Volatile Anesthetics and the Heart

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VOLATILE ANESTHETICS AND THE HEART

MECHANISMS OF SEVOFLURANE-INDUCED CARDIOPROTECTION

Robert Arthur Bouwman

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VOLATILE ANESTHETICS AND THE HEART

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Chapter 1

GENERAL INTRODUCTION:
VOLATILE ANESTHETICS AND THE HEART

R. Arthur Bouwman, René J.P. Musters, Christa Boer, Jaap J. de Lange

Submitted for publication

ABSTRACT

During surgical procedures and in intensive care units anesthetics are widely used to induce and maintain anesthesia and to sedate patients. Most anesthetics negatively affect the hemodynamical profile via negative inotropic effects on the heart. These effects may be detrimental for patients with cardiovascular pathology. However, at clinical relevant concentrations volatile anesthetics may also exert beneficial effects during ischemia and reperfusion, and are able to improve post-ischemic contractile function and reduce infarct size. These beneficial effects rely on a cardioprotective signaling cascade including several mediators, like protein kinase C, reactive oxygen species and adenosine triphosphate-sensitive K^+ channels in analogy to ischemic preconditioning. These cardioprotective properties may alter the importance of volatile anesthetics in the development of clinical cardioprotective strategies and may therefore become an important therapeutic tool. However, the cellular mechanisms of this phenomenon have not been elucidated. The purpose of this review is to summarize the current knowledge regarding the mechanisms and the possible clinical implications of volatile anesthetic-induced cardioprotection.

INTRODUCTION

Anesthetics are used to induce and maintain anesthesia in order to perform surgical procedures or to sedate patients in the intensive care unit (ICU). In addition to their effects on consciousness, most anesthetics reduce cardiac function due to negative inotropic properties with possible swings in the hemodynamic profile as result.

The contractile force of the heart is determined by myocardial Ca^{2+} handling, Ca^{2+} sensitivity of the contractile elements and the cellular length just before contraction (preload). Volatile anesthetics have profound inhibitory effects on cardiac Ca^{2+} homeostasis leading to depression of cardiac function. These effects might be detrimental for patients with reduced myocardial function due to cardiovascular pathology.

Interestingly, recent evidence shows that volatile anesthetics exert cardioprotective properties and may protect cardiomyocytes against ischemia and reperfusion (I/R)-injury (1). In particular, the myocardium contains intrinsic protective signaling pathways, which can be triggered by a variety of stimuli, including volatile anesthetics. This cardioprotective effect is intracellular mediated via interaction between reactive oxygen species (ROS), protein kinase C (PKC) and adenosine triphosphate-sensitive mitochondrial K^+($mitoK^+_{ATP}$) channels (2-4). However, the sequence of events in the signaling cascade is complex and potential end-effector proteins of this cardioprotective strategy remain to be elucidated.

Nevertheless, volatile anesthetics may become an important therapeutic tool in the induction of protection against I/R-injury and may provide possible clues for the development of clinical cardioprotective strategies. Therefore, the purpose of this review is to discuss the effects of volatile anesthetics on myocardial contractile function in relation to I/R-injury and anesthetic-induced cardioprotection (APC).

EXCITATION-CONTRACTION COUPLING IN THE HEART

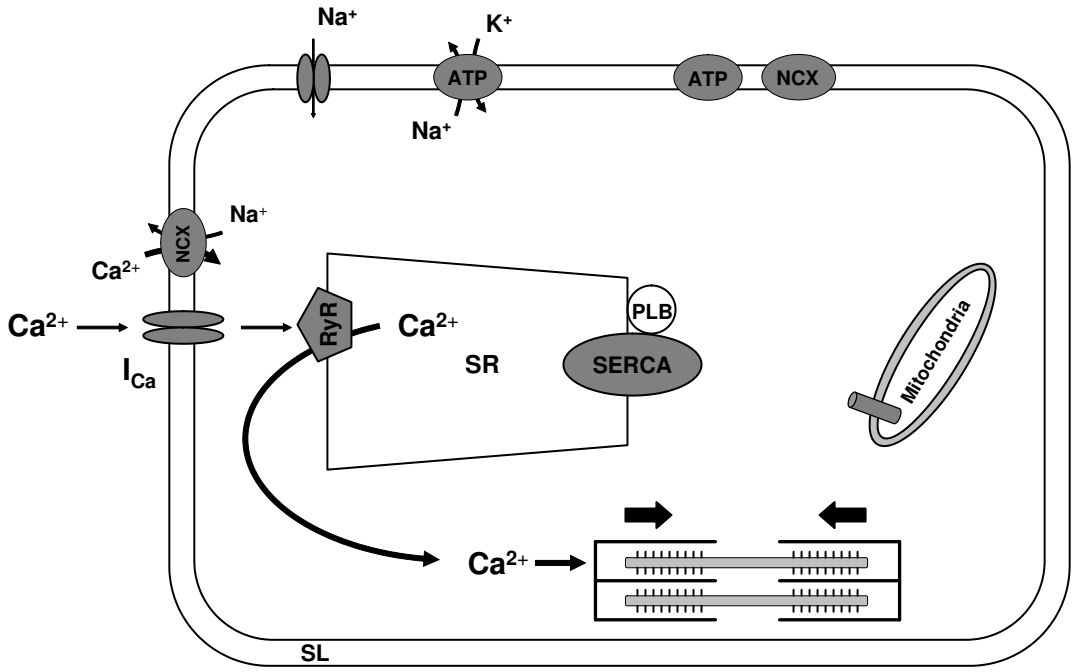
The heart ensures adequate circulatory blood flow and closely matches cardiac output to the metabolic demands of the various organs. Cardiac output is modulated by cardiac contractile

function, which is determined by force development of individual cardiomyocyte. The contractile machinery of cardiomyocytes consists of contractile proteins arranged in myofibrils containing thick (myosin) and thin (actin) filaments. During contraction the contractile filaments slide over each other due to formation of crossbridges between myosin heads and myosin binding sites on the actin filament, followed by force-generating conformational changes (powerstroke). The process of cross-bridge cycling is dependent on adenosine triphosphate (ATP) and has been extensively reviewed (5-7). Actomyosin interaction is closely regulated by the proteins tropomyosin and troponin (Tn). During cardiac rest (diastole) tropomyosin blocks cross-bridge interaction by interfering with myosin-binding sites on actin. Due to Ca^{2+} binding to the TnC subunit the inhibitory TnI subunit is released, allowing tropomyosin to move over the actin filament. Consequently, myosin-binding sites on actin are exposed and cross-bridge cycling is initiated.

In addition to activation of the contractile apparatus, Ca^{2+} is importantly involved in the regulation of cardiac electrical activity. Therefore, Ca^{2+} is an important second-messenger in the coupling of cardiomyocyte excitation to contraction. The amplitude of the developed force is dependent on the Ca^{2+} availability for activation of the contractile system. As a consequence, Ca^{2+} handling is tightly regulated and contractile dysfunction due to pathophysiologic conditions is mostly due to alterations in Ca^{2+} homeostasis.

When an action potential is generated, Ca^{2+} enters the cardiomyocyte via the voltage-gated L-type Ca^{2+} channels during the plateau phase of the action potential. This relatively small Ca^{2+} influx triggers massive Ca^{2+} release from the sarcoplasmic reticulum (SR) via activation of the ryanodine-sensitive Ca^{2+} release channels (ryanodine-receptors, RyRs). This amplification of Ca^{2+} influx is known as Ca^{2+}-induced Ca^{2+}-release (CICR) and provides the necessary Ca^{2+} for activation of the contraction. In order to allow for diastolic filling of the heart, cardiomyocyte relaxation is initiated by termination of Ca^{2+} release from the SR and subsequent reduction of intracellular [Ca^{2+}] ($[Ca^{2+}]_i$). During relaxation, Ca^{2+} is removed from the cytosol by Ca^{2+}-reuptake into the SR via the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and via extrusion from the cardiomyocyte in exchange for Na^+ entry through the Na^+/Ca^{2+}-exchanger (NCX) (see figure 1.1). The amplitude of developed force by the myocardium can be changed by: 1) altering the amplitude or duration of the Ca^{2+} transient, or 2) altering the sensitivity of the myofilaments for Ca^{2+}.

A) Contraction



B) Relaxation

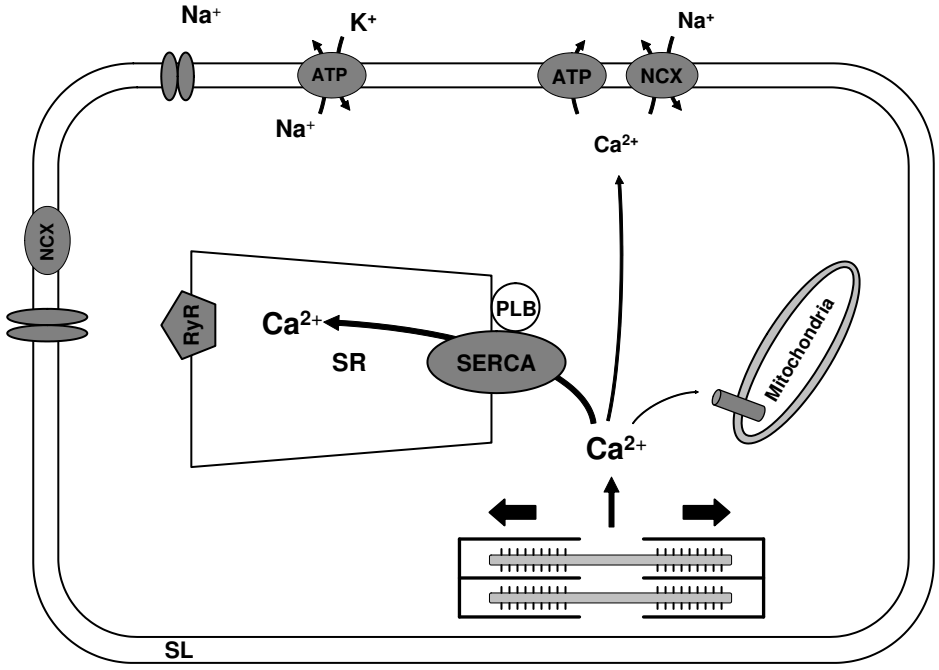


Figure 1.1 Calcium fluxes during excitation-contraction coupling in contraction (A) and relaxation (B). ATP=ATPase, NCX=Na^+/Ca^{2+}-exchanger, I_{Ca}=Ca^{2+} influx via voltage dependent L-type Ca^{2+} channels, RyR=ryanodin receptor, SR=sarcoplasmic reticulum, SERCA=sarco-endoplasmic reticulum Ca^{2+} ATPase, PLB=phospholamban, SL=sarcolemma.

Changes in the Ca^{2+} transient can be due to alterations in the Ca^{2+} influx as well as Ca^{2+} (re)uptake and Ca^{2+} release from the SR. Ca^{2+} sensitivity is influenced by various factors like myofilament length, temperature, pH and phosphorylation status of the myofilaments (contractile proteins).

EFFECT OF VOLATILE ANESTHETICS ON EXCITATION-CONTRACTION COUPLING

The molecular structures of modern inhalational anesthetic agents are based on ether (desflurane, enflurane, isoflurane and sevoflurane) or chloroform (halothane), which were used as the first general anesthetics. The mechanism of their anesthetic effects remains to be elucidated, but based on the lipid-solubility of these agents alterations in membrane properties are expected to be involved. Volatile anesthetics reduce myocardial contraction by both reducing the amount of Ca^{2+} available for contraction (a reduction in the Ca^{2+} transient) and reducing the myofilament sensitivity to Ca^{2+}. In an extensive review, Hanley *et al*. (2004) described the current knowledge regarding the negative inotropic effects of volatile anesthetics (8). The key elements of these mechanisms will be summarized here.

Several electrophysiological studies showed that volatile anesthetics reduce the whole-cell Ca^{2+} inward current due to inhibition of the voltage dependent L-type Ca^{2+} channels. Cardiac contractility will diminish because less Ca^{2+} is available for CICR. As a consequence, diminished Ca^{2+} influx via the L-type Ca^{2+} channels will shorten the plateau phase of the cardiac action potential. Additional opening of the ATP-sensitive sarcolemmal K^+-channels ($sarcK^+_{ATP}$) also contributes to action potential shortening.

The anesthetic effects on the SR Ca^{2+} content seem to be different between various anesthetic agents. Halothane decreases SR-Ca^{2+} content, as determined by caffeine-induced contractions. Halothane seems to facilitate opening of the SR Ca^{2+} release channel (RyR) causing Ca^{2+} to leak into the cytosol (9). As a consequence, halothane exposure transiently increases the cardiac contractility due to Ca^{2+} filling of the cytosol (10). In contrast, during sevoflurane-exposure SR Ca^{2+} content is increased, suggesting that negative inotropy is due to a reduction

of CICR in combination with a reduced cellular Ca^{2+} efflux (9). Hannon and Cody (2002) suggested that sevoflurane reduces Ca^{2+} efflux via inhibition of the sarcolemmal Ca^{2+}-ATPase (9). Others showed inhibitory effects of volatile anesthetics on sarcolemmal Ca^{2+}-transport via the NCX (11,12). In contrast, we recently demonstrated in isolated rat trabeculae that sevoflurane facilitates NCX-dependent Ca^{2+} influx (reverse mode of the NCX) providing another potential mechanism of sevoflurane-induced increase in SR-load (13).

In addition to a reduction in the Ca^{2+} transient, volatile anesthetics affect the sensitivity of the contractile system for Ca^{2+}. This is supported by data showing that restoration of the Ca^{2+} transient, by applying additional extracellular Ca^{2+} during anesthetic exposure, does not completely restore force development (14). However, conflicting results have been reported (8), since Davies *et al*. (2000) showed that sevoflurane exerted only minimal inhibitory effects on Ca^{2+}-sensitivity (15). Recently, Graham *et al*. (2005) provided a possible explanation for these contradictory results and reported that effects of volatile anesthetics on the contractile myofilaments are dependent on the duration of exposure (16).

