, where all further breeding and analysis of the transgenic line occurred.

Propagation and morphometric analysis of MLC-2$_v$-sPLA$_2$ mice

To confirm integration of the transgenic construct, a genomic DNA PCR strategy was developed involving a 5 primer specific for the MLC-2$_v$ promoter and a 3 primer specifically directed to the type IIA sPLA$_2$ sequence. In Figure 3 a typical PCR result of a litter from the original male founder with a wildtype female FVB/N mouse is presented. As demonstrated, a highly specific detection of the transgene was obtained through this method of screening. In this way, the transgenic line was propagated until the third generation (F3). Two breeding pairs, both containing one transgenic and one wildtype parent of the F1 generation, were set up to yield 39 pups in 9 litters, of which 19 were PCR positive. Subsequently, a breeding strategy was followed involving two (heterozygous) transgenic parents of the F2 generation to obtain pups with double copies of the transgene (homozygous transgenic) in a Mendelian fashion. Morphometric analysis, copy number determination, Northern blot analysis and RT-PCR reactions were performed primarily on the litters derived from latter homozygous transgenic breeding pair (F3 generation).

Table I depicts the morphometric findings of homozygous transgenic MLC-2$_v$-sPLA$_2$ transgenic mice compared to their non-transgenic mice. No obvious differences in health, appearance and body weight were detected between transgenic and non-transgenic mice. Additionally, gross histological inspection of the heart

following removal from the thorax demonstrated no obvious abnormalities in cardiac morphology of transgenic animals, nor significant differences in heart weight compared to wildtype hearts.

Dot blot determination of copy number

To determine the absolute amount of integrated transgene copies into the haploid genome of the MLC-2_v-$sPLA_2$ transgenic line, a standard curve of serial diluted plasmid pMLCSVOA-PLA_2 containing the transgene was constructed following hybridization with a 300 bp probe directed against the coding region of type IIA $sPLA_2$ present in the heart (Xho I/Pst I fragment, see Materials and Methods). Taking into account that 100 pg of a 4,000 bp plasmid consists of 7.3×10^6 molecules (first dot) and 6.25 pg contains 4.6×10^5 molecules of the plasmid (last dot), a standard curve was fitted by plotting the amount of transgene copies against the signals as quantitated by the PhosphorImager analysis (Figure 4). In Figure 4A the triplo serial diluted pMLCSVOA-PLA_2 is depicted by numbers 1, 2 and 3. The genomic DNA derived from two PCR positive MLC-2_v-$sPLA_2$ animals (one homozygous and one heterozygous transgenic mouse) and a wildtype littermate are also shown. Figure 4B depicts the standard curve obtained by plotting the absolute amount of arbitrary PhosphoImager units against the absolute amount of plasmid molecules following hybridization with ^{32}P-labeled PLA probe. Table II depicts all arbitrary PhosphoImager units calculated from the dot blot analysis as demonstrated in Figure 4A. Using the equation that follows from the standard curve in Figure 4B, the absolute amount of $sPLA_2$ copies in the serial diluted genomic DNA are given. By dividing the absolute transgene copies by the absolute amount of haploid genome copies spotted in each dot, an estimation of the transgene copy number is obtained (Table II). Following latter analysis it was found that the wildtype nontransgenic mouse haploid genome contains only one copy of type IIA $sPLA_2$ (endogenous type IIA $sPLA_2$ gene), while heterozygous transgenic MLC-2_v-$sPLA_2$ animals contain 9 copies and homozygous transgenic animals about the double number of heterozygous transgenic animals, i.e. about 20 copies per haploid genome (Figure 4A, Table II).

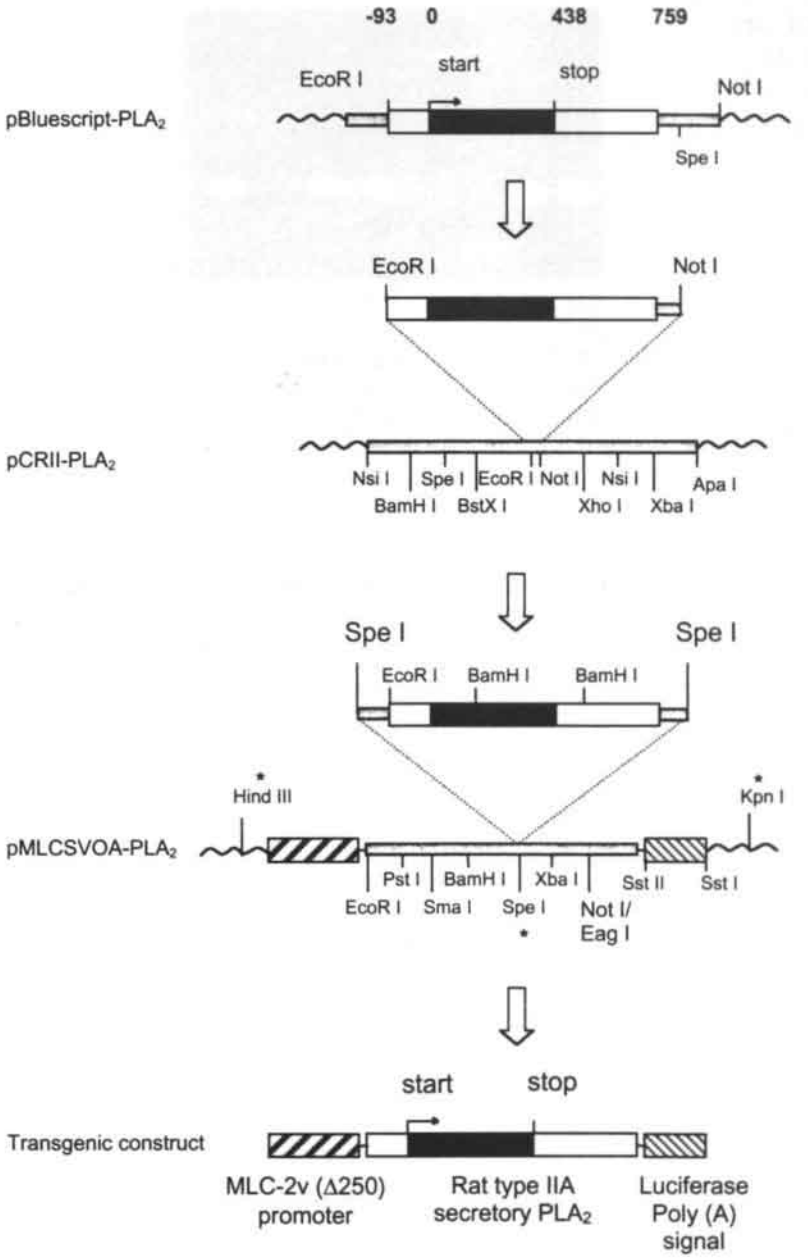

Figure 2. Cloning strategies to obtain a MLC-2v-sPLA$_2$ transgene vector. To obtain a MLC-2v-sPLA$_2$ expression vector to drive type IIA sPLA$_2$ specifically to the heart of mice, a 852 bp fragment containing the 5' and 3' UTF sequences and open reading frame of rat heart type IIA sPLA$_2$ was excised from a parental pBluescript vector and subcloned into a linearized pCRII vector. To this end, sPLA$_2$ was excised from a parental pBluescript vector (De Windt *et al.*, 1997) via an EcoRI/NotI double digest and ligated into an EcoRI/NotI linearized pCRII vector. Resulting pCRII-sPLA$_2$ was digested with SpeI to release a ~900 bp fragment containing the open reading frame of sPLA$_2$. Finally, this SpeI fragment was ligated into the multiple cloning site of pMLCSVOA, a vector containing the 250 bp truncated rat MLC-2v promoter upstream and the luciferase polyadenylation signal downstream of the multiple cloning site, respectively. A HindIII/KpnI digest removed the vector sequences from the transgenic construct for injection into oocytes. * indicates unique restriction site in vector.

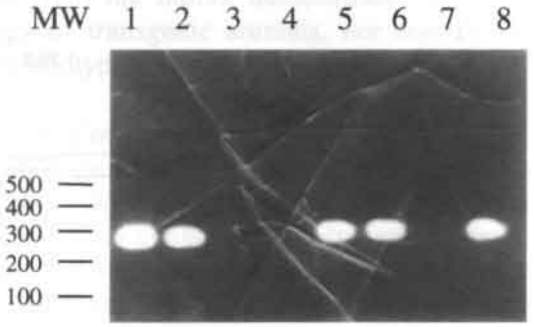

Figure 3. Example of MLC-2_v-$sPLA_2$ PCR genotyping. Lane 1 represents positive control as template (pMLCSVOA-$sPLA_2$ plasmid). Lanes 2-8 represent genotyping of 7 individual mice from a litter of the original founder mouse with a wildtype mate. The molecular weights are indicated on the left in bp.