CARDIOPROTECTION AGAINST ISCHEMIA/REPERFUSION INJURY

Ischemia/Reperfusion Injury

Ischemia is a condition in which the delivery of O_2 and metabolic nutrients is inadequate due to a reduction or cessation of myocardial blood flow. If blood flow is not restored, irreversible injury of cardiomyocytes will occur. However, restoration of blood flow is followed by additional harmful events and cellular injury is paradoxically increased. This phenomenon is also known as reperfusion injury. Thus, direct effects of ischemia as well as indirect effects through reperfusion contribute to cellular damage due to I/R-injury. After a brief reduction in blood flow (<20 minutes), myocardial viability is usually preserved, although contractile function will not fully restore after reperfusion. Over time, the myocardial contractility recovers to baseline levels and this gradual restoration is known as stunning (17). Prolonged ischemic periods are characterized by irreversible tissue damage due to necrotic as well as apoptotic cell death. Cellular Ca^{2+} overload and the production of reactive oxygen species (ROS) are both recognized as critical mediators in the development of the adverse sequelae of

I/R-injury.

Under normal conditions, the metabolic demand and supply of the heart are closely matched and reduced metabolic supply is quickly followed by physiological and metabolic changes in cardiomyocytes. Within seconds after coronary blood flow reduction, energy metabolism in cardiomyocytes shifts from aerobic to anaerobic glycolysis. Consequently, cardiomyocytes become energy depleted as the ATP production becomes less effective and depletion of cellular energy reserves (high-energy phosphates) starts within seconds after complete cessation of blood flow (18). These metabolic changes are accompanied by a reduction in cardiac contraction, which ultimately ceases completely. In addition to inadequate O_2-delivery and nutrient supply, metabolic products are not washed out due to decreased blood flow. Lactate and protons therefore accumulate and within minutes after the onset of ischemia intracellular acidosis is detected (19). In addition, intracellular Na^+ concentration will rise due to an increased Na^+-influx via the Na^+/H^+-exchanger (NHE) and reduced activity of the Na^+/K^+-ATPase. Furthermore, ischemic Na^+-overload alters the electrochemical driving force in favor of NCX-dependent Ca^{2+} influx (reverse mode of the NCX) leading to cytosolic accumulation of Ca^{2+} (20-22). Finally, in addition to these changes in intracellular ion-homeostasis, recent evidence indicated that ROS are also produced during ischemia itself and not only upon reperfusion. These ROS originate from the mitochondrial electron transport chain (ETC) despite low O_2 levels and contribute to ischemic cell death in addition to ATP-depletion and Ca^{2+} overload (23,24).

Upon reperfusion of the myocardium, rapid increases in ROS production as well as Ca^{2+} (over) loading during the first minutes of reperfusion have been convincingly demonstrated (25-29). During reperfusion, SERCA is quickly reactivated due to restoration of energy production (30). However, the amount of cytosolic Ca^{2+} exceeds the capacity of the SR, and as cellular Na^+-levels remain elevated due to increased Na^+-influx via the NHE cytosolic Ca^{2+} will continue to rise via the NCX in the reverse mode. In addition, energy is present for activation of the myofibrillar elements, but in the presence of high Ca^{2+}-levels, myofibrillar activation leads to sustained force development causing hypercontraction (31). According to Piper *et al*. (2004), the development of hypercontraction in combination with ischemia-induced cytoskeletal and sarcolemmal fragility results in cellular disruption followed by necrotic cell death. In addition, elevation of intracellular Ca^{2+} is also associated with

activation of a variety of protein kinases, phosphatases and proteases contributing to I/R-injury. Furthermore, mitochondrial function can be negatively affected as mitochondrial Ca^{2+} overload induces release of cytochrome C and pro-apoptotic factors into the cytosol (27).
In analogy with myocardial Ca^{2+} handling, ROS play an important role as signaling molecules in cell physiology and pathophysiology. However, during I/R-injury uncontrolled ROS production leads to sarcolemmal damage by lipid peroxidation, modification of protein structure and mitochondrial dysfunction (32-34). Particularly, application of exogenous ROS to cardiomyocytes induces similar metabolic and physiological alterations, like energy depletion, contracture development, reduced contractile function and Ca^{2+} overload (35). Therefore, ROS are strongly implicated in the pathogenesis of I/R-injury. However, ROS-scavenging during I/R-injury in animal studies has reported negative results and until now the clinical use of antioxidant therapy during I/R-injury have failed to show beneficial effects (28). Furthermore, as Ca^{2+} overload can increase ROS production (36,37) and ROS production promotes Ca^{2+} overload (35), the exact relationship and contribution of ROS and Ca^{2+} overload to I/R-injury remains to be established (31,38-41).

Ischemic Preconditioning (Classical Preconditioning)

Many experimental and clinical studies are focused on the development of cardioprotective strategies against the adverse sequelae of myocardial ischemia and subsequent reperfusion. Interestingly, the heart can resist brief ischemic periods remarkably well, and Verdouw *et al*. (1979) already showed that repeated brief ischemic periods favorably alter myocardial energy metabolism (42), whereas the possible cardioprotective effect of repeated brief ischemic periods was already suggested by Basuk *et al*. (1986) (43). The phenomenon of ischemic preconditioning (IPC) was introduced by Murry *et al*. (1986) who showed that four cycles of 5 minute periods of ischemia reduced the infarct size after prolonged I/R-injury (44). The cardioprotective effect of the preconditioning stimulus lasted for about 2 to 4 hours and beyond this time period protection was reduced (45,46). Interestingly, 24 hours after the initial preconditioning stimulus the heart developed an increased resistance against I/R-injury again (47,48). The delayed preconditioning response lasts for about 72 hours and this phenomenon is referred to as the second window of protection (49).
These observations clearly suggested that the myocardium possesses endogenous protective

mechanisms, which have been demonstrated in a variety of experimental studies. Due to the potential clinical benefit of an effective clinical cardioprotective strategy, the underlying cellular mechanisms of cardioprotection were extensively studied and the subject of many reviews (50-56). Inhibitory G-protein coupled receptor agonists, like adenosine, bradykinin, norepinephrin, opioids, angiotensin and endothelin, can trigger preconditioning of the myocardium. In addition, ROS, nitric oxide (NO) and Ca^{2+} are also able to trigger cardioprotection. Moreover, several protein kinases are recognized as important mediators of the protective response, like protein kinase C (PKC), tyrosine kinases (TK), mitogen activated protein kinases (MAPK) and phosphatidylinositol 3-kinase (PI3-kinase). The contribution of the mitoK^+_{ATP} channels to preconditioning is complex, as opening of the mitoK^+_{ATP} channels is involved as a trigger and mediator in the cardioprotective signaling cascade. In addition, among the possible end-effectors, the mitoK^+_{ATP} channels have been suggested to be involved in the preservation of mitochondrial function (57). However, interaction of the various intracellular signaling pathways during cardioprotection is complex and the exact mechanism and end-effector proteins remain to be elucidated.

ANESTHETIC-INDUCED PRECONDITIONING

Already in 1976, Bland and Lowenstein described the beneficial effect of halothane on the severity of ischemic injury (58). Subsequently, Davis *et al.* (1983) reported that halothane pretreatment reduced infarct size after I/R-injury (58,59). After the introduction of the concept of ischemic preconditioning by the study of Murry *et al.* (1986) (44), further research on the mechanism of anesthetic-induced protective signaling was greatly extended and summarized in several recent reviews (1,4,60-64).

In various experimental studies halothane, enflurane, isoflurane and sevoflurane all reduced infarct size after I/R (59,65-67). In addition to a reduction of cardiomyocyte cell death, several studies showed that volatile anesthetics also improved post-ischemic contractile function (2,68-70). Like ischemic preconditioning, volatile anesthetics also induce a second window of cardioprotection (71). Finally, in several experimental studies, application of volatile anesthetics solely during reperfusion proved to reduce infarct size as well (72). Only

two minutes of sevoflurane exposure during reperfusion produced maximal protection of the myocardium (73). This mimics the recently reported phenomenon of postconditioning by brief ischemic periods during reperfusion (74).

The exact mechanism of cardioprotection afforded by volatile anesthetics remains unresolved. However the protective effect seems to rely on the same intracellular signaling pathways as involved in ischemic preconditioning, like protein kinase C (PKC), ROS and mitoK^+_{ATP} channels (see figure 1.2). In the following section the involvement of the three most studied signaling molecules in anesthetic preconditioning will be discussed.

Protein Kinase C

PKCs can modify the function of a wide variety of target substrates by phosphorylation. Furthermore, they have been implicated in a range of (patho) physiological responses in the heart, like contraction, hypertrophy and apoptosis (75,76). PKC activation differs among the three subclasses of PKC-isoforms: conventional PKCs (α, β_1/β_2, γ) are activated by Ca^{2+}, phosphatidylserine (PS) and diacylglycerol (DAG), whereas the novel PKCs (δ, ε, η, θ) – and atypical PKCs (ζ ,λ, μ, ν) are Ca^{2+}-independent but are activated by DAG and PS (novel isoforms) or only PS (atypical isoforms). In the rat as well as human myocardium, expression of the PKC isoforms α, β_1/β_2, δ, ε has been demonstrated (76). PKCs are activated upon a variety of stimuli, including ischemia (77) and volatile anesthetics (78). This volatile anesthetic-induced PKC activation is essential for the preconditioning effect. Several studies showed that general inhibition of PKC abolished cardioprotection (2,65,79). Interestingly, considerable controversy exists which PKC-isoforms contribute to the anesthetic-induced preconditioning cascade. However, in analogy to ischemic preconditioning, PKC-δ and ε have been considered most important in volatile anesthetic-induced cardioprotection (3,80-85). In addition to the discussion regarding the exact contributing isoforms, the discrepancy extends also to the pattern of translocation of PKC-isoforms after preconditioning. Uecker *et al.* (2003) showed in isoflurane-treated rat hearts that PKC-δ translocated towards the nuclei and PKC-ε towards the sarcolemma (84). In an in vivo rat model, Weber *et al.* (2005) reported translocation of PKC-ε towards the sarcolemma, whereas PKC-δ was not primarily involved (3).

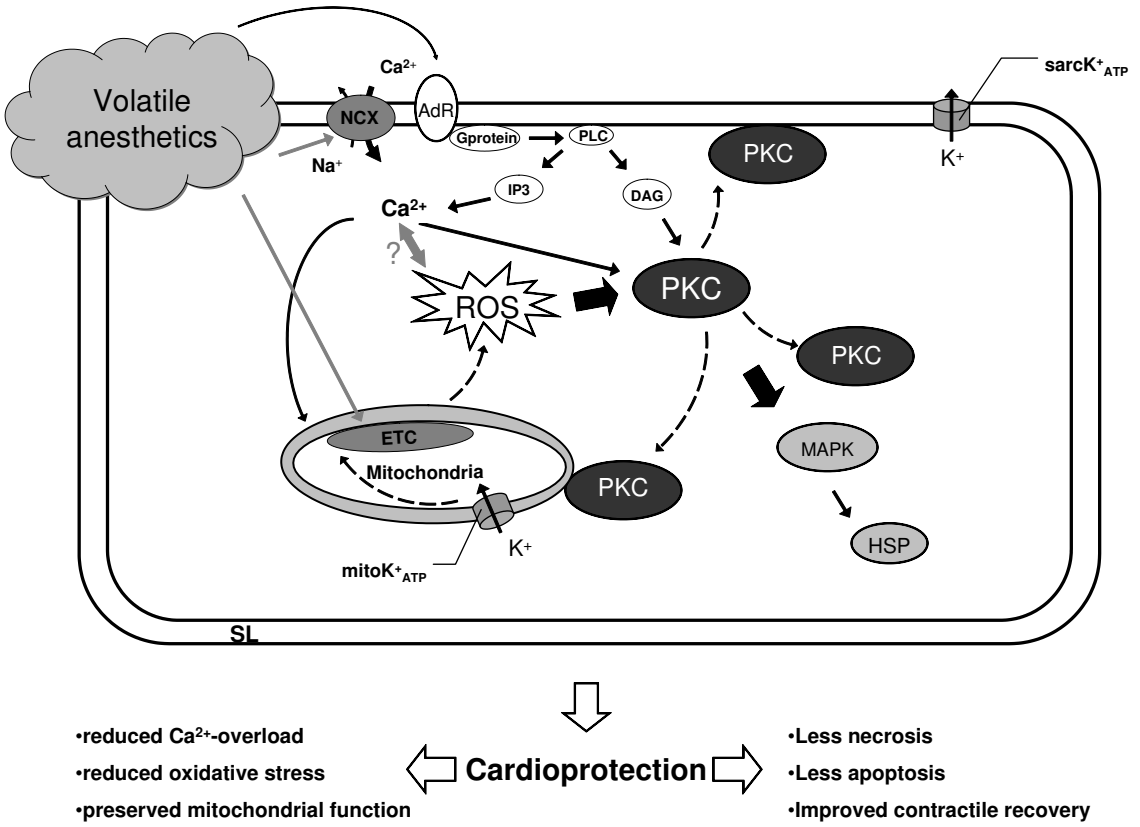


Figure 1.2 Cardioprotective signaling pathways involved in volatile anesthetic-induced preconditioning. Central in the volatile anesthetic-induced cardioprotective signaling cascade is activation of PKC. PKC activation relies on the production of ROS most likely due to direct inhibitory effects of volatile anesthetics on the mitochondrial respiratory chain. Other activation pathways are also considered to be involved, like activation of G-protein coupled receptors, phospholipase C and diacylglycerol. Interestingly, we recently demonstrated that the reverse mode of the Na^+/Ca^{2+}-exchanger precedes PKC activation, presumably via facilitation of Ca^{2+} influx (151). The exact sequence of events with respect to the NCX and ROS production remains to be elucidated. Among other isoforms, PKC-δ and PKC-ε have been considered most important. However, in isolated trabeculae PKC-α activation seemed also to be involved in the preconditioning cascade (unpublished observations). After activation, PKC translocates towards several subcellular structures, like the mitochondria, sarcolemma, intercalated disks and the cytoskeleton and modifies protein function due to phosphorylation. Although down-stream signaling targets remain to be elucidated, ATP-sensitive mitochondrial K^+ channels, ATP-sensitive sarcolemmal K^+ channels, the sarcoplasmic reticulum and heat shock proteins are considered to be potential candidates. Opening of ATP-sensitive mitochondrial K^+ channels is considered a possible end-effector in cardioprotection via preservation of mitochondrial bioenergetics. However, additional ROS production in response to channel opening is considered to participate as possible feed forward mechanism in further amplification of the cardioprotective signaling cascade. Other possible end-effects are shortening of the action potential due to ATP-sensitive sarcolemmal K^+ channel opening, heatshock protein-induced stabilization of the cytoskeleton and preserved sarcoplasmic reticular function. AdR=adenosine receptor, PLC=phospholipase C, DAG=diacylglycerol, IP3=inositol triphosphate, ROS=reactive oxygen species, ETC=electron transport chain, PKC=protein kinase C, $mitoK^+_{ATP}$=ATP-sensitive mitochondrial K^+ channels, $sarcK^+_{ATP}$=ATP-sensitive sarcolemmal K^+ channels, MAPK=mitogen activated protein kinase, HSP=heat shock protein, NCX=Na^+/Ca^{2+}-exchanger.

These observations contrast our immunofluorescent observations in isolated rat trabeculae, showing PKC-δ translocation towards the sarcolemmal membrane in response to sevoflurane. Ludwig *et al*. (2004) confirmed PKC-δ translocation towards the sarcolemma and additionally showed PKC-ε in the mitochondria after isoflurane. Interestingly, in human atrial biopsies, a sevoflurane-induced translocation of PKC-δ into the sarcolemma and PKC-ε to the intercalated disks was confirmed (85). Recently, several studies addressed the down-stream signaling effects of PKC and in particular the involvement of mitogen-activated protein kinases (MAPKs), like p38, JNK and ERK (81,86). New insights were revealed by Weber *et al*. (2005), who showed that heat shock protein (HSP)-27 formed a downstream target of PKC-ε and p38 during xenon-induced preconditioning, which resulted in cytoskeletal changes (87). In contrast, others found no involvement of MAPKs as part of the downstream-signaling of anesthetic-induced preconditioning (88). In the future, additional targets for PKCs should be extensively explored like the sarcoplasmic reticulum (89), contractile myofilaments (90) and sarcK^+_{ATP} channels and mitoK^+_{ATP} channels (see also paragraph: "*mechanisms of protection: end-effector proteins"*) (79,91,92).

Reactive Oxygen Species

Anesthetic-induced production of ROS has been implicated as important trigger of protective signaling. Oxygen free radicals are oxygen derived species characterized by the presence of one or several unpaired electrons with a very high reactivity. Especially superoxide ($O_2^{\cdot-}$), nitric oxide ($NO^{\cdot}$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) and hydroxyl radicals ($OH^{\cdot}$) are assumed to be involved in cardioprotective signaling. In a review, Kevin *et al*. (2005) summarized the contribution of ROS in the pathogenesis of cardiac injury and as mediator of protection (93). Exogenous application of ROS was shown to trigger PKC activation and to reduce the extent of necrosis after I/R-injury (94). Several studies provided evidence that increased levels of ROS are present during the preconditioning stimulus with volatile anesthetics (25,95). In addition, anesthetic preconditioning with volatile anesthetics can be abolished, when ROS is scavenged during the preconditioning stimulus (2,95-98). Interestingly, scavenging of ROS during preconditioning with volatile anesthetics attenuated anesthetic-induced PKC activation (3). This indicates that the production of ROS precedes

PKC activation and implies that elevated ROS during preconditioning serves as trigger in cardioprotective signaling (83).

The mechanism as well as the source of ROS during volatile anesthetic exposure has not completely been resolved, but a major contribution of the mitochondria has been suggested. Volatile anesthetics may temporarily attenuate the mitochondrial electron transport chain (ETC) (see figure 1.3) (93). Experiments in isolated cardiac mitochondria showed that volatile anesthetics may inhibit complex I as well as complex III of the ETC, providing a possible source of ROS (99,100). In addition, opening of the mitoK^+_{ATP} channels contributes to ROS production and indeed, some studies provided evidence that anesthetic-induced ROS production is due to opening of the mitoK^+_{ATP} channels (101), although this was recently opposed by others (see also paragraph *ATP-sensitive K^+ channels*) (25). Riess *et al*. (2004) showed that sevoflurane-induced inhibition of the mitochondrial respiratory complexes in isolated mitochondria was partly ROS dependent (102). This suggests that cardioprotective signaling may rely on ROS-induced ROS production via feed forward signaling steps and illustrates the complexity of cardioprotective signaling cascades.

ATP-sensitive K^+ channels

Cardiomyocytes contain two types of ATP-sensitive K^+ channels, which are expressed in the sarcolemma (sarcK^+_{ATP} channels) and mitochondria (mitoK^+_{ATP} channels), respectively. These channels are pore-forming transmembrane channels and allow cellular K^+-efflux (via sarcK^+_{ATP} channels) or mitochondrial K^+ influx (via mitoK^+_{ATP} channels) in response to alterations in ATP and ADP levels. SarcK^+_{ATP} channels as well as mitoK^+_{ATP} channels are both involved in cellular responses to metabolic alterations, and both have been implicated as end-effector proteins of cardioprotective signaling (see paragraph: "*mechanism of protection: end-effector proteins"*). However, a triggering role especially for mitoK^+_{ATP} channels has been suggested (103). The production of ROS is suggested as the link between opening of mitoK^+_{ATP} channels and cardioprotective signaling (57). However, studies on the role of mitoK^+_{ATP} channels in relation with ROS production have been conflicting, as previously mentioned (23,101).

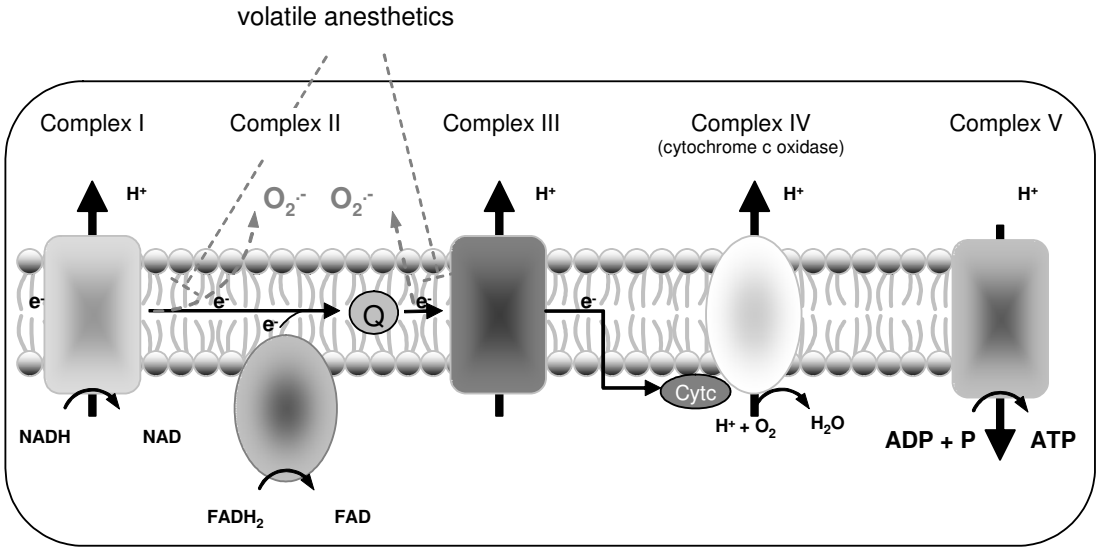


Figure 1.3 Effect of volatile anesthetics on the electron transport chain leading to formation of reactive oxygen species. Oxidation of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) by respectively complex I and complex II provide electrons to reduce O_2 to H_2O. ATP synthesis occurs at complex V via the proton gradient established via active proton transport to the intermembrane space through complex I, III and complex IV. Volatile anesthetics are reported to inhibit complex I and complex III. Electrons will leak from the tightly controlled respiratory chain and will form reactive oxygen species. NADH=nicotinamide adenine dinucleotide, $FADH_2$=flavin adenine dinucleotide, Q=ubiquinone, CytC=cytochrome C.

Other Important Mediators of Cardioprotective Signaling

In addition to ROS, PKC and mitoK^+_{ATP} channels, volatile anesthetic-induced cardioprotective signaling seems to rely on a variety of other signaling molecules. These include G-protein coupled receptors (like adenosine receptor (104), opioid receptors (105) and α- and β-adrenoreceptors (106)), G-proteins (79,107) in addition to other kinase dependent pathways like the phosphatidylinositol-3-kinase (PI3-kinase)/protein kinase B (PKB) signaling pathway (108,109), nitric oxide (NO) synthase/NO/cyclic guanine monophosphate (cGMP)/protein kinase G (PKG)-pathway (79) and protein tyrosine kinase signaling pathway (110,111). At this moment the exact interaction between the different protective signaling pathways remains unclear.

Mechanisms of Protection: End-effector Proteins

The end-effector mechanisms that indeed result in cardioprotection as induced by volatile anesthetics remain unknown and speculative. However, reduction of cellular Ca^{2+} overload, ROS production and preservation of mitochondrial function have been associated with improved contractility, metabolic function and a reduction of infarct size, and may form the main end-effector targets of cardioprotective signaling pathways (25,68,96,97,112).

Reduced cellular Ca^{2+} loading due to volatile anesthetics has been attributed to the depressant effects on the various elements of the myocardial Ca^{2+} homeostasis (see: "*effect of anesthetics on excitation-contraction coupling"*). Volatile anesthetics reduce cardiac SR Ca^{2+} release and thereby prevent SR-mediated Ca^{2+} oscillations with subsequent Ca^{2+} overload (113). Interestingly, Yamamura *et al.* (2005) showed that PKC activation resulted in a decrease in SR Ca^{2+} load, and therefore suggested this as an end-effector mechanism in cardioprotective signaling pathways (89). Inhibition of the L-type Ca^{2+} channels as well as facilitated opening of the sarcK^{+}_{ATP} channels have been demonstrated to occur after anesthetic exposure and limits the influx of Ca^{2+} via the sarcolemma due to shortening of the action potential (114,115). Interestingly, it has recently become evident that the effects of volatile anesthetics on sarcK^{+}_{ATP} are also dependent on activation of PKC (116), even after washout of the anesthetic agent (91).

In contrast to elevation of ROS during anesthetic preconditioning, ROS production during and after I/R is demonstrated to be reduced in preconditioned hearts and may contribute to anesthetic-induced cardioprotection (25,97). During I/R the mitochondria are the major source of ROS (117,118). Therefore, preservation of mitochondrial bioenergetics is suggested as the underlying mechanism for reduced production of ROS in preconditioned hearts. However, the effects of other sources of ROS like xanthine oxidase or neutrophils cannot entirely be excluded. Indeed, sevoflurane and isoflurane inhibit NADPH-oxidase in neutrophils, leading to reduced neutrophil-induced ROS production during I/R, thereby contributing to anesthetic-induced cardioprotection (119).

As mentioned before, another possible target for cardioprotective signaling pathways is the preservation of mitochondrial function. Intact mitochondrial membranes are a prerequisite for optimal cardiomyocyte function. During ischemia, mitochondrial Ca^{2+} accumulation is accompanied with increased oxidative stress, alterations in intermembrane and mitochondrial

matrix volume and reduced ATP production (57,120). Finally, a nonselective mitochondrial membrane permeabilization occurs via opening of the mitochondrial permeability transition pore (PTP), leading to dissipation of the mitochondrial membrane potential and subsequent cell death.

MitoK^+_{ATP} channels are involved in the regulation of mitochondrial volume, energy production, formation of ROS and the mitochondrial membrane potential. Opening of mitoK^+_{ATP} channels slightly reduces the mitochondrial membrane potential and therefore alters the driving force for mitochondrial Ca^{2+} uptake. Piriou *et al*. (2004) showed that this increased the resistance to Ca^{2+}-induced opening of the PTP via a mitoK^+_{ATP} channel-dependent mechanism (121). Interestingly, in preconditioned cardiomyocytes mitochondrial Ca^{2+} overload is reduced via a mitoK^+_{ATP} channel-dependent mechanism (122). Furthermore, mitoK^+_{ATP} channel opening favorably preserves the architecture of the mitochondrial intermembrane space augmenting the efficacy of energy production and ATP transport to the cytosol (57). As a consequence, ATP-availability is increased and production of ROS is reduced, which both contribute to cardioprotection. Indeed, anesthetic preconditioning with volatile anesthetics has been shown to preserve ATP synthesis (96) and to attenuate ROS production during reperfusion (25).

Finally, several other mechanisms providing cardioprotection have been proposed, like reduced membrane damage, reduced cytoskeletal fragility, reduced Ca^{2+} sensitivity thereby preventing hypercontracture, preservation of cardiac (SR) Ca^{2+} handling and a reduced activation of pro-apoptotic signaling pathways (50,112,123,124).

CLINICAL IMPLICATIONS

Despite the interesting progress on the mechanism of cardioprotective signaling in experimental animal studies, the most important question is whether the phenomenon of preconditioning can be used in the development of clinical cardioprotective strategies. The phenomenon of ischemic preconditioning in patients was soon reported after the introduction of the concept in animal studies (125,126). However, the choice of anesthetic agents during surgery was generally believed not to influence the occurrence of ischemic episodes (127).

Nevertheless, in vitro experiments in human isolated atrial trabeculae showed enhanced contractile recovery due to pretreatment with volatile anesthetics (104,106,128). Interestingly, the cardioprotective signaling cascade seems to rely on similar signal transduction elements as ischemic preconditioning, like PKC, ROS as well as mitoK^+_{ATP} channels (104,106,128-131). Several clinical studies that focus on the cardioprotective effect of volatile anesthetics were performed in patients scheduled for coronary artery surgery. As part of their treatment, these patients are subjected to myocardial ischemia during surgery providing the opportunity for anesthetic pretreatment before ischemia (aortic cross-clamp) occurs. Ramsay *et al*. (1994) reported that a volatile anesthetic-based anesthesia during cardiopulmonary bypass graft surgery (CABG) reduces the incidence of perioperative infarction (132). The first clinical studies evaluating a classical preconditioning stimulus with volatile anesthetics showed slight reduction in plasma markers of necrosis (troponin I and creatinin kinase MB), but failed to reach significance (133-135). However, in the study of Penta de Peppo *et al*. (1999) patients anesthetized with sevoflurane showed evidence of improved contractility of the left ventricle as demonstrated by pressure-area relations (135). De Hert *et al*. (2002) were the first to demonstrate that patients receiving sevoflurane during coronary artery surgery had a better post-surgical cardiac function and reduced plasmalevels of TnI compared to patients subjected to an intravenous anesthetic regimen (136). In subsequent studies, they showed that these beneficial effects were also detectable in high risk patients with reduced cardiac function (137). Furthermore, they found that anesthesia with sevoflurane reduced the length of stay in the ICU and in the hospital as compared to propofol anesthesia (138). It was advocated that the volatile anesthetic should be present for the whole duration of the procedure, since sevoflurane pretreatment or institution during reperfusion did not provide similar protection (139). However, Julier *et al*. (2003) included 72 patients in a multi-center study and showed that sevoflurane pretreatment reduced postoperative brain natriuretic peptide (BNP) plasma levels, suggestive for a reduction of postoperative LV dysfunction (85). In addition, in atrial appendages it was elegantly shown that after preconditioning, PKC-δ translocated to the sarcolemma and PKC-ε to the intercalated disks. However, sevoflurane-treated patients also received significantly more phenylephrine to maintain blood pressure. As α-adrenergic stimulation is cardioprotective via activation of PKC, these observations might be biased (140). Therefore, the recent study of Fraβdorf *et al*. (2005) in patients undergoing coronary

artery bypass grafting is the first study showing that sevoflurane pretreatment can reduce postoperative TnI plasma levels significantly, indicating a reduction in myocardial necrosis (141). The phenomenon of cardioprotection by volatile anesthetics may be relevant for clinical practice, as patients are anesthetized with this group of anesthetics as part of daily anesthetic practice. Interestingly, other commonly used agents during anesthesia, like propofol and opioids have also been reported to exert cytoprotective effects (93,142), but the clinical cardioprotective effect of propofol - when compared to volatile anesthetics - seems to be limited (136). Interestingly, in experimental studies morphine was shown to enhance the efficacy of the cardioprotective response induced by volatile anesthetics providing important clinical therapeutic options (105). These observations may significantly contribute to the development of balanced anesthesia techniques. Furthermore, it may be interesting to speculate how the anesthesia technique may exceed the purpose of cardioprotection and may be extended to modulate the general response to surgical stress or protect several organ systems. It is interesting to realize that the choice of the anesthetic regimen may modify the cytokine production in response to surgery (143), and therefore this protection may extend beyond the operating room. Interestingly, the observation that sevoflurane favorably alters the hemodynamic profile in septic rats (unpublished observations) may suggest that the choice of anesthetics may influence the management of septic patients in the operating room but also at the intensive care unit.