Northern blot analysis of cardiac type IIA $sPLA_2$ levels

Following confirmation of germline dependent transmission and copy number assessment of the transgene it was investigated by RNA blot analysis whether the MLC-2_v-$sPLA_2$ transgenic line demonstrated increased cardiac mRNA levels for type IIA $sPLA_2$. Figure 5 demonstrates a respresentative result of this analysis, demonstrating high levels of type IIA $sPLA_2$ mRNA in rat ileum (positive control tissue). A very weak signal for $sPLA_2$ mRNA was obtained in hearts derived from wildtype nontransgenic littermates (lane 1, 4 and 6; Figure 5). However, no difference was found in type IIA $sPLA_2$ mRNA signals in hearts derived from transgenic MLC-2_v-$sPLA_2$ animals (lane 2, 3 and 5; Figure 5). This result demonstrated that the MLC-2_v-$sPLA_2$ transgenic line was not overexpressing type IIA $sPLA_2$, despite the presence of multiple copies of the transgene.

Table I

Morphometric parameters of homozygous transgenic MLC-2_v-$sPLA_2$ mice and wildtype mice.

	Wildtype	*MLC-2_v-$sPLA_2$*
BW (g)	30.6 ± 4.6	29.9 ± 4.1
HW (mg)	207 ± 22	217 ± 12
HW/BW ($mg.g^{-1}$)	6.87 ± 1.16	7.32 ± 0.66
Age (weeks)	28 ± 5	28 ± 5
N	6	6

Data are expressed as means ± SD. BW, body weight; HW, heart weight; HW/BW, heart weight-to-body weight ratio.

RT-PCR

To further investigate this issue, an RT-PCR analysis of $sPLA_2$ transcripts was performed on total RNA isolated from wildtype and transgenic hearts. To ascertain linearity of the PCR reaction, PCR products were analyzed following 15, 20, and 25 cycles and subjected to agarose gel electrophoresis. It was found that 20 RT-PCR cycles were sufficient to detect a weak signal for $sPLA_2$ mRNA and still remain in the linear phase of PCR product generation (data not shown). No notable upregulation of mRNA transcripts were observed between wildtype and MLC-2_v-$sPLA_2$ hearts (data not shown). The latter result confirmed the observations obtained by using Northern blotting procedures that the MLC-2_v-$sPLA_2$ transgenic line did not overexpress type IIA $sPLA_2$ mRNA transcripts in the heart and, hence, was not suitable for studies to assess the role of PLA_2-mediated phospholipid degradation during cardiac ischemia and reperfusion

Cloning of α-MHC-$sPLA_2$ construct

Following the negative result of the MLC-2_v-$sPLA_2$ transgenic line, experiments were undertaken to obtain several transgenic lines of mice with differing amounts of $sPLA_2$ overexpressed and driven to the heart by a 5.5 kb α-MHC promoter construct. To this end, the cDNA coding for rat heart type IIA $sPLA_2$ was excised from the PCRII-$sPLA_2$ vector (Figure 6) by an EcoR I/Xba I double digest and cloned into EcoR I/Xba I linearized pBluescript vector. This subcloning stategy allowed excision of the full lenght rat heart type IIA $sPLA_2$ cDNA as an Xho I fragment, which allowed for cloning into clone 26 (generous gift from Prof. J Robbins, Children s Hospital Medical Center, Cincinnati OH, USA to Dr. PA Doevendans, Department of Cardiology, University Hospital Maastricht), a pBluescript based vector containing the mouse 5.5 kb α-MHC promoter upstream and the 0.6 kb human growth hormone poly adenylation site (HgH poly A) downstream of the multiple cloning site. Cloning of the Xho I $sPLA_2$ fragment into the Sal I site of clone 26 finally resulted in the vector pBluescript-α-MHC-$sPLA_2$. Resulting transgenic vector (pBluescript-α-MHC-$sPLA_2$) was subjected to extensive restriction digest and sequencing analysis to confirm correct orientation and integrity of the open reading frame of $sPLA_2$.

In a collaboration the transgenic vector was sent to Dr. Solito of the Institute Cochin de Genetique Moleculaire INSERM U-322, Paris, France, where the ~ 7.0 kb fragment containing the α-MHC promoter, $sPLA_2$ and the HgH poly A signal were released by Not I restriction digest. Subsequently, the transgenic construct was purified and injected into fertilized oocytes as described earlier (Hogan *et al.*, 1986).

Following one round of injections, three litters with a total of 20 pups were obtained. Screening of transgenic pups by PCR analysis as described in the Materials and Methods section demonstrated the presence of 5 PCR positive pups. The presence of the transgene was further confirmed by Southern blot analysis of genomic DNA using a probe to detect the HgH poly (A) signal of the transgenic vector (data not shown). The PCR-positive founder with the highest signal at the Southern blot level soon became ill between 1-2 weeks after birth and died several days later. Gross examination of the major organs revealed no apparent

abnormalities or deviations in organ wet weight and, more importantly, the heart did not show any morphological abnormalities or signs of hypertrophy (Personal communications E Solito).
Following overnight fixation in 10 % formalin, the heart of the animal was shipped to the Netherlands where total RNA was isolated in an attempt to establish whether transgenic type IIA $sPLA_2$ expression could be detected via Northern blot analysis. Unfortunately, the quality of the RNA was of such poor quality, probably because the animal was deceased for some time after detection of the carcass, that the cardiac RNA had already been degraded upon fixation of the tissues.

Discussion

In the present study two approaches were undertaken to overexpress type IIA $sPLA_2$ levels selectively in the murine myocardium *in vivo*. The first approach involved the generation of transgenic mice where the cDNA of type IIA $sPLA_2$ was placed under control of the 250 bp MLC-2_v promoter. Early studies indicated that this 250 bp fragment was sufficient to drive exogenous gene products specifically to both ventricles in the mouse (Lee *et al.*, 1992, Lee *et al.*, 1994, Hunter *et al* 1995). Following multiple rounds of oocyte injections, however, only one founder with 9 copies of the transgene incorporated into the haploid genome was obtained which, as indicated by Northern blot and RT-PCR analysis of myocardial RNA, did not show overexpression of the transgene. Hereafter experiments were initiated to overexpress type IIA $sPLA_2$ in a cardiac-restricted fashion under control of the α-MyHC promoter. By using this approach, multiple α-MHC-$sPLA_2$ transgenic founder mice were identified and await further characterization. Of interest, one founder with the highest transgene copy number died of unknown cause soon after birth, which might implicate that driving high type IIA $sPLA_2$ transgene levels in the heart is unfavorable for correct cardiac function in the adult mouse.

Although the promoter and cis-elements responsible for driving the cardiac-restricted expression patterns of MLC-2_v gene have been identified and characterized (Henderson *et al.*, 1989, Lee *et al.*, 1992, Lee *et al.*, 1994, Navankasattusas *et al.*, 1994, Hunter *et al.*, 1995, Ross *et al.*, 1996) only few reports have documented successful cardiac-restricted overexpression *in vivo* using the MLC-2_v promoter and involved the oncogene *H-ras* in the murine heart (Hunter *et al.*, 1995), two reporter genes, luciferase and β-galactosidase, in the mouse heart (Lee *et al.*, 1992, Lee *et al.*, 1994, Ross *et al.*, 1996) and the plasma membrane, calmodulin-dependent Ca^{2+} ATPase (PMCA) in the rat heart (Hammes *et al.*, 1998). One specific advantage of the MLC-2_v promoter is based in the ventricular specificity of the MLC-2_v gene in the animal *in vivo* with no detectable expression in the atria (Lee *et al.*, 1992, Lee *et al.*, 1994, Ross *et al.*, 1996). However, major drawbacks of the promoter are related to the developmental expression pattern of the MLC-2_v gene which itself is one of the earliest markers of cardiac morphogenesis (O Brien *et al.*, 1993).