However, in consideration of the existing controversies, as mentioned before, upon the clinical induction of protection by anesthetics, the need for large well-designed multi-center trials has been stressed by various investigators in order to develop effective clinical cardioprotective strategies (144,145).

FUTURE DIRECTIONS

The clinical relevance of preconditioning may be questioned for several reasons. Preconditioning-induced cardioprotection is elicited by applying a protective stimulus before I/R will occur. In clinical practice, the occurrence of ischemic episodes may most often not be predicted. This is the reason that many investigators now focus on postconditioning, thereby

applying the protective stimulus during the reperfusion phase. Recently, it was shown that brief ischemic periods during the first minutes of reperfusion afford the same amount of protection compared to precondition. Zhao *et al*. (2003) defined this phenomenon as 'postconditioning', although the beneficial effects of volatile anesthetics during reperfusion were already described in earlier studies (72,146,147). Postconditioning seems to depend on similar signaling pathways as preconditioning, and was recently demonstrated to be cardioprotective in a clinical study performed by Staat *et al*. (2005) (148). This study may be an important step in the development of clinical cardioprotective strategies that can be applied after the ischemic phase.

Preconditioning has been called a phenomenon of the healthy heart, as several studies showed that the diseased heart is not effectively protected by preconditioning (149,150). This is disappointing, as diseased myocardium would benefit the most from cardioprotective measures. Several studies aim to address this issue, however, our understanding remains limited. Therefore, studies with specific focus on the mechanism of cardioprotection in the diseased heart would greatly enhance the clinical potential.

In summary, the last two decades of cardiac research have greatly improved our understanding regarding the interaction of volatile anesthetics and the ischemic heart. This group of anesthetics has been shown to trigger distinct cardioprotective signaling cascades, which provides the heart with an increased resistance against ischemic episodes. Despite the increased understanding of the responsible mechanisms, our knowledge has to be extended in order to implement volatile anesthetics for this purpose in the clinical situation. However, the results remain promising, and suggest a possible new therapeutic action for volatile anesthetics.

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AIM OF THIS THESIS

In the present thesis the mechanisms of the cardioprotective properties of the volatile anesthetic sevoflurane were investigated. The benefical effects of sevoflurane preconditioning on cardiac function during ischemia an reperfusion (I/R) were studied in an *in vitro* experimental setup consisting of isolated rat trabeculae subjected to a standardized I/R-protocol. We specifically focused on the role and interaction of the production of reactive oxygen species (ROS), protein kinase C (PKC) and the ATP sensitive mitochondrial K^+ ($mitoK^+_{ATP}$) channels. The specific aims of the chapters of this thesis are briefly summarized here.

Outline of the Dissertation

In **chapter 2** the involvement of production of ROS, PKC and $mitoK^+_{ATP}$ channels in sevoflurane-induced preconditioning was studied in two different experimental ischemic protocols: metabolic inhibition with cyanide *versus* simulated ischemia with hypoxia. The aim of this study was to investigate whether the ischemic stimulus may alter the contribution of specific signaling molecules to the protective signaling cascade.

The study described in **chapter 3** focused on the contribution of PKC-δ (a novel PKC-isoform) in sevoflurane-induced protective signaling. We used pharmacological inhibition in combination with immunofluorescent analysis of PKC-δ activation and ROS production to establish the role of the interaction between PKC-δ and production of ROS in sevoflurane-induced preconditioning.

We subsequently show in **chapter 4** that PKC-α, a Ca^{2+}-depedendent PKC-isoform, is also essentially involved in sevoflurane-induced preconditioning. We show evidence that PKC-α activation involves ROS production and is independent of PKC-δ and $mitoK^+_{ATP}$ channels, suggesting parallel activation of the PKC isoforms.

Chapter 5 demonstrates that an active Na^+/Ca^{2+}-exchanger (NCX) is essential in the protective signaling cascade elicited by sevoflurane preconditioning. In this chapter we specifically show that sevoflurane is able to facilitate Ca^{2+} influx via the reverse mode of the NCX and that sevoflurane-induced PKC-δ activation is preceded by the reverse mode of the NCX.

In **chapter 6** we characterize the contractile function of trabeculae of insulin-resistant rats. To increase clinical relevance, insulin-resistance/type II diabetes was induced in normal rats subjected to a long-term high-fat diet. In this chapter we present our preliminary data on the efficacy of sevoflurane-induced cardioprotection in these hearts.

Chapter 7 describes the main conclusions and discusses the results and clinical implications of the data presented in this thesis.

Chapter 2

THE MECHANISM OF SEVOFLURANE-INDUCED CARDIOPROTECTION IS INDEPENDENT OF THE APPLIED ISCHEMIC STIMULUS IN RAT TRABECULAE

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ABSTRACT

Sevoflurane protects the myocardium against ischemic injury through protein kinase C (PKC) activation, adenosine triphosphate-sensitive mitochondrial K^+ ($mitoK^+_{ATP}$) channel opening and reactive oxygen species production (ROS). However, it is unclear whether the type of ischemia determines the involvement of these signaling molecules. We therefore investigated whether hypoxia (HYP) or metabolic inhibition (MI), which differentially inhibit the mitochondrial electron transport chain (ETC), are comparable concerning the relative contribution of PKC, $mitoK^+_{ATP}$ channels and ROS in sevoflurane-induced cardioprotection.

Rat right ventricular trabeculae were isolated and isometric contractile force (F_{dev}) was measured. Trabeculae were subjected to HYP (hypoxic glucose-free buffer; 40 minutes) or MI (glucose-free buffer, 2 mM cyanide; 30 minutes), followed by 60 minutes recovery. Contractile recovery ($F_{dev,rec}$) was determined at the end of the recovery period and expressed as a percentage of F_{dev} before hypoxia or MI, respectively. Chelerythrine (6 μM), 5-hydroxydecanoic acid (100 μM) and n-(2-mercaptopropionyl)-glycine (300 μM) were used to inhibit PKC, $mitoK^+_{ATP}$ channels and ROS, respectively.

$F_{dev,rec}$ after HYP was reduced to 47±3% (P<0.001 *versus* Time control; n=5) whereas MI reduced $F_{dev,rec}$ to 28±5% (P<0.001 *versus* Time control; n=5). A 15 minutes period of preconditioning with sevoflurane (3.8%) equally increased contractile recovery after HYP (76±9%; P<0.05 *versus* HYP) as well as MI (67±8%; P<0.01 *versus* MI). Chelerythrine, 5-hydroxydecanoic acid as well as n-(2-mercaptopropionyl)-glycine abolished the protective effect of sevoflurane in both ischemic models. Trabeculae subjected to HYP or MI did not demonstrate any apoptotic or necrotic markers.

PKC, $mitoK^+_{ATP}$ channels and ROS are involved in sevoflurane-induced cardioprotection after HYP or MI, suggesting that the means of mitochondrial ETC inhibition does not determine the signal transduction of cardioprotection by anesthetics.

INTRODUCTION

The volatile anesthetic sevoflurane protects the heart against ischemia-induced adenosine triphosphate (ATP) depletion, Ca^{2+} overload and oxidative stress through activation of protein kinase C (PKC), opening of adenosine triphosphosphate sensitive mitochondrial K^+ ($mitoK^+_{ATP}$) channels and the production of reactive oxygen species (ROS) (1-6).

We previously simulated ischemia by cyanide-induced metabolic inhibition (MI) (4,7). Cyanide inactivates cytochrome c oxidase in complex IV of the mitochondrial electron transport chain (ETC), resulting in ATP depletion and ROS formation (figure 2.1) (8,9).

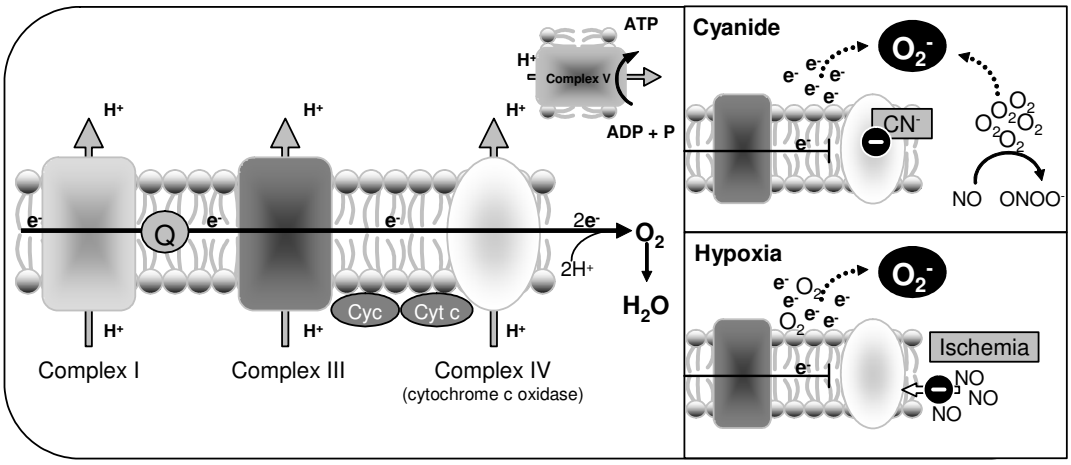

Figure 2.1 Simplified scheme of the mitochondrial electron transport chain (ETC) and the effects of cyanide and hypoxia on electron transport, adenosine triphosphate (ATP) and reactive oxygen species (ROS) production. The ETC is located in the inner mitochondrial membrane and consists of different complexes, which contain the different elements of the chain. The ETC simultaneously transfers electrons and protons, resulting in oxidative phosphorylation and ATP synthesis at complex V. In complex IV, electrons are donated to O_2, which reacts with $2H^+$ into H_2O. Electron transport and oxidative phosphorylation are coupled, thus inhibition of electron transport leads to ATP depletion. Cyanide (CN^-) combines with the oxidized heme iron in cytochrome c oxidase, thereby preventing reduction of this oxidase. This results in the inhibition of the ETC and thus ATP depletion and Ca^{2+} overload. The O_2 molecules react with free electrons derived from the ETC, resulting in the generation of ROS like O_2^- (superoxide). During ischemia, the availability of the electron acceptor O_2 is reduced. This prevents complex IV from donating electrons and therefore inhibits ATP production. Furthermore, the lower availability of O_2 leads to more nitric oxide (NO), which also inhibits complex IV and reduces O_2 consumption, resulting in the generation of superoxide and $ONOO^-$ (peroxynitrite).

In contrast, hypoxia is associated with a reduction in the O_2 concentration and thereby prevents complex IV from donating electrons, finally leading to ATP depletion. Furthermore,

hypoxia coincides with more mitochondrial nitric oxide (NO), which also inhibits complex IV and reduces mitochondrial O_2 consumption, the latter resulting in superoxide generation (10,11). Finally, hypoxia, but not MI, is followed by reoxygenation, which may cause additional cardiac injury (12).

It has already been shown that the type of preconditioning stimulus determines the relative contribution of distinct signaling molecules in the cardioprotective process (13) and thus it may very well be possible that the type of metabolic deprivation induces variation in the signal transduction pathways involved in sevoflurane-induced cardioprotection.

We therefore investigated whether two different stimuli of ischemic injury, i.e. MI and hypoxia, alter the relative contribution of PKC, mitoK^+_{ATP} channels and ROS in the signal transduction of sevoflurane-induced cardioprotection in isolated right ventricular rat trabeculae (4). This study provides more insight in the effect of sevoflurane on altered mitochondrial respiratory function during cardiac ischemia.

MATERIALS AND METHODS

Animals

Male Wistar rats (250-400 gram, Harlan, Horst, the Netherlands) were used according to the Institutional Animal Care and Use Committee of the VU University Medical Center. Rats were anesthetized with sodium pentobarbital (80 mg/kg, intraperitoneally, Nembutal®, Sanofi Sante BV) and intravenously heparinized with 1000 units of heparin (Leo Pharma, Breda, Netherlands). Subsequently, the heart was quickly removed and perfused through the aorta with Tyrode buffer (120 mM NaCl, 1.22 mM $MgSO_4.7H_20$, 1.99 mM NaH_2PO_4, 27.0 mM $NaHCO_3$, 5.0 mM KCl, 1 mM $CaCl_2$ and 10 mM glucose; 95% O_2 / 5% CO_2, pH 7.4). The myocardium was protected during dissection by adding 30 mM 2,3-butanedione monoxime and 15 mM KCl to the buffer solution (14). A suitable right ventricular trabecula (length 2-5 mm, diameter<0.2 mm) was carefully dissected (15).