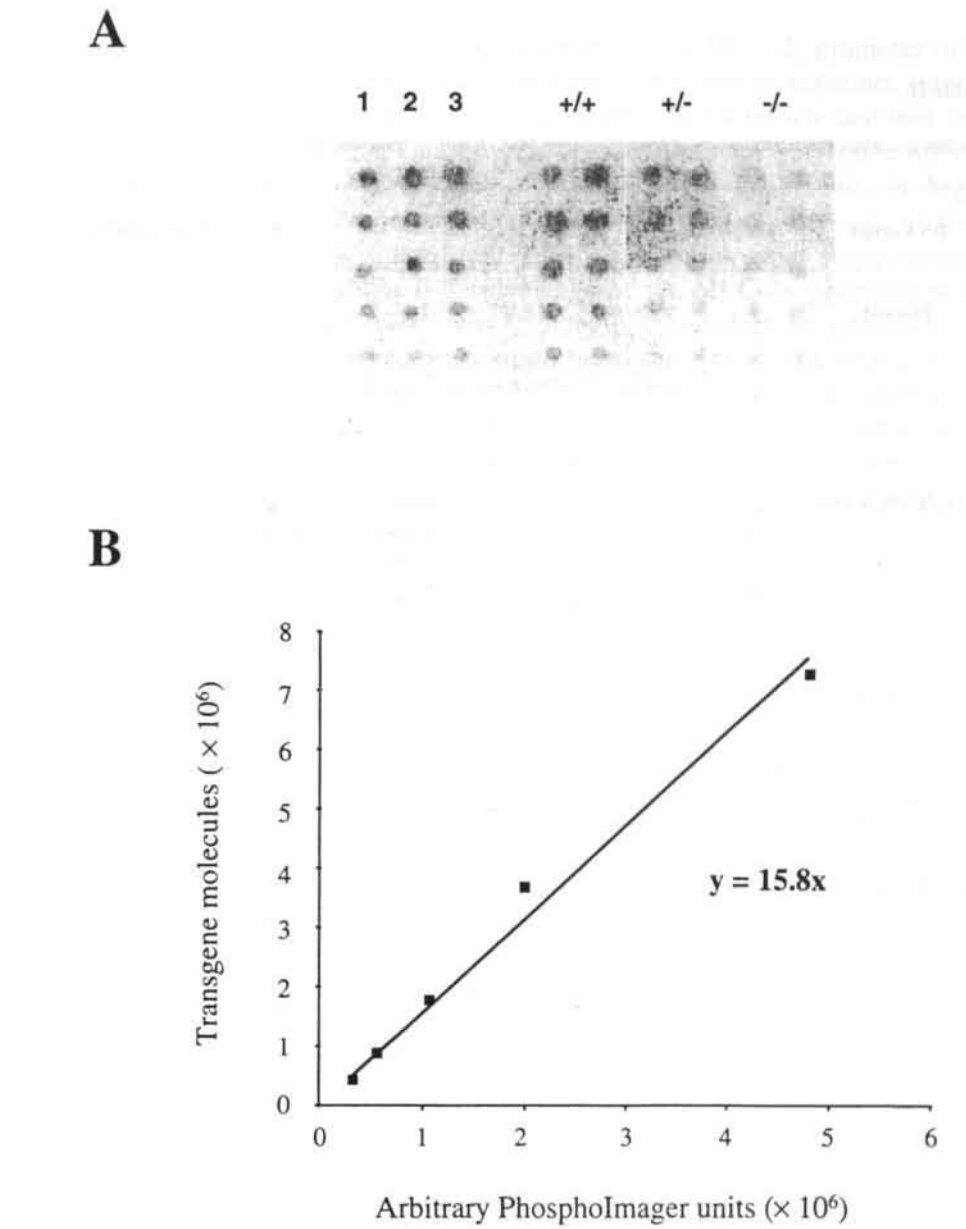

Figure 4. Demonstration of copy number analysis in MLC-2$_v$-sPLA$_2$ transgenic mice. ***A***, Genomic DNA isolated from wildtype (-/-), heterozygous (+/-) and homozygous (+/+) transgenic animals was two-fold serial diluted and spotted in duplicate next to two-fold serial dilutions of the plasmid containing the transgene (pMLCSVOA-sPLA$_2$; Figure 2). As a radiolabeled probe a 275 bp fragment of the coding region of rat heart type IIA sPLA$_2$ was used (De Windt *et al.*, 1997). ***B***, Taking into account that 100 pg of a 4,000 bp plasmid consists of 7.3×10^6 molecules (first dot) and 6.25 pg contains 4.6×10^6 molecules of the plasmid (last dot), a standard curve was fitted by plotting the number of transgene copies against the signals as quantitated by the PhosphorImager analysis using the arbitrary PhosphoImager units obtained with the serial diluted plasmid and the absolute number of plasmid molecules.

Table II

Dot blot procedure for determination of copy number in MLC-2,-sPLA$_2$ transgenic mice.

DNA origin	Dot #	A	B	C	D	Copy number	E
Plasmid	1	478880	$7.3.10^6$	-	-	-	
	2	198025	$3.7.10^6$	-	-	-	
	3	105167	$1.8.10^6$	-	-	-	
	4	55329	$0.9.10^6$	-	-	-	
	5	31830	$0.45.10^6$	-	-	-	
MLC-2,-sPLA$_2$ (+/+)	1	454915	-	7187657	340000	21	
	2	264867	-	4184899	170000	25	
	3	121522	-	1920048	85000	23	
	4	43707	-	690571	42500	16	
	5	23538	-	371900	21300	17	20
MLC-2,-sPLA$_2$ (+/-)	1	161740	-	2555492	340000	8	
	2	80618	-	1273764	170000	7	
	3	49564	-	783111	85000	9	
	4	28798	-	455008	42500	11	
	5	12944	-	204515	21300	10	9
MLC-2,-sPLA$_2$ (-/-)	1	19702	-	311292	340000	1	
	2	9752	-	154082	170000	1	
	3	4936	-	77989	85000	1	
	4	2279	-	36008	42500	1	
	5	-	-	-	21300	-	1

Depicted are the mean arbitrary PhospholImager units (column A) following hybridization with a sPLA$_2$-specific probe. Data are expressed as means from triplicate (plasmid) or duplicate (MLC-2,-sPLA$_2$) experiments. DNA was spotted in serial 2-fold dilutions starting with 100 pg or 1 µg (dot 1) and ending with 6.25 pg or 62.5 ng (dot 5) for plasmid and genomic DNA respectively. Corresponding number of plasmid molecules (B) or number of haploid genomes (D) are indicated. In C the unit obtained in A are multiplied using the correction factor from the standard curve of Figure 4, i.e. 15.8. Following division of C by D the copy number of transgene integration into the haploid genome is obtained. Column (E) depicts the mean of the data presented in column (Copy number) and represents the mean copy number per haploid genome.

As a consequence, the truncated 250 bp fragment of the MLC-2$_v$ promoter drives not only high transgene expression levels prenatally, but also in a distinct anterior-posterior gradient, leading to robust expression in the right ventricle and less or no expression in the left ventricle.This distinct feature, although only once reported using a MLC-2$_v$-β-galactosidase fusion gene (Ross *et al.*, 1996), is highly undesirable for projects where left ventricular ischemic vulnerability is to be tested in the adult animal using an isolated left ventricular ejecting heart preparation (De Windt *et al.*, 1999). An additional disadvantage of the MLC-2v promoter is that relatively large numbers of transgenic founder lines need to be generated and screened before ventricular overexpression, in the absence of ectopic expression, is truly achieved (personal communication KR Chien). Regarding latter difficulties, the MLC-2$_v$ promoter is clearly a less apropiate device to generate animal models with a cardiac-specific gain-of-function. It should be noted that at the time of generating the MLC-2v-sPLA$_2$ transgenic mice in the present study, this information (Ross *et al.*, 1996) had not been published about this particular promoter construct.

Although less restricted in its expression pattern than the MLC-2$_v$ promoter, the α-MHC promoter has been used successfully to drive overexpression of a large number of transgenes to the myocardium. The α– and β-MHC genes are a group of muscle-specific genes which are expressed in both the cardiac and skeletal muscle compartments. In the mouse, β-MHC expression is first detected 8 days post-coitum in the primitive heart tube and as the heart undergoes looping and forms distinct atrial and ventricular chambers, the expression of β-MHC becomes restricted to the ventricular region, whereas α-MHC is restricted to the atrial region. Subsequently, β-MHC expression is down-regulated in the ventricular compartment and within 1 week after birth α-MHC transcripts accounts for 95 % of the MHC transcripts in the heart (Lyons *et al.*, 1990). Similar to the spatio-temporal expression pattern of the endogenous α-MHC gene in rodents, transgenes under control of the 5.5 kb mouse α-MHC promoter fragment are expressed atrial-specifically at very low levels during embryonic development. About one week after birth a copy number dependent overexpression pattern in both ventricles, both atria and the pulmonary myocardium is routinely observed (Subramanian *et al.*, 1991). The α-MHC promoter generally results in a large numbers of founder animals per injection, a relatively large number of which are likely to demonstrate germline-dependent overexpression of the transgene (about 35 % of founders). Although the exact reason for the high successrate of the α-MHC promoter remains to be established, the size of the transgene (generally over 7 kb), which normally integrates into a chromosomal locus as a complex of concatemers, and the large number of cardiac muscle specific enhancers present within the promoter (Molkentin *et al.*, 1996) probably contribute to the observed efficiency of this promoter. In line with this notion, it is of interest to note that recent efforts to reconstruct the original MLC-2$_v$ promoter by increasing the size of the construct (up to 5 kb) have resulted in a high succesrate in obtaining transgenic founders expressing reporter enzymes at very

high levels in a ventricular-specific fashion without the presence of an anterior-posterior gradient (personal communications J Robbins).

The observation that the one founder with the highest copy number died soon after activation of the α-MHC promoter might indicate that high type IIA $sPLA_2$ expression levels specifically in the heart is incompatible with life, resulting in selection of transgenic animals with relatively lower levels of the enzyme. This might also be an alternative explanation for the low successrate of generating MLC-2v-$sPLA_2$ founders. However, the above considerations are in contrast with the observation that type IIA $sPLA_2$ transgenic mice have been successfully generated with overexpression in the heart, albeit not in a cardiac-specific overexpression pattern (Grass *et al.*, 1996, Nevelainen *et al.*, 1997, Fox *et al.*, 1996).

In summary, it was attempted to create an animal model with a cardiac-specific overexpression of type IIA $sPLA_2$ by generating mice with rat type IIA $sPLA_2$ under control of either of the MLC-2_v or the α-MHC promoter. Following multiple rounds of micro-injections, only one transgenic founder line of MLC-2_v-$sPLA_2$ was obtained and propagated. Although germline transmission was observed, no overexpression of type IIA $sPLA_2$ could be detected in the heart. Subsequently, type IIA $sPLA_2$ was placed under control of the α-MHC promoter and following one round of oocyte injections multiple transgenic mice were identified. The availability of an animal model with a gain-of-function for cardiac PLA_2 activity, in combination with an isolated perfused heart model to perform cardiac ischemia/reperfusion studies (De Windt *et al*, 1999), will provide more insight into the question whether membrane phospholipid degradation is a critical step during the transition from reversible to irreversible damage.

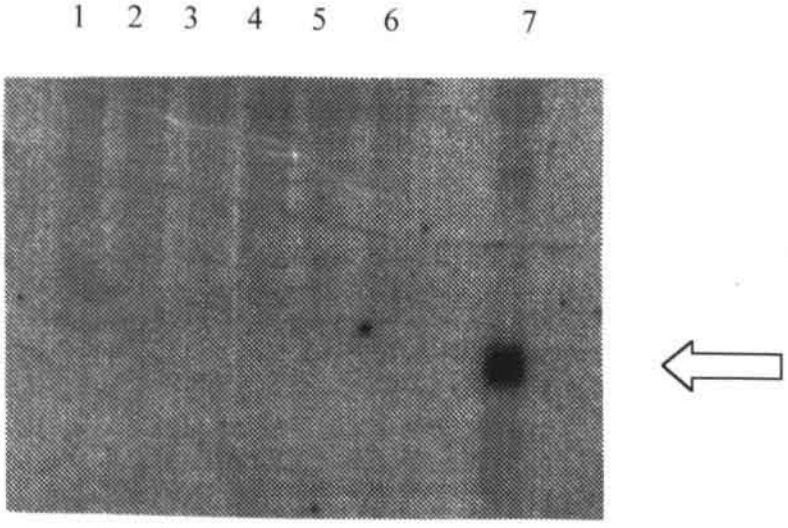

Figure 5. Northern blot analysis of total RNA isolated from homozygous transgenic MLC-2_v-$sPLA_2$ hearts (lane 2, 3, 5) and wildtype non-transgenic littermates (lanes 1, 4, 6) using a P^{32}-labeled type IIA $sPLA_2$ probe (De Windt *et al.*, 1997). As a positive control and reference for size total RNA derived from rat ileum was included in the analysis (Lane 7). Arrow indicates size of type IIA $sPLA_2$ mRNA. To check for equality in loading, the RNA was visualized with methylene blue (data not shown).

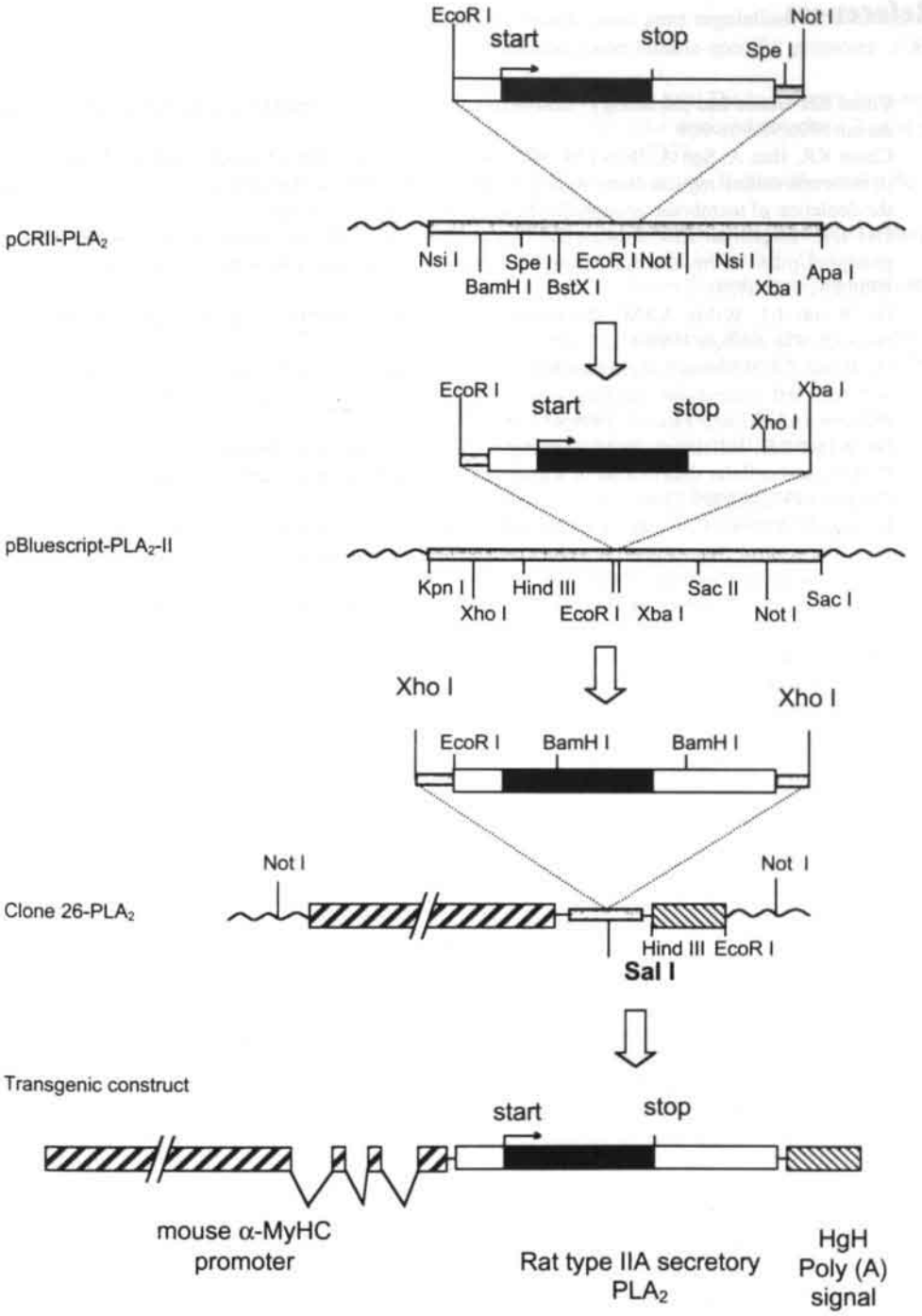

Figure 6. Cloning strategies followed to place the full length cDNA coding for rat heart type IIA sPLA$_2$ under control of the 5.5 kb murine α-MHC promoter. The full length cDNA was excised from the pCRII-sPLA$_2$ vector (see Figure 2) by an EcoRI/XbaI double digest and subcloned into an EcoRI/XbaI linearized pBluescript vector. This subcloning strategy allowed excision of the full length rat heart type IIA sPLA$_2$ as an Xho I fragment, which allowed for ligation into the Sal I site of clone 26, a pBluescript based vector containing the murine 5.5 kb α-MHC pomoter upstream and the 0.6 kb human growth hormone polyadenylation signal downstream of the multiple cloning site. Cloning of the XhoI sPLA$_2$ fragment into the SalI site of clone 26 resulted in the vector pBluescript-α-MHC-sPLA$_2$. A NotI digest susbsequently removed the vector sequences from the transgenic construct for injection into fertilized murine oocytes.

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Chapter 10

General Discussion

The central hypothesis of this study stated that type IIA secretory phospholipase A_2 activity plays a role in ischemia and reperfusion-induced membrane phospholipid degradation, eventually leading to cardiac muscle cell injury and deterioration of cardiac function. To address this issue the presence and abundance of type IIA secretory phospholipase A_2 in the heart was established. Secondly, an *ex vivo* perfused, left ventricular ejecting mouse heart model was developed to specifically study ischemia/reperfusion related phenomena in this organ, puting placing emphasis on cardiac phospholipid metabolism. Thirdly, phospholipid homeostasis and cardiac ischemia tolerance were investigated in a mutant inbred mouse strain that lack type IIA secretory phospholipase A_2 to explore whether absence of this phospholipase isoform would attenuate ischemia and reperfusion-induced cardiac phospholipid degradation, cellular injury and functional deterioration. In addition, attempts were made to create transgenic mice overexpressing type IIA phospholipase A_2 in the heart to study the significance of increased phospholipase A_2 activity on cardiac ischemia tolerance in this animal species.

Ischemia/reperfusion-induced damage to the isolated mouse heart

Functional aspects

To study ischemia/reperfusion-induced alterations in hemodynamic function, cardiac energy metabolism and lipid homeostasis in the isolated mouse heart, the assisted mode perfusion set-up, previously developed for the isolated rat heart, was downscaled to the level of the mouse heart. It was demonstrated in *Chapter 5* that it is feasible to obtain hemodynamic values of the isolated working mouse heart that resemble findings of the anesthetized mouse heart *in vivo* (Lembo *et al.*, 1996, Lorenz *et al.*, 1997, Milano *et al.*, 1994). The major improvement of the present model is that the impedance characteristics of the artificial outflow tract is matched with the *in vivo* outflow tract of the mouse heart so that physiologically relevent measurements are obtained. In this experimental model the hemodynamic function of the mouse heart is quite stable, as evidenced by minor changes in cardiac output over an extended period of time. The possibility to construct Frank-Starling curves (at different extracellular Ca^{2+} concentrations) makes this model suitable to properly evaluate cardiac function in transgenic or gene targeted models with deteriorated heart function or heart failure.