Experimental Setup

The muscle was mounted between a force-transducer (AE801, SensoNor, Norway) and a micromanipulator in an airtight organ bath. After mounting, superfusion was started with normal Tyrode buffer at 27ºC (2 ml/min), and trabeculae were stimulated (5 ms duration, stimulation frequency 0.5 Hz). Subsequently, trabeculae were stretched until passive force (F_{pas}) was approximately 8% of developed force (16). After 40 minutes of stabilization, the stimulation frequency and temperature were decreased to 0.2 Hz and 24ºC, respectively, followed by another 20 minutes of stabilization. After this period, initial developed force of contraction ($F_{dev,start}$) and maximal force ($F_{max,start}$), as determined by a post-extrasystolic potentiation protocol (PESP), were recorded. PESP determines the contractile reserve of trabeculae by maximal Ca^{2+} filling of the sarcoplasmic reticulum (17). Trabeculae failing to stabilize, failing to show PESP or spontaneously contracting trabeculae were excluded.

Experimental Protocol

Figure 2.2 shows the experimental groups to which trabeculae were assigned. Trabeculae (except for time controls; n=5) were either subjected to a period of hypoxia (HYP; n=5) or metabolic inhibition (MI; n=5). During hypoxia, trabeculae were superfused with hypoxic Tyrode (95% N_2 and 5% CO_2, $pO_2 \leq 4$ mm Hg), whereas during MI muscles were superfused with buffer containing 2 mM sodium cyanide. During hypoxia or MI, glucose was omitted from the buffer and the stimulation frequency was increased to 1 Hz. Shortly after starting hypoxia or MI, contractile force decreased to 0 followed by an increase in F_{pas}. When F_{pas} increased to 50% of $F_{max,start}$, trabeculae were subjected for another 40 minutes to hypoxia or for 30 minutes to MI. Subsequently, muscles were perfused with normal oxygenated buffer for 60 minutes to allow contractile recovery. The recovery of F_{dev} ($F_{dev,rec}$), F_{max} ($F_{max,rec}$), time to peak, time to half relaxation, and rate of contraction (+dFdt) and relaxation (-dFdt) were determined and expressed as a percentage of the initial values during the start of experiment. Except for time and inhibitor control experiments, trabeculae were preconditioned for 15 minutes with normal Tyrode saturated with 3.8vol % vaporized sevoflurane (Sevorane®, Abbott, Hoofddorp, the Netherlands) 30 minutes prior to hypoxia or MI. The volume percentage of sevoflurane in the gas phase above the Tyrode was continuously monitored by a calibrated infrared anesthetic monitor (Capnomac Ultima, Datex, Helsinki, Finland). After

washout of sevoflurane, trabeculae were superfused for 15 minutes with normal Tyrode until hypoxia or MI. In the inhibitor groups, trabeculae were additionally superfused with either the PKC-catalytic site inhibitor chelerythrine (CHEL; 6 µM; n=5; Sigma-Aldrich), the mitoK^+_{ATP} channel blocker 5-hydroxydecanoic acid (5-HD; 100 µM; n=5; Sigma-Aldrich) or the ROS-scavenger n-(2-mercaptopropionyl)-glycine (MPG; 300 µM n=5; Sigma-Aldrich) 10 minutes prior to preconditioning until the washout period before hypoxia or MI (see figure 2.2). The effects of inhibitors alone on $F_{dev,rec}$ were studied in separate inhibitor control experiments for both ischemic conditions.

Determination of Necrosis and Apoptosis

The extent of necrosis and apoptosis was evaluated by cell morphology studies using

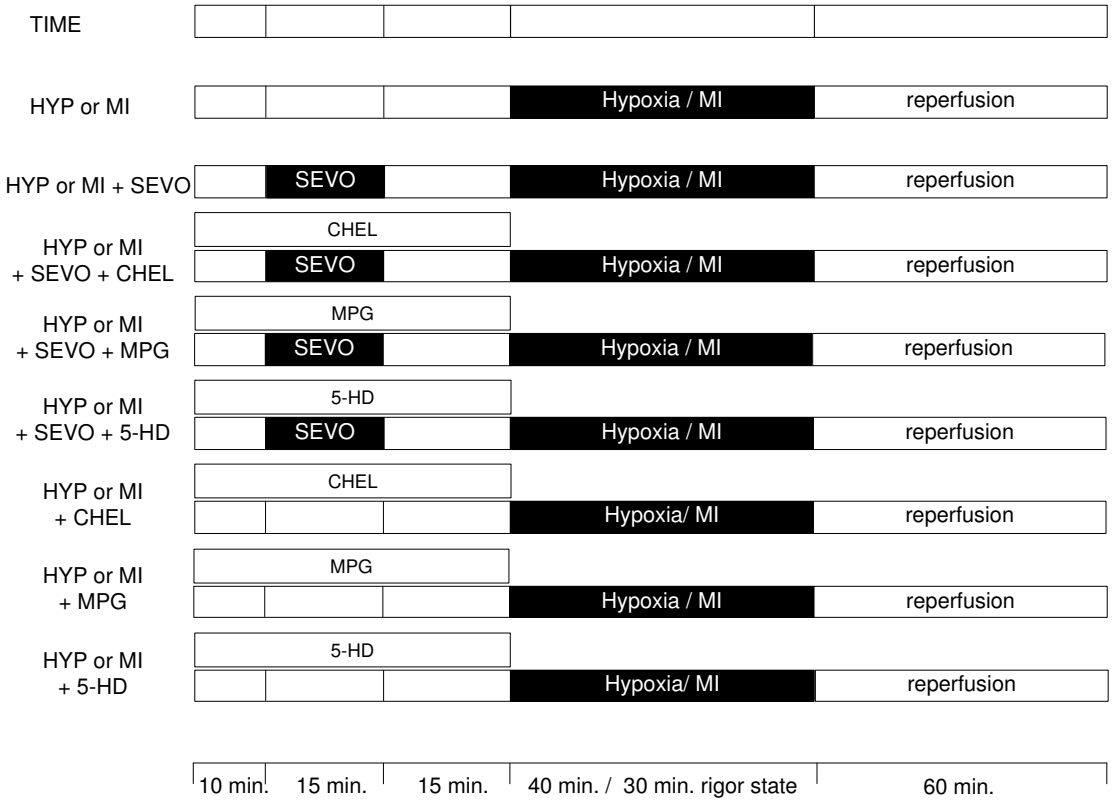

Figure 2.2 Scheme for the 15 experimental groups of randomized isolated trabeculae. HYP=control hypoxia and recovery (sustained for 40 min after the time to rigor), MI=control metabolic inhibition (sustained for 30 min after the time to rigor), SEVO=sevoflurane, CHEL=chelerythrine, MPG=N-(2-mercaptopropionyl)-glycine, 5-HD=5-hydroxydecanoic acid, TIME=time control.

hematoxilin eosin staining, histochemical staining of myoglobin and TdT-mediated dUTP nick-end labeling (TUNEL) staining for evaluation of DNA fragmentation. All trabeculae subjected to these measurements were immediately embedded in gelatin after 60 minutes of recovery following either hypoxia or MI, frozen in liquid nitrogen and stored in -80°C until use. Cross-sections (5 μm) were histochemically stained for myoglobin as previously described by Lee-de Groot, des Tombe and van der Laarse (1998) (18). In a separate set, TUNEL staining was performed with the Dead End Fluorometric TUNEL labeling kit (Promega). These sections were counterstained with 10% (v/v) wheat germ agglutinin (WGA; W-7024, Molecular probes, Invitrogen, Breda, The Netherlands) to provide sarcolemmal staining and mounted on glass cover slips using 4',6 diamidino-2-phenylindole-containing medium (H1200, Vectashield, Vector Laboratories, Burlingame, USA) to stain the nuclei. Sections stained for hematoxilin eosin and TUNEL were analyzed by Digital Imaging fluorescence Microscopy (DIM) equipped with a ZEISS Axiovert 200 Marianas^{TM} inverted digital imaging microscopy workstation. Images were recorded with a cooled CCD camera (Cooke Sensicam [Cooke Co., Tonawanda, NY, USA], 1280x1024 pixels). The digital imaging microscopy workstation was under full software control (SlideBook^{TM} software version 3.11, Intelligent Imaging Innovations, Denver, CO, USA).

Statistical Analysis

The sample size of each experimental group was 5, except the inhibitor control groups (n=4). Data were tested for normal distribution and one way analysis of variance (ANOVA) followed by a Tukey post-hoc analysis or a Students T-test, when appropriate, was performed to determine differences between the experimental groups. A P-value$\leq$0.05 was considered to reflect a significant difference. All values are given as means±SEM.

RESULTS

General Characteristics

Time to rigor development was similar for both the HYP and MI group and was only minimally (not significantly) affected by sevoflurane pretreatment or the inhibitors (table 2.1). In the time control group, F_{dev} was reduced after 3 hours reduced to 80±9%. Figure 2.3 shows the injury induced by hypoxia or MI. $F_{dev,rec}$ was reduced to 47±3% (P<0.05 *versus* [Time Control]) and to 27±6% (P<0.001 *versus* [Time Control]) in hypoxia and MI-groups, respectively.

Table 2.1 Time to Rigor Development in the Different Experimental Groups.

	Time to rigor (minutes)
TIME CONTROL	-
HYP	26 ± 4
HYP + SEVO	29 ± 4
HYP + SEVO + CHEL	26 ± 1
HYP + CHEL	24 ± 2
HYP + SEVO + 5HD	25 ± 2
HYP + 5HD	27 ± 1
HYP + SEVO + MPG	35 ± 4
HYP + MPG	32 ± 5
MI	30 ± 3
MI + SEVO	34 ± 5
MI + SEVO + CHEL	34 ± 4
MI + CHEL	28 ± 3
MI + SEVO + 5HD	36 ± 2
MI + 5HD	27 ± 4
MI + SEVO + MPG	30 ± 1
MI + MPG	31 ± 6

Data presented as mean±SEM, n=5 per group. The time to rigor development was not different between HYP and MI. The inhibitors (CHEL, and 5HD and MPG) itself did not affect the time to rigor. HYP=hypoxia, SEVO=sevoflurane, CHEL=chelerythrine, 5HD=5-hydroxydecanoic acid, MPG=n-(2-mercaptopropionyl)-glycine.

Preconditioning with Sevoflurane

Preconditioning with sevoflurane equally reversed the hypoxia- as well as the MI-induced decrease in $F_{dev,rec}$ to 76±9% for hypoxia (P<0.05; [HYP+SEVO] *versus* [HYP]) and 72±7% for MI (P<0.001; [MI+SEVO] *versus* [MI]) (see Fig 2.3). PKC inhibition by chelerythrine completely abolished the protective response of sevoflurane during hypoxia (37±5%; P<0.01 for [HYP+SEVO+CHEL] *versus* [HYP+SEVO] and during MI (31±5%; P<0.001 for [MI+SEVO+CHEL] *versus* [MI+SEVO]) (figure 2.3). In addition, either inhibition of mitoK^+_{ATP} channels by 5-HD or scavenging ROS by MPG completely abolished sevoflurane-induced cardioprotection during hypoxia as well as MI.

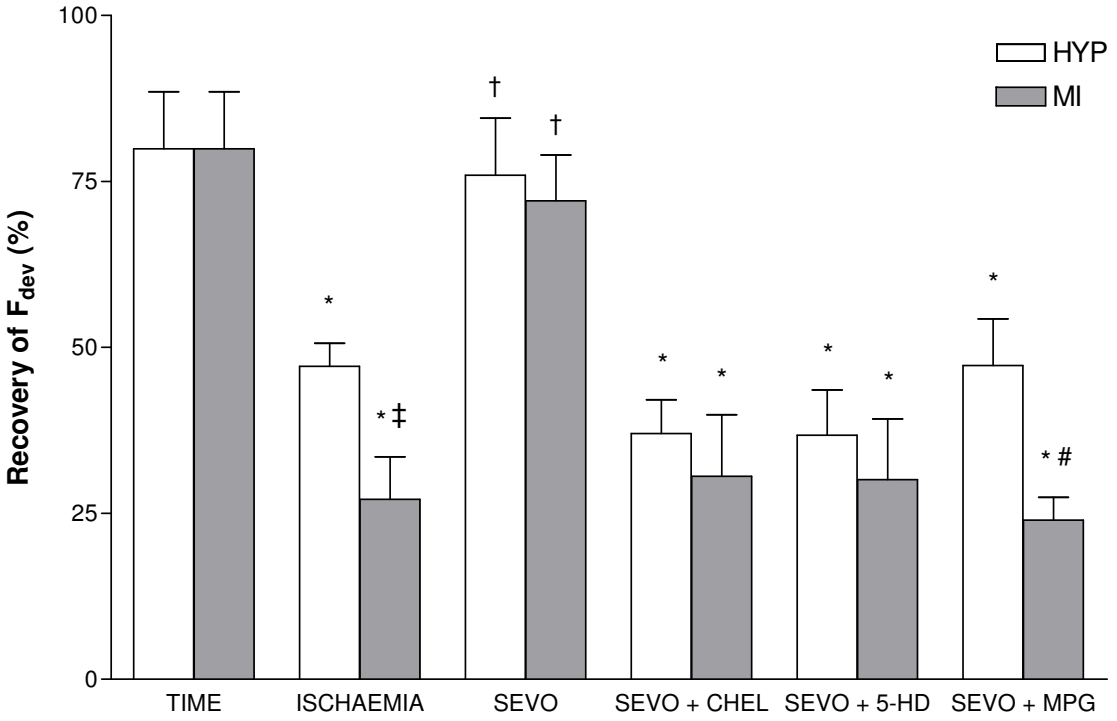

Figure 2.3 Developed force after recovery as a percentage of the initial developed force before the start of the experimental protocol ($F_{dev,rec}$) of HYP, HYP+SEVO, MI and MI+SEVO groups in combination with chelerythrine (CHEL; 6 μM), 5-hydroxydecanoic acid (5-HD; 100 μM) and n-(2-mercaptopropionyl)-glycine (MPG; 300 μM). The HYP protocol as well as the MI protocol reduced the $F_{dev,rec}$, and preconditioning of the trabeculae before metabolic deprivation completely restored the $F_{dev,rec}$ in both groups. The protective effect of sevoflurane was abolished in both HYP and MI groups by CHEL, 5-HD and MPG. HYP=hypoxia, MI=metabolic inhibition, F_{dev}=developed force, SEVO=sevoflurane. * indicates P<0.05 compared to TIME CONTROL, † indicates P<0.05 compared to ISCHEMIA, SEVO+CHEL, SEVO+5-HD and SEVO+MPG, ‡ indicates P<0.05 compared to HYP, # indicates P<0.05 compared to HYP+SEVO+MPG.

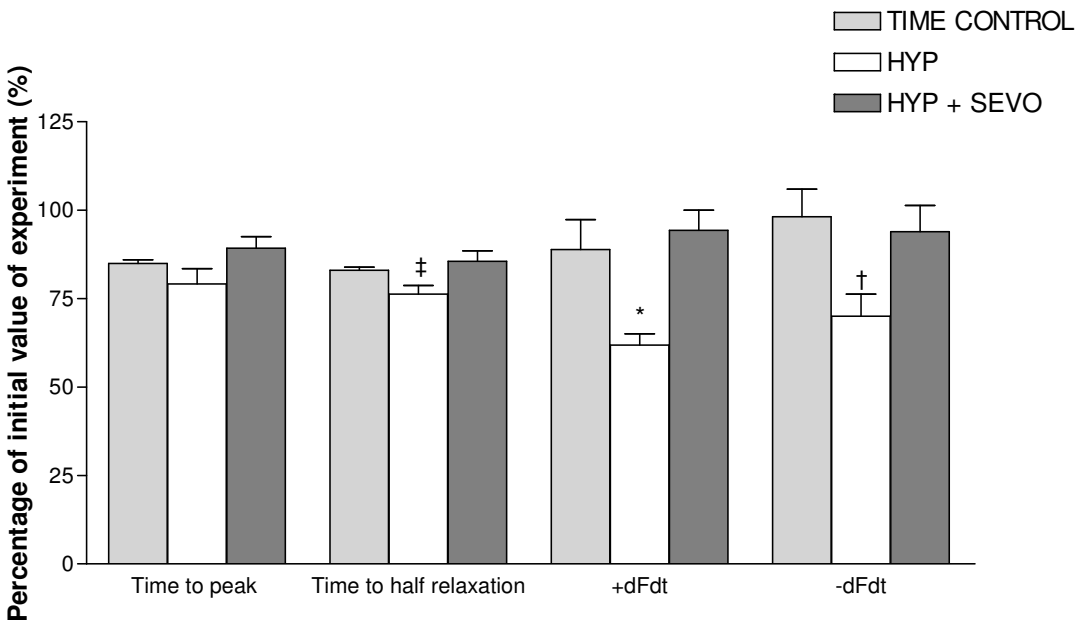

Figure 2.4 Time to peak, time to half relaxation, +dFdt and -dFdt expressed as a percentage of the initial values at the start of the experimental protocol in time controls and the HYP and HYP+SEVO groups. HYP tended to reduce the time to peak and time to half relaxation. Pretreatment with sevoflurane slightly prolonged the time to half relaxation. Interestingly, preconditioning with sevoflurane completely restored the decrease in positive dFdt because of HYP. * indicates $P<0.05$ compared to TIME CONTROL and HYP+SEVO, † indicates $P<0.05$ compared to TIME CONTROL, ‡ indicates $P<0.05$ compared to HYP+SEVO.

The inhibitors established no intrinsic cardioprotective effects and did not affect the F_{dev} (data not shown).

Figure 2.4 shows additional contractile parameters of the hypoxia group including time to peak, time to half relaxation and the rate of contraction (+dFdt) and relaxation (-dFdt). Values are expressed as a percentage of the initial value at the start of the experiment. Hypoxia did not reduce the time to peak (79±4% [HYP] *versus* 85±1% [Time Control]; $P>0.05$) and the time to half relaxation (76%±2% [HYP] *versus* 83±1% [Time Control]; $P>0.05$). However, preconditioning increased the time to half relaxation slightly, but significantly (85±3% [HYP+SEVO] *versus* 76±2% [HYP]; $P<0.05$). Interestingly, hypoxia reduced the +dFdt (62±3% [HYP] *versus* 89±8% [Time Control]; $P<0.01$) as well as the -dFdt (70±6% [HYP] *versus* 98±8% [Time Control]; $P<0.05$). The decrease in +dFdt was completely reversed by sevoflurane (94±6%; $P<0.01$ *versus* [HYP]). Although the decrease in –dFdt tended to be higher in preconditioned trabeculae, this difference was not significant compared to the hypoxia group (94±7%; $P>0.05$ *versus* [HYP]).

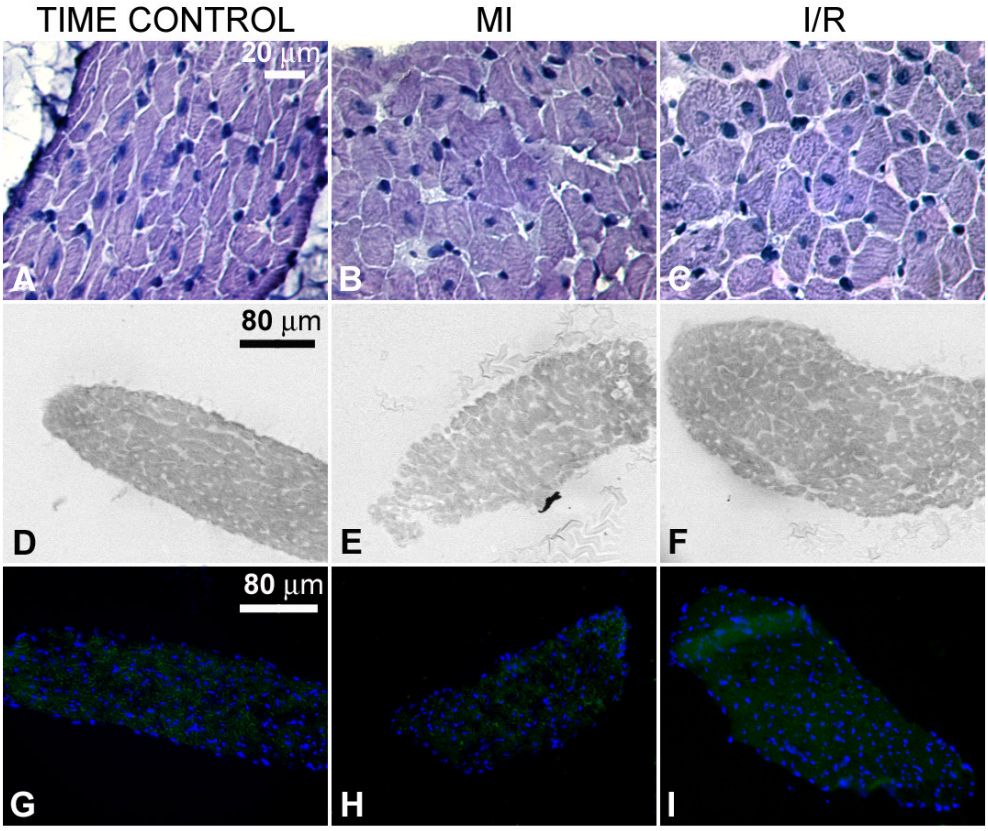

Figure 2.5 Microscopic analysis of markers for necrosis and/or apoptosis in cross-sectional sections of isolated trabeculae. Hematoxilin eosin staining in panel A through C showed in all sections an intact cellular morphology with no signs of disruptions of the plasma membranes, cytosolic vacuolization or pyknosis of nuclei. In panels D through F, the histochemical staining the amount of myoglobin correlates with the shades of gray. No difference in myoglobin content was detected in trabeculae subjected either to MI or HYP compared to time controls. Representative images of the TUNEL analysis are shown in panel G through I. Blue fluorescence shows DAPI staining of the nuclei. Double-stranded DNA breakage in this TUNEL-analysis is detected by green fluorescence. In trabeculae subjected to MI or HYP showed no altered numbers of positive nuclei compared to time controls, as is shown by the absence of nuclei displaying both blue and green fluorescence.

Determination of Necrosis and Apoptosis

Figure 2.5 shows markers of apoptosis and necrosis in embedded trabeculae. There was no difference between the experimental groups in cell morphology as shown by hematoxilin eosin staining (Figure 2.5, panel A trough C). In all sections, cardiomyocytes showed intact sarcolemmal membranes, no cytosolic vacuoles and no pyknosis of nuclei. Panel D through F shows the myoglobin histochemical staining, in which myoglobin is indicated by shades of gray. There was no difference in the amount of myoglobin between the different experimental

groups of trabeculae. This suggests that the sarcolemmal integrity of trabeculae is preserved after either hypoxia or MI.

Panels G through I show TUNEL staining of trabeculae. Blue indicates the nuclei stained by DAPI. Green fluorescence indicates the presence of DNA strand breaks. Panel G shows a TUNEL staining of a time control trabecula and only a faint cytosolic background green fluorescent signal is detected and the nuclei are stained blue by DAPI. Neither trabeculae subjected to MI (panel H) nor in trabeculae following hypoxia (panel I), green fluorescence was detected in the nuclei, indicating absence of double stranded DNA breakage and thus absence of apoptosis.

DISCUSSION

We demonstrated that the relative contribution of three common signaling molecules in sevoflurane-induced cardioprotection is not dependent on the type of applied ischemic stimulus. PKC activation, opening of mitoK^+_{ATP} channels and ROS are essential in conferring sevoflurane-induced protection against ATP-depletion and Ca^{2+} overloading as induced by hypoxia or metabolic inhibition through cyanide. Furthermore, trabeculae subjected to hypoxia or metabolic inhibition showed no markers for apoptosis and necrosis, indicating cardiac contractile dysfunction following recovery after hypoxia or metabolic inhibition as a result of altered Ca^{2+} handling and/or myofilament Ca^{2+} sensitivity rather than loss of cardiomyocytes.

Cyanide has frequently been used to model hypoxia. Recent studies show that clear differences exist in physiological responses to chemical anoxia and hypoxia (19,20). Additionally, isolated cardiomyocytes subjected to cyanide became earlier necrotic and had more LDH-release compared to cells subjected to hypoxia (21). Differences between hypoxia and metabolic inhibition may rely on (1) distinct sources of ROS formation like xanthine dehydrogenase-oxidase, myoglobin or NADPH-oxidase, as cyanide predominantly inhibits complex IV, (2) higher concentrations of ROS formation during metabolic inhibition as high levels of O_2 are present as a result of inhibition of complex IV and (3) a different modulation of the O_2-dependent regulation of mitochondrial redox signaling by NO during hypoxia (see

also figure 2.1) (11). Thus, the contribution of ROS and NO signaling pathways is relatively different in hypoxia or metabolic inhibition-induced ischemic injury. It has been demonstrated that the production of ROS during sevoflurane preconditioning involves inhibition of the mitochondrial ETC (22). Interestingly, Riess *et al*. (2005) recently showed that inhibition of sevoflurane-induced preconditioning with either ROS- or NO-scavengers was associated with reduced attenuation of the mitochondrial ETC (23). This suggests that this sevoflurane-induced inhibition of the ETC is mediated via ROS as well as NO and therefore implies that differences in ROS and NO signaling between hypoxia and metabolic inhibition may account for variation in the cardioprotective signaling induced by volatile anesthetics. However, we demonstrated that in both hypoxia and metabolic inhibition, preconditioning with sevoflurane equally preserves contractile function of myocardial tissue. Moreover, in both models PKC, mitoK^+_{ATP} channels and ROS are likewise involved. This indirectly shows that the mechanism of preconditioning with sevoflurane involves mainly the protection against adverse effects of mitochondrial ETC inhibition during ischemia, i.e. the generation of ROS, ATP-depletion and Ca^{2+} overload. However, it is important to note that the recovery of preconditioned trabeculae treated with the ROS-scavenger MPG was different in the hypoxia and metabolic inhibition group (figure 2.3). The underlying mechanism cannot be explained from the present data, but it may reflect that differences in the actual mechanism of protection against oxidative stress due to hypoxia or metabolic inhibition indeed may exist despite equal involvement of the signal transduction molecules in sevoflurane-induced cardioprotective signaling. However, this remains speculative at this time and should be addressed in future studies.

Recently, it has been demonstrated that preconditioning with sevoflurane improved mitochondrial function during hypoxia, as demonstrated by increased ATP synthesis and reduced ROS formation in addition to a reduced Ca^{2+} load (3,6). This afforded protection depended on opening of the mitoK^+_{ATP} channels. Akao *et al*. (2003) showed that hypoxia-induced ROS production results in a loss of mitochondrial membrane potential ($\Delta\Psi_m$), which serves as an indicator of mitochondrial function (24). This loss of $\Delta\Psi_m$ was attenuated by specific mitochondrial K^+ channels openers, and thereby confirmed the importance of mitochondrial integrity preservation for prevention of cellular injury. Similarly, volatile anesthetics augment the open probability of mitoK^+_{ATP} channels via a PKC-dependent

pathway (25), suggesting that they might preserve mitochondrial function via PKC and mitoK^+_{ATP} channels.

In our study, trabeculae subjected to either hypoxia or metabolic inhibition did not demonstrate any markers for necrosis or apoptosis. However, after 60 minutes of recovery following metabolic inhibition or hypoxia, apoptotic and necrotic markers may not yet be visible. This was also found in other studies showing that 60 minutes of ischemia or less is not sufficient to induce significant apoptotic or necrotic characteristics (26). Our data suggest that in both models of metabolic deprivation the reduction of active force development might be provoked by post-ischemic cardiomyocyte contractile dysfunction because of altered Ca^{2+} handling and/or Ca^{2+} sensitivity, rather than by a loss of cardiomyocytes. Currently, several mechanisms have been proposed to be involved in ischemic cardiomyocyte dysfunction, like the generation of ROS and Ca^{2+} overload, both leading to damage of proteins involved in either contraction or Ca^{2+} homeostasis (27). Interestingly, our data suggest that sevoflurane might protect SR function as is demonstrated by alterations in the rate of contraction (+dFdt) and relaxation (-dFdt). Sevoflurane preserves the +dFdt and –dFdt compared to hypoxia alone, indicating preserved Ca^{2+} handling and Ca^{2+} availability (28). These data correspond with Ca^{2+} measurements performed in isolated trabecula, showing that the Ca^{2+} re-uptake capacity of the SR was improved in pharmacologically preconditioned trabeculae subjected to metabolic inhibition (7).

In this study we used isolated right ventricular trabeculae to elucidate mechanisms of sevoflurane-induced preconditioning. We extensively applied this well-defined model for intracellular signaling as well as for functional studies in several previous studies (4,7,16). However several limitations have to be taken into account in the interpretation of the present results. Experiments were performed at 24°C to maintain a stable preparation over several hours and to prevent oxygen limitation. These relative hypothermic conditions may affect (cardioprotective) signaling processes. With regard to the production of ROS, the effect of hypothermia is difficult to predict, as reduced as well as increased production of ROS has been reported (29,30). However, activation of other signaling molecules, like PKC and mitoK^+_{ATP} channels, has been demonstrated to occur under hypothermic conditions (31,32). Furthermore, this study depends on the specificity of the used pharmacological inhibitors.