Isolated Swiss mouse hearts were found to be highly sensitive to an ischemic insult with a narrow time interval between almost complete hemodynamic recovery and severe impairment of functional recovery (*Chapter 6*). An ischemic period of 10 min resulted in hardly any impairment of function and only a limited degree of cellular injury following reperfusion. These findings indicate that this mouse heart preparation allows distinction between small differences in ischemia/reperfusion insults as far as functional and cell injury are concernes. This notion is substantiated by the finding that the myocardium of the IGF-1 heterozygous knockout mouse is more sensitive to ischemia and reperfusion-induced damage than the myocardium of wildtype mice following 15 min of global ischemia. When the ischemic period was extended by 5 min to 20 min, however, the

function of IGF-1 heterozygous knockout hearts were deteriorated to the same extent as those of wildtype littermates. Also, subtle differences in cardiac ischemia tolerance were observed between hearts derived from different mouse strains. Wildtype C57BL/6 hearts tolerated 15 min of ischemia substantially better (*Chapter 8*) than Swiss mouse hearts (*Chapter 6*). It is doubtful whether such subtle differences in ischemia tolerance would have been detected in the less physiological Langendorff perfused heart model, indicating the advantage of the present ejecting heart model. Collectively, these findings are in line with the notion of a relative susceptibility of the mouse heart to ischemia/reperfusion damage as compared to hearts derived from other species such as the rat (Headrick *et al.*, 1998, De Groot *et al.*, 1993).

As indicated by the observations in *Chapter 8*, the model is also sufficiently sensitive to detect subtle differences in murine baseline systolic and diastolic cardiac function (i.e. before the ischemic insult) as a result of lowered levels of IGF-1 in a gene targeted mouse model and in different mouse strains. Hearts derived from Swiss mice (*Chapter 5 and 6*), C57BL/6 mice (*Chapter 8*) and 129/Sv mice (*Chapter 7*), mouse strains that are extensively used in cardiovascular studies, demonstrated some differences in baseline systolic function and contractility. More specifically, isolated C57BL/6 hearts displayed higher baseline values for systolic left ventricular pressure and contractility (*Table II, Chapter 8*) than hearts derived from Swiss (*Table I, Chapter 5*) and 129/Sv mice (*Table II, Chapter 7*). Using an isolated working mouse heart preparation, Hewett and colleagues demonstrated markedly elevated contractility in BALB/c and DBA mice, as compared to such strains as Swiss Webster, C57BL/6 and CD-1 (Hewett *et al.*, 1994), resulting from higher levels of α-skeletal actin in the BALB/c heart due to a duplication event in the promoter of the gene (Garner *et al.*, 1986, Alonso *et al.*, 1990). It can be concluded that the isolated ejecting mouse heart preparation may be of value in future studies to detect differences in cardiac hemodynamic function of various knockout and transgenic models.

Lipid changes in the ischemic-reperfused mouse heart

Disruption of the sarcolemma has been proposed as a critical event of ischemia/reperfusion-induced cellular injury (Jennings *et al.*, 1986). The initial weakening of the plasma membrane may be related to several mechanisms. Impaired anchoring of the plasma membrane to the cytoskeleton and increased osmotic forces have been postulated to play a role in ischemia/reperfusion-induced weakening of the sarcolemma (Steenbergen *et al.*, 1987). In addition, the Ca^{2+}-induced loss of assymmetric bilayer behavior of phospholipid species, such as phosphatidylserine and phosphatidylethanolamine, leading to phase segregation and subsequent destabilization of the sarcolemma (Post *et al.*, 1998, Musters *et al.*, 1993, Post *et al.*, 1995), and enhanced hydrolysis of membrane phospholipids due to activation of cardiac phospholipases A_2 (Van der Vusse *et al.*, 1982, Chien *et al.*, 1984, Prinzen *et al.*, 1984, Das *et al.*, 1986, Van Bilsen *et al.*, 1989, Prasad *et al.*, 1991) have also been proposed as alternative mechanisms. Irrespective of the mechanism, subsequent disruption of the sarcolemma, most probably caused by physical forces imposed on the cardiac myocyte (Jennings *et al.*, 1986), leads to

disruption of the weakened sarcolemma and release of cytoplasmic constituents, representing a hallmark of irreversible cell injury.

Exposure of Swiss mouse hearts to ischemia either or not followed by reperfusion did not lead to appreciable changes in the tissue content of phospholipids or triacylglycerols. However, an initial small but significant decrease in cardiac unesterified fatty acid content and shifts in the composition of the cardiac fatty acid pool relative to the pre-ischemic fatty acid pool were observed (*Chapter 6*). When the ischemic period was extended (i.e., 20 min) the tissue fatty acid content started to rise. This increase in fatty acid content was accompanied by a significant increase in the amount of such (poly)-unsaturated fatty acids as arachidonic acid. Reperfusion was associated with a substantial rise in the cardiac fatty acid content, the absolute amount depending on the duration of the ischemic period. The relatively high contribution of poly-unsaturated fatty acid species, such as arachidonic acid and docosahexaenoic acid, to the (unesterified) fatty acid pool indicates a disbalance between the deacylation and reacylation rates of the cardiac membrane phospholipids during reperfusion. These findings are in line with earlier observations in the ischemic or reperfused canine heart (Van der Vusse *et al.*, 1982, Prinzen *et al.*, 1984, Chien *et al.*, 1984) and rat heart (Van Bilsen *et al.*, 1989) and support the notion that the underlying mechanisms of disturbances of cardiac membrane phospholipid homeostasis are similar in the mouse heart.

A linear correlation was found between post-ischemic tissue fatty acid accumulation on the one hand and the extent of irreversible cell injury, as assessed by the cumulative release of cytoplasmic enzymes or post-ischemic recovery of mechanical behavior as determined by the percentage recovery of cardiac output, on the other (*Figure 8 in Chapter 6 and Figure 5 in Chapter 7*). These findings suggest a close relation between membrane phospholipid degradation and loss of cellular integrity and, hence, mechanical recovery of the ischemic-reperfused myocardium. It should be noted that the elevation in post-ischemic fatty acid content, although substantial, still represents only about 2.5 % of the total murine cardiac phospholipid content. At first glance this may be a discrepancy between the number of cells irreversibly damaged (about 10 % following 20 min of global ischemia) and the amount of (unesterified) fatty acids accumulated. However, if the hydrolysis of membrane phospholipids is confined to a limited number of cells or only to phospholipids of the sarcolemma, which represent a small percentage of total phospholipids, cellular integrity might well be compromised by the observed extent of phospholipid degradation (Van der Vusse *et al.*, 1989). It should be noted that the increased tissue arachidonic acid levels could also be a reflection of reduced reacylation activity of the post-ischemic heart (Van Bilsen *et al.*, 1989). Collectively, these findings lend support to the notion that derangements in cardiac membrane phospholipid homeostasis, possibly mediated by the activation of cardiac phospholipases A_2, is a causal factor in the transition from reversible to irreversible cellular damage following ischemia and reperfusion.

Since accumulation of polyunsaturated fatty acids in cardiac tissue reflects an imbalance between membrane phospholipid hydrolysis and resynthesis, it has been hypothesized that activation of phospholipase A_2 (relative to the activity of enzymes involved in phospholipid resynthesis) plays a major role in ischemia and

reperfusion-induced net phospholipid degradation. Circumstantial evidence for the involvement of cardiac phopholipases A_2 in the loss of cellular integrity in the ischemic-reperfused myocardium is provided by the present findings in the more ischemia vulnerable, heterozygous IGF-1 knockout mouse. It was demonstrated that total post-ischemic cardiac arachidonic acid content correlated well with recovery of cardiac output and cellular viability in this mouse model, suggesting a relationship between ischemia tolerance and phospholipid homeostasis (*Figure 5, Chapter 7*). At present at least three different classes of phospholipase A_2 have been identified in cardiac tissue: (I) the small molecular mass type IIA and V secretory phospholipases A_2; (II) the high molecular mass, type IV cytosolic phospholipase A_2; and (III) the plasmalogen-specific type VI phospholipase A_2 (Chen *et al.*, 1994, De Windt *et al.*, 1997, Larsson *et al.*, 1998, Liu *et al.*, 1998, Ma *et al.*, 1999, Murakami *et al.*, 1998, Pickard *et al.*, 1999, Underwood *et al.*, 1998).

Type IIA secretory phospholipase A_2

Type IIA secretory phospholipase A_2 was chosen as a likely candidate for cardiac ischemia and reperfusion-induced phospholipid degradation based on a number of earlier studies and its enzymatic characteristics. First, in the present study in mice it was demonstrated (*Chapter 6*) that like in the ischemic-reperfused rat heart (Van Bilsen *et al.*, 1989b) not only arachidonic acid but also other (un)saturated fatty acid species accumulate during ischemia. This argues in favor of the aspecific type IIA secretory phospholipase A_2. Secondly, the observation that fatty acids continue to rise in the reperfusion phase points toward a role for type IIA secretory phospholipase A_2 in the process, since reperfusion is known to be accompanied by a substantial influx of calcium, potentially leading to the activation of the Ca^{2+}-dependent type IIA secretory phospholipase A_2. Thirdly, pretreatment of isolated hearts with antibodies raised against a snake venom type II secretory phospholipase A_2 effectively blocked the degradation of membrane phospholipids and mitigated the release of cytoplasmic proteins in the acute reperfusion phase (Prasad *et al.*, 1991). Anti-type IIA secretory phospholipase A_2 antibodies were also found to decrease phospholipid degradation in homogenates of rat hearts that had been previously subjected to a period of hypoxia and reoxygenation (Kikuchi-Yanoshita *et al.*, 1993). Fourthly, peroxidation of membrane phospholipids due to enhanced oxygen free radical production occurring during reperfusion (Myers *et al.*, 1985), may also play a role, because peroxidation-damaged phospholipids are more vulnerable to type IIA secretory phospholipase A_2 attack (Dan *et al.*, 1990). Finally, increased expression levels of type IIA secretory phospholipase A_2 have been found in rat brain after severe forebrain ischemia (Laurtizen *et al.*, 1994) and in the rat small intestinal mucosa after ischemia and revascularization (Otamiri *et al.*, 1987). On the basis of its Ca^{2+}-sensitivity and the profile of fatty acids accumulating in ischemic/reperfused myocardium, it was proposed that type IIA secretory phospholipase A_2 is involved in phospholipid hydrolysis in the flow-deprived and reperfused heart.

In this thesis the presence of type IIA secretory phospholipase A_2 in the myocardium was confirmed by cloning of its cDNA from a rat heart cDNA library and detection of its mRNA in cardiac tissue as well as isolated myocytes. The full length cDNA predicts a mature protein of 146 amino acids residues, including a typical N-terminal stretch of 21 amino acid sequence representing a signal peptide for secretion. As such it resembles mammalian type IIA secretory phospholipases A_2 present in other tissues. Analysis of the tissue distribution of type IIA secretory phospholipase A_2 mRNA (*Chapter 3*) confirmed its ubiquitous presence (Ishizaki *et al.*, 1989, Kennedy *et al.*, 1995). The abundance of the transcript demonstrates large differences between the tissues examined, with the highest levels in rat ileum and relatively low levels in the myocardium, spleen and soleus muscle. Using primary cultures of rat neonatal ventricular cardiomyocytes it was further demonstrated that type IIA secretory phospholipase A_2 was stimulated at the level of gene transcription by pathophysiologically relevant stimuli such as TNF-α and phenylephrine. In this respect, it is of interest to note that in the infarcted human myocardium high levels of TNF-α have been reported (Maury *et al.*, 1989), while α_1-adrenergic stimulation of cardiomyocytes is associated with hypertrophic growth (Sadoshima and Izumo, 1997), suggesting a role for type IIA secretory phospholipase A_2 both in myocardial infarction and in the processes leading to cardiac hypertrophy and failure.

In the present study attempts were made to assess the protein levels of type IIA secretory phospholipase A_2 in various different tissues via Western blotting techniques. The enzyme was found to be below the detection level in cardiac tissue via regular immunoblotting techniques, in contrast to rat ileum and rat and human platelets in which the protein could be readily detected (*Chapter 4*). The presence of a N-terminal signal peptide for secretion in the enzyme raises the possibility that type IIA secretory phospholipase A_2 exerts its (patho)physiological functions both intra- and extracellularly. Recent findings that components of the extracellular matrix bind to and influence type IIA secretory phospholipase A_2 activity are of interest and support the notion of an extracellular localization of this particular enzyme (Murakami *et al.*, 1996, Romano *et al.*, 1998). Whether type IIA secretory phospholipase A_2 is also secreted by cardiac myocytes and/or is localized on the extracellular side of the sarcolemma and what the physiological consequences might be of this observation remain to be determined.

Ischemia and reperfusion-induced cardiac phospholipid degradation: involvement of type IIA secretory phospholipase A_2

To address the issue whether (increased) phospholipase A_2 activity in general is involved in the transition from reversible to irreversible cardiac cell injury and loss of cardiac function, attempts were made to create an animal model with a gain-of-function of type IIA secretory phospholipase A_2 in the heart. Unfortunately, one transgenic line, in which type IIA secretory phospholipase was placed under control of the ventricular myosin light chain promoter, did not demonstrate transgene expression in the heart. In a follow-up approach to the latter attempt, a number of

transgenic founders was produced by placing type IIA secretory phospholipase A_2 under control of the α-myosin heavy chain promoter. Interestingly, one founder animal with a relatively high copy number died at young age, possibly indicating that high level cardiac overexpression of type IIA secretory phospholipase A_2 might be lethal in the mouse. Future characterization of the remaining founders may result in transgenic lines with different levels of type IIA secretory phospholipase A_2 overexpression.

To more specifically investigate the role of type IIA secretory phospholipase A_2 in cardiac ischemia/reperfusion-induced damage, the ischemia tolerance of hearts from two closely related inbred mouse strains was investigated. No significant differences in post-ischemic tissue arachidonic acid or total fatty acid accumulation, ischemia/reperfusion-induced cellular injury or loss of cardiac function was observed between two 86 % genetically identical inbred mouse strains, except for the fact that in one strain no functional type IIA secretory phospholipase A_2 was present (Kennedy *et al.*, 1997). These findings argue against a significant role for cardiac type IIA secretory phospholipase A_2 in sarcolemmal phospholipid degradation as observed during ischemia and reperfusion in the isolated heart. It should be noted that these findings do not exclude a role for e.g. circulating type IIA secretory phospholipase A_2 in sarcolemmal phospholipid degradation in the infarcted heart *in vivo*.

Future directions

It is becoming increasingly clear that ischemia and reperfusion-associated irreversible cell death is a multifactorial process including free radical generation, intracellular Ca^{2+} overload and alterations in the sarcolemmal composition. To more specifically define whether phospolipase A_2 mediated hydrolysis of (sarcolemmal) membrane phospholipids plays a crucial role in the sequence of events leading to ischemic cardiac cell death, a definition of the individual roles and interplay of the growing family of cardiac phospholipase A_2 is needed. Knowledge about the significance of ischemia and reperfusion associated phospholipase A_2-mediated cardiac cell damage could be of therapeutic significance, and might lead to application of specific pharmacological inhibition of ischemia and reperfusion-induced cellular injury. Regarding the finding that cellular type IIA secretory phospholipase A_2 probably does not play a dominant, or rather a redundant, role in cardiac ischemia/reperfusion-induced cell death (*Chapter 7*), reconsideration of the pathophysiological roles of the other phospholipase A_2 candidates is required.

A major future obstacle in defining the individual roles of cardiac phospholipases A_2 is posed by the identification of novel members and isoforms of the (cardiac) phospholipase A_2 family in recent years. Currently several splice isoforms of type VI calcium-independent, plasmalogen-specific phospholipase A_2 have been detected in cardiac muscle, human pancreatic islets and Chinese hamster ovary cells (Larsson *et al.*, 1998, Liu *et al.*, 1998, Ma *et al.*, 1999). Calcium-independent phospholipases A_2 have been reasoned to play dominant pathophysiological roles in cardiac muscle where contractions cause large

fluctuations in calium concentrations, and, hence, the need exists to regulate phospholipase A_2 activity via calcium-independent processes. Additionally, the family of calcium-dependent, arachidonoyl-specific type IV cytosolic phospholipase A_2 consists of at least three distinct members, one of which, type IV cPLA$_2$-γ, was demonstrated to be abundantly present in heart and muscle in a membrane-bound state (Underwood *et al.*, 1998, Pickard *et al.*, 1999). Finally, the identification of the novel small molecular mass, Ca^{2+}-dependent type V secretory phospholipase A_2 with limited specificity towards fatty acids esterified at the *sn-2* position of membrane phospholipids and functions redundant to type IIA secretory phospholipase A_2 remains an attractive candidate for ischemia/reperfusion-associated membrane phospholipid hydrolysis, especially as this type of phospholipase A_2 is abundantly present in the heart (Chen *et al.*, 1994, Murakami *et al.*, 1998). In this light, the recent findings of Murakami and colleagues demonstrating cross-talk between type IV cytosolic phospholipase A_2, on the one hand, and type IIA and type V secretory phospholipase A_2, on the other, using co-transfection assays of several phospholipase A_2 families, are of interest. It was observed that (*I*) type IV cytosolic phospholipase A_2 was required for proper action of both type V and type IIA phospholipases A_2, which act in a functionally redundant manner, and (*II*) that type IV cytosolic phospholipase A_2 and type IIA and type V secretory phospholipases A_2 cooperate in the generation of arachidonic acid metabolites (Murakami *et al.*, 1998). Furthermore, studies in mice genetically deficient of type IV cytosolic phospholipase A_2 demonstrated important roles for this enzyme in such processes as ischemia-induced cell death in the forebrain and asthmatic processes (Bonventre *et al.*, 1997, Uozumi *et al.*, 1997).