Chelerythrine, MPG and 5-HD are commonly used inhibitors in preconditioning studies and the used concentrations are comparable with existent literature.

In conclusion, our study shows that hypoxia or metabolic, are suitable to study ischemic cardiac injury and the cardioprotective of sevoflurane. Although hypoxic injury may be more relevant to the clinical situation, the effects of cyanide on complex IV of the mitochondrial electron transport chain highly resemble carbon monoxide poisoning (33). The activated intracellular signal transduction pathway involved in sevoflurane-induced cardioprotection is not dependent on the applied ischemic stimulus. This suggests that a common trigger, like ROS production, may be responsible for the onset of the protective process in cardiomyocytes.

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Chapter 3

REACTIVE OXYGEN SPECIES PRECEDE PROTEIN KINASE C-δ ACTIVATION INDEPENDENT OF ADENOSINE TRIPHOSPHATE-SENSITIVE MITOCHONDRIAL POTASSIUM CHANNEL OPENING IN SEVOFLURANE-INDUCED CARDIOPROTECTION

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ABSTRACT

In the current study we investigated the distinct role and relative order of protein kinase C (PKC)-δ, adenosine triphosphate-sensitive mitochondrial K^+ (mitoK^+_{ATP}) channels and reactive oxygen species (ROS) in the signal transduction of sevoflurane-induced cardioprotection and specifically addressed their mechanistic link.

Isolated rat trabeculae were preconditioned with 3.8% sevoflurane and subsequently subjected to an ischemic protocol by superfusion of trabeculae with hypoxic, glucose-free buffer (40 minutes) followed by 60 minutes of reperfusion. In addition, the acute effect of sevoflurane on PKC-δ and PKC-ε translocation and nitrotyrosine formation was established using immunofluorescent analysis. The inhibitors chelerythrine (6 μM), rottlerin (1 μM), 5-hydroxy decanoic acid sodium (100 μM) and n-(2-mercaptopropionyl)-glycine (300 μM) were used to study the particular role of PKC, PKC-δ, mitoK^+_{ATP} channels, and ROS in sevoflurane-related intracellular signaling.

Preconditioning of trabeculae with sevoflurane preserved contractile function after ischemia. This contractile preservation was dependent on PKC-δ activation, mitoK^+_{ATP} channel opening, and ROS production. In addition, on acute stimulation by sevoflurane, PKC-δ but not PKC-ε translocated to the sarcolemmal membrane. This translocation was inhibited by PKC-inhibitors, ROS scavenging but not by inhibition of mitoK^+_{ATP} channels. Furthermore, sevoflurane directly induced nitrosylation of sarcolemmal proteins, suggesting the formation of peroxynitrite. In sevoflurane-induced cardioprotection, ROS release but not mitoK^+_{ATP} channel opening precedes PKC-δ activation. Sevoflurane induces sarcolemmal nitrotyrosine formation, which might be involved in the recruitment of PKC-δ to the cell membrane.

INTRODUCTION

The volatile anesthetic sevoflurane makes the heart more resistant to ischemia and reperfusion (I/R) damage in experimental models (1-3) as well as in humans (4,5). We and others have shown that protein kinase C (PKC), adenosine triphosphate-sensitive mitochondrial K^+ ($mitoK^+_{ATP}$) channels, and reactive oxygen species (ROS) are necessary in the signal transduction of volatile anesthetic-induced cardioprotection (3,6,7). Volatile anesthetics directly activate PKC (8) and induce intracellular ROS production (7), either through modulation of $mitoK^+_{ATP}$ channel function (9) or modulation of the electron transport chain (10). ROS modulates PKC function directly *via* oxidative modification or indirectly *via* tyrosine phosphorylation (11,12). In addition, $mitoK^+_{ATP}$ channels can be directly activated by ROS (13). Consequently, a complex mechanistic link exists between these three signaling molecules, and so far, their relative order in anesthetic-induced cardioprotection has not been established.

Cardiomyocytes express a variety of PKC isoforms modifying the function of a wide range of (end-effector) target-substrates by phosphorylation. The involved PKC isoforms that contribute to preconditioning differ between species and the type of the preconditioning stimulus (14,15). In particular, the class of novel PKCs, *i.e.* PKC-δ and PKC-ε, are involved in several preconditioning stimuli (16-20). Recently, an essential role for PKC-δ activation was acknowledged in the signal transduction of isoflurane-induced cardioprotection in isolated rat hearts (21). Furthermore, it has been demonstrated that PKC-ε activation is necessary as signaling molecule in sevoflurane-induced cardioprotection in guinea pig hearts (22). In addition, different translocation patterns in response to volatile anesthetics have been shown for these PKC-isoforms. In response to isoflurane, PKC-δ translocates to mitochondria whereas PKC-ε translocates to the intercalated discs in rat myocardium (21). However, in human atrial tissue, sevoflurane induced PKC-δ translocation towards the sarcolemma in addition to a translocation of PKC-ε to nuclei, intercalated discs and mitochondria (23).

In the current study we studied the contribution of the PKC-δ and PKC-ε isoforms in isolated rat trabeculae in the signal transduction of sevoflurane-induced cardioprotection. Second, we investigated whether PKC isoform translocation and/or activation appears to be upstream or

downstream of ROS formation and opening of mitoK^+_{ATP} channels. Our study provides more insight in the sequence of intracellular signaling involved in sevoflurane-induced cardioprotection.

MATERIALS AND METHODS

Animals

The study was performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the VU University Medical Center (Amsterdam, Netherlands). Male Wistar rats (weight, 250-400 gram; Harlan Netherland, Horst, the Netherlands) were anesthetized with sodium pentobarbital (80 mg/kg, intraperitoneally; Ceva Sante Animale BV, Maassluis, Netherlands). After 1,000 units heparin (Leo Pharma, Breda, the Netherlands) were intravenously injected, the heart was quickly removed and subsequently perfused through the aorta with Tyrode buffer (95% O_2 and 5% CO_2, pH 7.4) at room temperature. Standard solution contained 120 mM NaCl, 1.22 mM $MgSO_4.7H_20$, 1.99 mM NaH_2PO_4, 27.0 mM $NaHCO_3$, 5.0 mM KCl, 1 mM $CaCl_2$ and 10 mM glucose. During dissection 30 mM 2,3-butanedione monoxime and 15 mM KCl was added to the solution in order to protect the myocardium (24). Subsequently, a suitable right ventricular trabecula (length 2-5 mm, diameter<0.2 mm) (25), running from the free ventricle wall to the tricuspid valve, was carefully dissected.

Experimental Setup for Isolated Trabeculae

Muscles were mounted between a force-transducer (AE801, SensoNor, Horten, Norway) and a micromanipulator in an airtight organ bath. After mounting, superfusion was started with normal Tyrode buffer at 27^{o}C with a flow of 2 ml/min, and trabeculae were stimulated with two platinum field electrodes (5 ms duration, stimulation frequency 0.5 Hz). The stimulation voltage was set at two times the stimulation-threshold. Subsequently, the length of individual trabeculae was set at 95% of the maximal length as determined by a force-length relation, followed by a stabilization period of 40 minutes. The stimulation frequency and temperature were then decreased to 0.2 Hz and 24^{o}C, respectively, followed by another 20 minutes of

stabilization. After this stabilization period the initial developed contractile force ($F_{dev,start}$) and maximal force ($F_{max,start}$), as determined by a post-extrasystolic potentiation protocol, were recorded. Post-extrasystolic potentiation determines the contractile reserve of trabeculae by maximal filling the sarcoplasmic reticulum with Ca^{2+} (26). Trabeculae failing to stabilize, spontaneously contracting trabeculae and trabeculae with a saturation ($=F_{dev}/F_{max}$) < 25% or >75% were excluded. The contractile forces measured by the force-transducer were digitized and recorded with a sampling rate of 500 Hz.

Measurements of Contractile Force

Trabeculae were randomly assigned to distinct experimental groups as represented in figure 3.1. Trabeculae (except for time controls) were subjected to simulated ischemia (SI) by switching to superfusion with hypoxic glucose-free Tyrode (95% N_2 and 5% CO_2, $pO_2 \leq 4$ mmHg) and by increasing the stimulation frequency to 1 Hz. Ischemia decreased F_{dev} to zero followed by an increase in passive force after starting SI. When the passive force increased to 50% of $F_{max,start}$ (=start of rigor), trabeculae were subjected to SI for another 40 minutes and were subsequently reperfused for 60 minutes with normal oxygenated buffer solution. After reperfusion, recovery of F_{dev} ($F_{dev,rec}$) and F_{max} ($F_{max,rec}$) were determined and expressed as percentages of $F_{dev,start}$ and $F_{max,start}$, respectively. Except for the time and inhibitor control experiments, trabeculae were preconditioned for 15 minutes with normal Tyrode saturated with 3.8vol% vaporized sevoflurane (Sevorane, Abbott B.V., Hoofddorp, Netherlands) 30 minutes before the onset of SI. The volume percentage of sevoflurane in the gas phase above the Tyrode was continuously monitored by a calibrated infrared anesthetic monitor (Capnomac Ultima; Datex, Helsinki, Finland). Sevoflurane was washed out 15 minutes prior to the onset of SI.

In four additional groups, trabeculae were superfused with the PKC-catalytic site inhibitor chelerythrine (6 μM; Sigma-Aldrich Chemie BV, Zwijndrecht, Netherlands), the specific inhibitor of PKC-δ rottlerin (1 μM; Sigma-Aldrich Chemie BV, Zwijndrecht, Netherlands), the ROS-scavenger n-(2-mercaptopropionyl)-glycine (MPG; 300 μM; Sigma-Aldrich Chemie BV, Zwijndrecht, Netherlands) or the mitoK^+_{ATP} channel inhibitor 5-hydroxy decanoic acid (5-HD; 100 μM; Sigma-Aldrich Chemie BV, Zwijndrecht, Netherlands) 10 minutes before preconditioning with sevoflurane until the onset of SI (see figure 3.1). The intrinsic effects of

the distinct inhibitors on $F_{dev,rec}$ after SI were studied in individual inhibitor control experiments.

Immunohistochemical Analysis

Intracellular translocation and/or activation of distinct signaling molecules was visualized by immunofluorescent staining and subsequent three-dimensional digital imaging microscopy. Isolated trabeculae were subjected to similar protocols as compared with the force measurement protocols including the application of inhibitors. However, trabeculae were immediately embedded in gelatin, rapidly frozen in liquid nitrogen and stored in –80°C until further use after the inhibitor washout period. For immunofluorescent staining, sections were paraformaldehyde fixated and incubated with a primary antibody (rabbit) raised against rat PKC-δ (662-673) or PKC-ε (728-737; both obtained from Research & Diagnostic Antibodies, Benicia, CA, USA). Sections were then incubated with a fluorescein isothiocyanate-labeled swine anti-rabbit secondary antibody (F0205; DakoCytomation BV, Heverlee, The

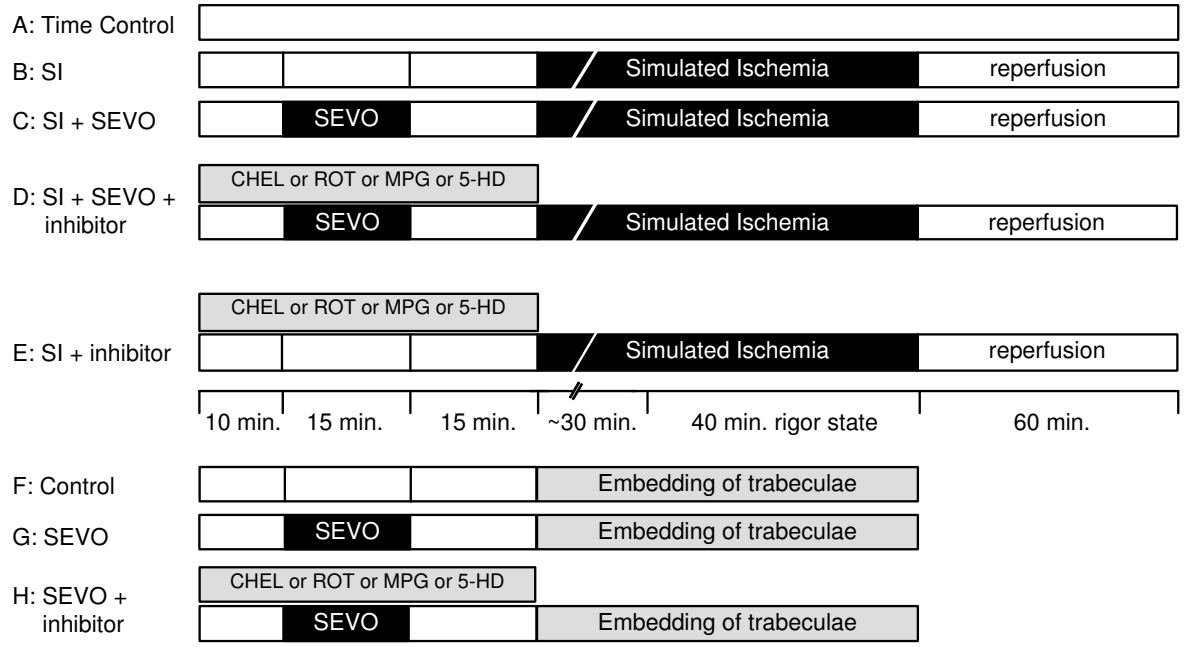

Figure 3.1 Overview of the applied experimental protocols for 17 experimental groups of randomized isolated trabeculae. The protocols for functional contractile experiments are indicated as protocols A - E and protocols for immunofluorescent staining are depicted in F - H. 5-HD=5-hydroxy decanoic acid; CHEL=chelerythrine; MPG=n-(2-mercaptopropionyl)-glycine; ROT=rottlerin; SI=simulated ischemia.

Netherlands). Simultaneously, sections were counterstained for the sarcolemma with 10% (vol/vol) wheat germ agglutinin (WGA) (W-7024; Molecular probes, Invitrogen, Breda, The Netherlands). Finally, nuclei were stained using 4',6 diamidino-2-phenylindole (DAPI)-containing mounting medium (H1200, Vectashield; Vector Laboratories, Burlingame, CA, USA). Separate sections were incubated with a primary antibody (rabbit) raised against rat nitrotyrosine (A-21285; Molecular probes, Invitrogen, Breda, The Netherlands) and then incubated with a secondary antibody as described above.