Future *in vitro* studies on cultured cardiomyocytes, using adenoviral overexpression and sense/antisense technologies of the various types of phospholipase A_2 in combination with an *in vitro* hypoxia-reoxygenation model, should provide a means to investigate the individual role or potential cross-talk between the various phospholipase A_2 members. An advantage of adenoviral mediated gene transfer is that the low transfection efficiency of cultured cardiomyocytes can be circumvented. Such *in vitro* technologies provide a relatively less expensive and time consuming approach than the generation of genetically altered mouse models to obtain data *in vitro* regarding hypoxia-reoxygenation-mediated irreversible cell injury. This model, however, would not provide relevant data on the hemodynamic consequences of ischemia and reperfusion-induced alterations in the whole organ. Future experimental efforts should also concentrate on testing the cardiac ischemic vulnerability of mice genetically engineered to overexpress type IIA or type V secretory phospholipase A_2 in a cardiac-restricted manner (*Chapter 9*) using the *ex vivo* perfused mouse heart model as described in the present thesis. In addition, other transgenic mouse models of type IIA secretory phospholipase A_2 overexpression, albeit not in a cardiac-restricted manner (Grass *et al.*, 1996, Nevelainen *et al.*, 1997, Fox *et al.*, 1996), and of the type IV cytosolic phospholipase A_2 deficient mouse model, could be tested in the isolated ejecting murine heart model to provide more insight into the significance of phospholipase A_2-mediated membrane phospholipid hydrolysis during experimental ischemia and reperfusion and to determine the dominant

phospholipase A_2 member(s) in this particular pathological process. The generation of mouse models with a gain-of-function and a loss-of-function of each of the cardiac phospholipase A_2 members and testing the ischemic tolerance of their hearts in e.g. an *ex vivo* perfusion setup as described in the present thesis will constitute an example of molecular physiological approaches that will give more insight into the pathophysiological processes in the heart. In either way, the mouse as a mammalian species, complex enough to give relevant information about human (patho)physiology but still allowing routine genetic manipulations, will be the future animal model of choice to obtain insight into role of the major players in ischemia/reperfusion-induced cardiac muscle injury.

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Samenvatting

In **Hoofdstuk 1** worden de achtergrond en het doel van de huidige studie beschreven. Het hart is een holle spier die circa 70 maal per minuut samentrekt en daarmee het lichaam van bloed voorziet. Tijdens iedere contractie voorziet het hart zichzelf van zuurstof en voedingstoffen via de kransslagaderen. Tijdens een hartinfarct als gevolg van een vernauwing van een kransslagader of tijdens een kransslagader bypass operatie lijdt de hartspier onder een periode van zuurstoftekort (ischemie), wat kan leiden tot afsterving van de hartspiercellen. Herstel van de bloedtoevoer (reperfusie) kan slechts ten dele de afsterving van hartspiercellen voorkomen en in sommige gevallen zelfs het proces versterken. Dit fenomeen van ischemie en reperfusie geïnduceerde hartspiercelschade wordt ten dele veroorzaakt door veranderingen in de celmembraan, de natuurlijke barriëre van de hartspiercel. Uit experimentele studies is gebleken dat enzymatische afbraak van de belangrijkste componenten van de celmembraan, namelijk de fosfolipiden, mogelijk een belangrijke factor speelt in ischemie en reperfusie geïnduceerde celschade. Het lichaam bevat een familie van enzymen, fosfolipases A_2 genaamd, die gespecialiseerd zijn in de afbraak van fosfolipiden en die verschillen in werking en lokalisatie. In het huidige proefschrift werd de rol van het type IIA secretoire fosfolipase A_2 enzym (type IIA $sPLA_2$) nader onderzocht. In **hoofdstuk 2** wordt ingegaan op de theoretische overwegingen voor de keuze van dit lid van de fosfolipase A_2 familie. Het type IIA $sPLA_2$ werd gekozen op basis van zijn werkingsprofiel en omdat dit bepaalde enzym gevoelig is voor calcium, een mineraal dat in verhoogde concentratie voorkomt in het hart tijdens ischemie en reperfusie.

In **hoofdstuk 3** werd via moleculair biologische technieken onderzocht of het type IIA $sPLA_2$ in het hart voorkomt. Daartoe werd het hart van de rat onderzocht op de aanwezigheid van de moleculaire voorloper van het enzym, het messenger RNA. Onderzoek wees uit dat het hart inderdaad het type IIA $sPLA_2$ messenger RNA bevat en dat de genetische code zeer sterk geconserveerd is vergeleken met diverse andere celtypen en diersoorten. Tevens bleek dat de hoeveelheid van het type IIA $sPLA_2$ messenger RNA in het hart zeer gering zijn.

In **hoofdstuk 4** werd de genetische code van het enzym zodanig aangepast dat het enzym in grote hoeveelheden in de *E. Coli* bacterie geproduceerd kon worden. Tegen dit recombinante type IIA $sPLA_2$ enzym werden vervolgens in de konijn polyclonale antistoffen opgewekt. De mogelijkheid van deze antilichaam fractie om het type IIA $sPLA_2$ enzym aan te tonen werd getest naast andere antilichamen die waren opgewekt tegen andere typen fosfolipase A_2. Al de geteste antilichamen bleken in meer of minder mate het type IIA $sPLA_2$ enzym te herkennen in diverse celtypen en organen. Geen van de geteste antilichamen konden het type IIA $sPLA_2$ enzym in het hart echter aantonen, waaruit geconcludeerd kon worden dat dat dit enzym in geringe hoeveelheden in het hart voorkomt, aangezien de onderste detectiegrens van het meetsysteem niet overschreden werd.

Hoofdstuk 5 beschrijft de ontwikkeling van een geïsoleerd, met buffer geperfundeerd muizenhart model om de hemodynamische functie van het intacte hart in detail te bestuderen. Door speciale aandacht te besteden aan de aorta cannule, de temperatuur van het geïsoleerde hart en de samenstelling van de perfusiebuffer, bleek het mogelijk te zijn hemodynamische metingen te verrichten die vergelijkbare waarden opleverden met hartfunctiemetingen in de intacte, levende muis.

Dit model werd verder gebruikt in **hoofdstuk 6** om de ischemie tolerantie van het muizenhart te testen. Door metingen te verichten aan de hoeveelheid intracellulaire eiwitten die verschijnen in de perfusiebuffer na een periode van ischemie, kon een schatting gemaakt worden van het percentage hartspiercellen dat onherstelbaar beschadigd was door de voorafgaande periode van ischemie. Ook werd na afloop van

het experiment in het hart de stapeling gemeten van o.a. arachidonzuur, een meervoudig onverzadigd vetzuur dat vrijkomt bij de afbraak van membraanfosfolipiden tengevolge van fosfolipase A_2 activiteit. Het bleek dat de afname in hemodynamische hartfunctie na een periode van ischemie gecorreleerd kon worden aan enerzijds het percentage beschadigde hartspiercellen en anderzijds aan de afbraak van membraan fosfolipiden. Ook wees deze studie uit dat al na een relatief korte periode van zuurstoftekort (circa 15 minuten) het muizenhart een sterke afname in hemodynamische functie vertoont, hetgeen duidt op een grote ischemie gevoeligheid van dit orgaan.

Verder werd in **hoofdstuk 7** de ischemie tolerantie gemeten van harten die afkomstig waren van muizen die zodanig genetische gemodificeerd waren dat ze verminderde hoeveelheden van de groeifactor insulin-like growth factor-1 (IGF-1) bevatten. Eerdere experimentele studies hebben uitgewzen dat IGF-1 een protectief effect heeft op het hart na een periode van zuurstoftekort. De verwachting is dat harten met een verminderde hoeveelheid IGF-1 gevoeliger zouden zijn voor een periode van zuurstoftekort. De harten van IGF-1 deficiënte muizen bleken inderdaad na een periode van ischemie en reperfusie slechter hemodynamisch te herstellen, en verhoogde celschade en grotere stapeling van vrij arachidonzuur in het hart te vertonen. Deze bevindingen tonen aan dat het model ontwikkeld en beschreven in hoofdstuk 5 en 6 inderdaad gevoelig genoeg is om subtiele verschillen in ischemie tolerantie aan te tonen van het geïsoleerde, intacte hart. Verder toont deze studie aan dat fosfolipase A_2-gemedieerde afbraak van celmembranen van de hartspiercel mogelijk een rol kan spelen in ischemie en reperfusie geïnduceerde harspiercelschade in de IGF-1 deficiente muis.

Om de mogelijke rol van het type IIA $sPLA_2$ te testen in ischemie en reperfusie geïnduceerde afbraak van celmembranen, werd in **hoofdstuk 8** de ischemie tolerantie getest van muizen die een chromosomale mutatie bevatten in de genetische code voor het type IIA $sPLA_2$. Deze muizenstam bevat als gevolg van deze mutatie geen type IIA $sPLA_2$ in het hart. De ischemie tolerantie van harten van deze mutante muizenstam werd vergeleken met die van een nauw verwante muizenstam die normale hoeveelheden type IIA $sPLA_2$ in het hart bevat. Interessant genoeg werden geen verschillen gevonden in arachidonzuur stapeling in het hart, de hoeveelheid celschade of de afname in hemodynamische functie na een periode van ischemie gevolgd door reperfusie. Deze bevindingen tonen aan dat het type IIA $sPLA_2$ in het geïsoleerde muizenhart zeer waarschijnlijk geen dominante rol speelt in ischemie en reperfusie gemedieerde afbraak van celmembranen en dat andere leden van de fosfolipase A_2 familie mogelijk een belangrijkere rol in dit fenomeen vervullen.

Om meer inzicht te verkrijgen in de rol van fosfolipase A_2 aktiviteit op zich in ischemie en reperfusie geassocïerde membraanafbraak in het hart, werden in **hoofdstuk 9** pogingen ondernomen om een transgeen muismodel te crëeren, dat zodanig genetisch gemodificeerd was dat het hart meer fosfolipase A_2 bevat. Dit resulteerde in een transgene muizenstam die weliswaar meer kopiën van het gen voor fosfolipase A_2, maar geen aantoonbare hogere hoeveelheden fosfolipase A_2 in het hart bevatte. Een vervolgpoging werd gedaan met andere DNA konstrukten en verscheidene transgene muizen werden gecrëerd die meerdere kopiëen van het fosfolipase A_2 gen bevatten. Eén van de transgene muizen die een groot aantal kopiën bevatte stierf snel na geboorte, wat mogelijk inhoudt dat grote hoeveelheden fosfolipase A_2 niet verenigbaar zijn met normale hartfunctie. De beschikbaarheid van muismodellen met grotere of verminderde hoeveelheden fosfolipase A_2 in het hart, in combinatie met een geïsoleerd muizenhartmodel om de ischemie tolerantie van het hart van genetisch gemodificeerde muizen te testen, kan in toekomstige studies uitwijzen welk lid van de fosfolipase A_2 familie een dominante rol speelt in ischemie en reperfusie geïnduceerde hartspierschade.

Summary

In **chapter** 1 the background to the present thesis and the general aim of our study are presented. The heart is a muscle that contracts approximately 70 times per minute and supplies the body with blood. During each contraction the heart supplies itself with oxygen and nutrients through the coronary arteries. During a myocardial infarction as a result of an occlusion of coronary arteries or during aorta-coronary bypass surgery, the heart is temporarily devoid of oxygen (ischemia), what eventually leads to cardiac muscle cell death. Restoration of the cardiac blood supply (reperfusion) is only partially able to reduce cardiac cell death and may even exacerbate the injuring process. This phenomenon of ischemia and reperfusion induced cardiac muscle cell death is partially caused by a disruption of the cellular membrane, which forms the natural barier of the cardiac cell. Experimental studies have indicated that enzymatic breakdown of the major components of the cellular membrane, the phospholipids, might play an important role in the transition from ischemia and reperfusion induced reversible to ireversible cell damage, eventually leading to cardiac dysfunction. Our body contains a family of enzymes, phospholipases A_2, which are specialized in hydrolyzing membrane phopholipids and differ amongst each other in their activation profile and subcellular localization. In the present thesis the role of one particular member of this family, type IIA secretory phospholipase A_2 (type IIA $sPLA_2$) in ischemia and reperfusion induced cell damage was investigated in more detail. **Chapter 2** presents a review of literature and theoretical background of the rationale behind the choice for this particular enzyme. In brief, type IIA $sPLA_2$ was chosen on the basis of its activation profile and its dependency on calcium, a mineral the intracellular level of which is increased during cardiac ischemia and reperfusion.

In **chapter 3** it was investigated whether type IIA $sPLA_2$ is present in the heart using molecular biological techniques. To this end, the rat heart was investigated for the presence of the messenger RNA of type IIA $sPLA_2$, the molecular precursor of the protein itself. It was demonstrated that the heart indeed contains this messenger RNA and that the genetic code of cardiac type IIA $sPLA_2$ was highly conserved amongst different species and cell types. It was also demonstrated that the amount of type IIA $sPLA_2$ messenger RNA is very low in the heart.

In **chapter 4** high amounts of recombinant type IIA $sPLA_2$ were produced and purified from the *E. Coli* bacteria. Against this purified enzyme polyclonal antibodies were produced in the rabbit and used to detect type IIA $sPLA_2$ protein in different tissues. The ability of the latter antibody to detect type IIA $sPLA_2$ was compared with four other anti-phospholipase A_2 antibodies. It was found that all antibodies were able to detect the enzyme with different sensitivity. None of the antibodies, however, were able to detect the enzyme in cardiac tissue, providing further indication that the protein level of type IIA $sPLA_2$ is very low in cardiac muscle.

Chapter 5 describes the development and characterization of an isolated, buffer perfused mouse heart model to measure hemodynamic cardiac function devoid of neuro-humoral stimulation. By paying special attention to the artificial aortic outflow tract, the temperature of the isolated heart and the composition of the perfusion buffer, it was found that this model was able to sensitively monitor hemodynamic function, that resembled cardiac function of the mouse heart *in vivo*.

This model was subsequently used in **chapter 6** to study ischemia and reperfusion phenomena in the mouse heart. By measuring the leakage of intracellular enzymes into the coronary outflow an estimate could be obtained of the percentage cells irreversibly damaged during the preceding ischemic period. Biochemical analysis of cardiac tissue after experimentation allowed measurements of the accumulation of unesterified fatty acids such as arachidonic acid, which is a sensitive marker for phospholipase A_2 activity. A strong

correlation was found between the tissue accumulation of arachidonic acid on the one hand and the percentage irreversibly damaged cardiac cells or the post-ischemic recovery of hemodynamic function on the other. In addition, it was found that the mouse heart shows a relatively high sensitivity towards global ischemia and reperfusion.

This model was subsequently used in **chapter 7** to measure the ischemia tolerance of hearts derived from mice that were genetically engineered to contain less insulin-like growth factor-1 (IGF-1) in their bodies. Previous studies provide evidence that IGF-1 has a potent protective effect on the cardiac muscle during ischemia and reperfusion. As such, IGF-1 deficient hearts would be expected to be more vulnerable towards ischemia and reperfusion induced damage. IGF-1 deficient hearts subjected to a period of ischemia followed by reperfusion indeed demonstrated a significant increase in cellular damage, lower hemodynamic recovery and increased accumulation of unesterified fatty acids, such as arachidonic acid. These findings provided further indications that the model described in chapter 5 and 6 was sensitive enough to detect subtle differences in ischemia tolerance of isolated mouse hearts. In addition, these observations further point toward a possible role of phospholipase A_2-mediated hydrolysis of membrane phospholipids in the suequela of events leading to cardiac ischemia and reperfusion induced cell death.

To study the possible role of type IIA $sPLA_2$ in cardiac ischemia and reperfusion-induced membrane damage and cell death, in **chapter 8** the ischemia tolerance was tested of hearts derived from mice with a chromosomal mutation in the gene of type IIA $sPLA_2$. As a result of the mutation this mouse strain is unable to produce the type IIA $sPLA_2$ enzyme in the heart. The ischemia tolerance of mutant mouse hearts was compared with hearts derived from a mouse strain that was closely related but did not contain the mutation, and, hence, has normal cardiac type IIA $sPLA_2$ levels. Interestingly, following ischemia and reperfusion no differences were found in the accumulation of arachidonic acid, the amount of irreversible cell damage or recovery of hemodynamic function. These findings indicate that type IIA $sPLA_2$ activity most probably is not a major factor in cardiac ischemia and reperfusion-induced membrane damage in the isolated mouse heart and that other members of the phospholipase A_2 family may have more dominant roles in this phenomenon.

To gain more insight in the role of phospholipase A_2 activity during cardiac ischemia and reperfusion, attempts were made in **chapter 9** to create a transgenic mouse model that was genetically modified to contain increased type IIA secretory phospholipase A_2 activity in the heart. This resulted in a transgenic mouse strain that contained more copies of the phospholipase A_2 gene in its chromosomes, but did not demonstrate a detectable increase in the amount of the enzym. In a follow up experiment other DNA constructs were used and multiple transgenic mice were obtained. Interestingly, one mouse that contained a high number of copies of the phospholipase A_2 gene died soon after birth, possibly indicating that large quantities of phospholipase A_2 may not be compatible with normal cardiac function. The availability of genetically engineered mice containing either more or less of the different members of the phospholipase A_2 in the heart, in combination with an isolated mouse heart model to test the cardiac ischemia tolerance, may indicate in the future whether or not phospholipase A_2 mediated membrane hydrolysis plays a role in the transition of reversible to irreversible cardiac myocyte injury and which of the members of the phospholipase A_2 family plays a dominant role in this process.

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Curriculum vitae

September 1988 – April 1994	Undergraduate studies Biology, University of Utrecht.
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List of Publications

Papers

1. **De Windt LJ**, Pöpping S, Willemsen PHM, Reneman RS, Van der Vusse GJ, Van Bilsen M. Cloning and cellular distribution of a group II phospholipase A_2 expressed in the heart. *J Mol Cell Cardiol* 1997;29:2095-2106.

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14. **De Windt LJ**, Molkentin JD, Doevendans PA. Mouse models of human heart disease: potential for gene discovery arrays. Submitted *Eur Heart J*.

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