Sections were qualitatively analyzed using a inverted digital imaging microscopy workstation (Axiovert 200 Marianas; Carl Zeiss, Sliedrecht, Netherlands) equipped with a motorized stage and multiple fluorescent channels. A cooled charge-coupled device camera (Cooke Sensicam; Cooke Co., Eugene, OR, USA) was used to record images. Exposures, objective, montage and pixel binning were automatically recorded and stored in memory. Dedicated imaging and analysis software was obtained from Intelligent Imaging Innovations (Denver, CO, USA) and included advanced deconvolution techniques.

Table 3.1 General Characteristics of the Isolated Trabeculae.

	CSA (mm^2)	$F_{dev,start}$ (mN/mm^2)	$F_{dev,start}/F_{max,start}$ (%)	F_{pas}/F_{max} (%)	Time to Rigor (minutes)
Time	0.060 ± 0.02	51 ± 9.0	62 ± 5	3.1 ± 0.27	-
SI	0.038 ± 0.01	50 ± 11	47 ± 7	3.0 ± 0.32	26 ± 3
SI + SEVO	0.053 ± 0.02	59 ± 13	45 ± 6	3.8 ± 1.3	30 ± 3
SI + SEVO + CHEL	0.059 ± 0.01	26 ± 4.2	49 ± 8	2.9 ± 0.26	25 ± 2
SI + SEVO + ROT	0.077 ± 0.03	55 ± 22	44 ± 6	3.4 ± 0.52	20 ± 3
SI + SEVO + MPG	0.041 ± 0.008	45 ± 5.1	49 ± 8	3.4 ± 0.46	42 ± 5*
SI + SEVO + 5-HD	0.048 ± 0.01	73 ± 0.34	54 ± 3	3.6 ± 0.53	26 ± 1
SI + CHEL	0.061 ± 0.02	55 ± 22	48 ± 13	3.9 ± 0.45	25 ± 1
SI + ROT	0.073 ± 0.02	52 ± 8.9	56 ± 6	5.0 ± 1.1	20 ± 3
SI + MPG	0.054 ± 0.02	60 ± 14.0	64 ± 3	3.1 ± 0.21	36 ± 6
SI + 5-HD	0.0452±0.02	52 ± 14	43 ± 10	5.9 ± 1.2	33 ± 7

* $P<0.05$ compared with the experimental group SI+SEVO+ROT and SI+ROT. CSA=cross-sectional area; $F_{dev,start}$=initial developed force; $F_{dev,start}/F_{max,start}$=ratio between initial developed force and initial maximal force as determined by post-extra systolic potentiation (PESP); F_{pas}/F_{max}=ratio between initial passive force and initial maximal force as determined by PESP; 5-HD=5-hydroxy decanoic acid; CHEL=chelerythrine; MPG=n-(2-mercaptopropionyl)-glycine; ROT=rottlerin; SEVO=sevoflurane; SI=simulated ischemia; ROT=rottlerin.

Statistical Analysis

The sample size of each experimental group was 6, except for the inhibitor control groups (n=5) and the groups for immunofluorescent analysis (n=3). Data were evaluated for normal distribution and a one-way analysis of variance was performed followed by a Tukey *post-hoc* analysis to determine differences between the experimental groups. A *P*-value less than 0.05 was considered to reflect a significant difference. All values are represented as means±SEM.

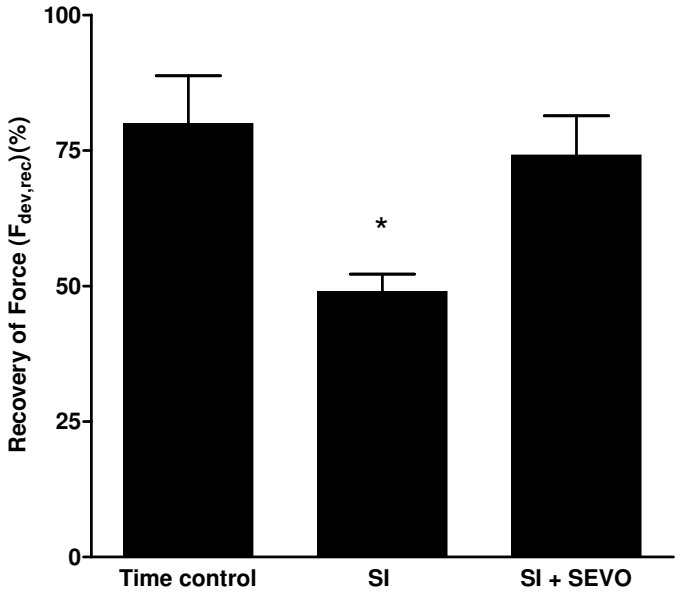

Figure 3.2 The recovery of force after simulated ischemia (SI) for trabeculae subjected to sevoflurane (SEVO) preconditioning. Recovery of force ($F_{dev,rec}$) is expressed as percentage of initial force ($F_{dev,start}$) for different groups. SI decreased $F_{dev,rec}$ compared with time controls, which can be restored by preconditioning with sevoflurane. * P<0.05 *versus* Time Control and SI+SEVO.

RESULTS

General Characteristics

Table 3.1 shows the initial general trabeculae characteristics for each experimental group. Minor variation was detected in trabeculae cross-sectional areas among groups. $F_{dev,start}/F_{max,start}$ was similar between experimental groups, implying that trabeculae had at 95% of the maximal length on average comparable contractile force reserves.

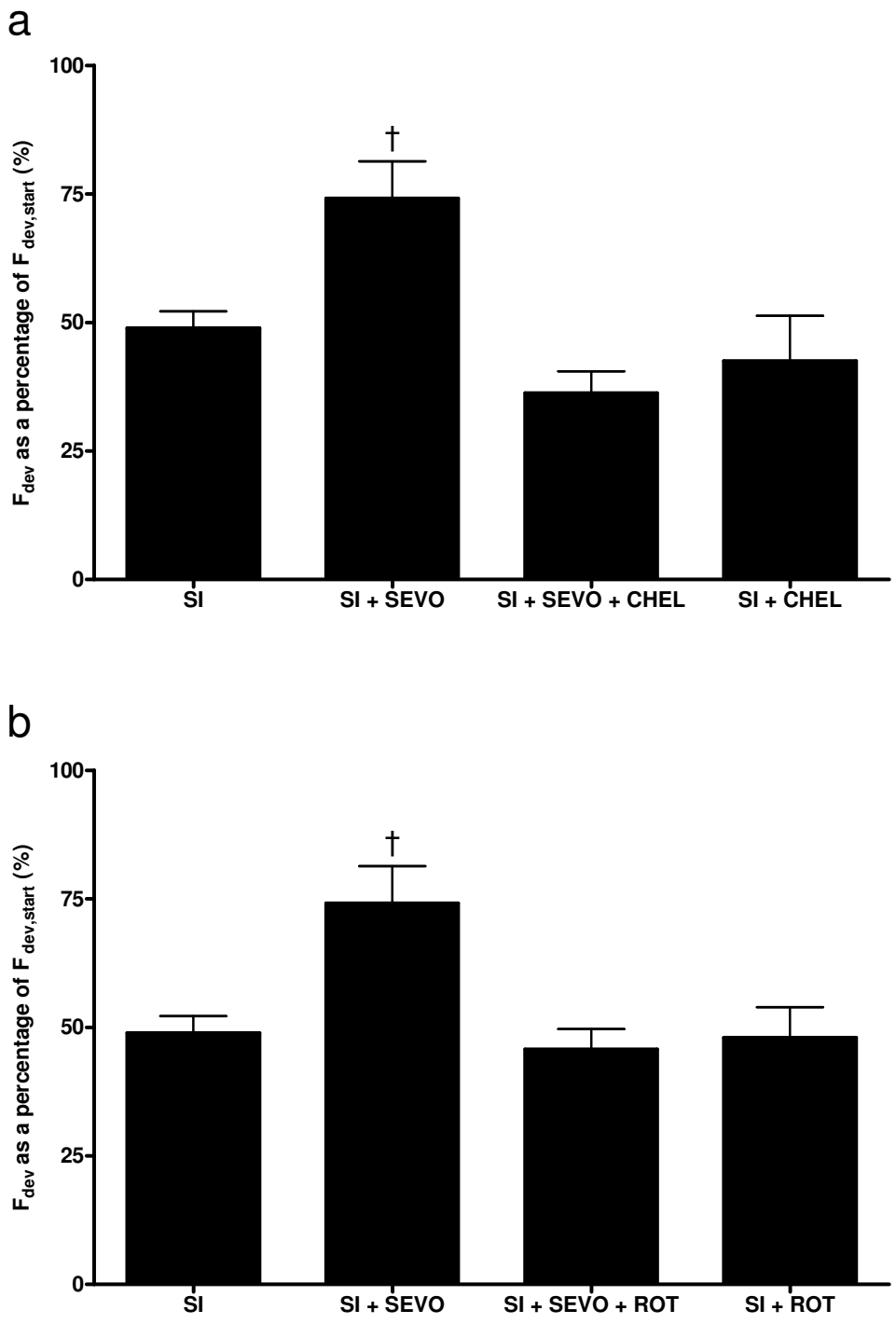

Figure 3.3 The role of PKC-δ in sevoflurane (SEVO)-induced cardioprotection in functional experiments. Recovery of force ($F_{dev,rec}$) is expressed as percentage of initial force ($F_{dev,start}$) for different groups. (a) Chelerythrine (CHEL) abolished the protective effect of sevoflurane on contractile function, but showed no intrinsic protective effect. (b) Similar results were observed for the PKC-δ specific inhibitor rottlerin (ROT). † $P<0.05$ *versus* Simulated Ischemia (SI), SI+SEVO+inhibitor and SI+inhibitor.

Furthermore, passive force expressed as percentage of $F_{max,start}$ was similar between groups before the onset of SI. SI resulted in an immediate decrease of F_{dev} to zero, followed by an increase in passive force, ultimately leading to a rigor state. On average, the time to the onset of the rigor state was 28 minutes. MPG increased the time to rigor, but we did not find a significant correlation between time to rigor and $F_{dev,rec}$ or $F_{max,rec}$.

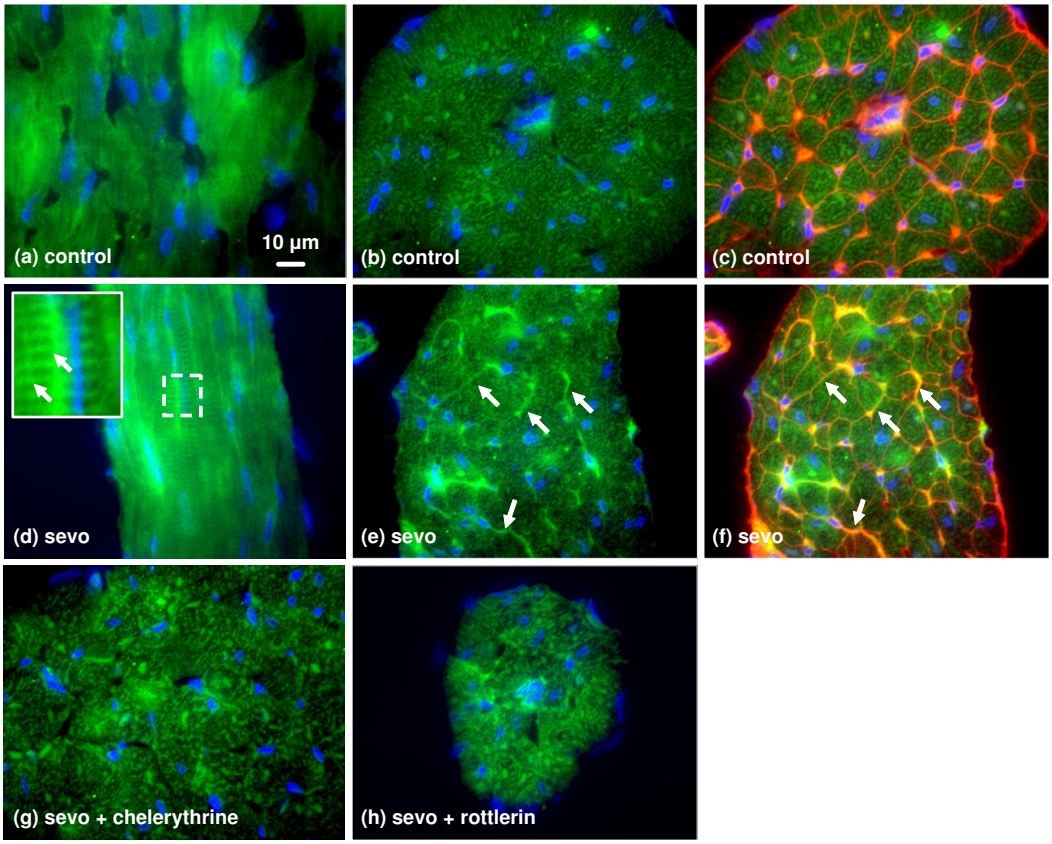

Figure 3.4 Three-dimensional digital imaging microscopy analysis of protein kinase (PKC)-δ translocation patterns in response to sevoflurane (sevo). In all panels, *green* represents specific PKC-δ staining, and *blue* the nuclear 4',6 diamidino-2-phenylindole staining. In *panel* c and f, *red* represents the wheat germ agglutinin staining of the sarcolemmal glycocalyx. Control trabeculae show a cytosolic location of PKC-δ in longitudinal sections (*a*) as well as in cross-sectional sections (*b* and *c*). In trabeculae exposed to sevoflurane, PKC-δ is localized in striation-like patterns and linear structures in longitudinal sections (*d*, *inset*, *white arrows*). In cross-sectional sections, PKC-δ is translocated to linear, patchy structures (*e*, *white arrows*). The *red* sarcolemmal counterstain is changed into *yellow*, indicating sevoflurane-induced co-localization of PKC-δ in the cardiomyocyte sarcolemmal membrane (*f*). This specific translocation of PKC-δ in response to sevoflurane was abolished by chelerythrine (*g*) and rottlerin (*h*).

Sevoflurane-induced Cardioprotection

Figure 3.2 depicts the effects of sevoflurane on recovery of force after SI. In time controls, F_{dev} and F_{max} were decreased after about 170 minutes to 80±9% and 81±2% of $F_{dev,start}$ and $F_{max,start}$, respectively. SI reduced the recovery of force ($F_{dev,rec}$) to 49±3% ($P<0.01$ *versus* Time Control). In addition, the recovery of F_{max} was decreased to 65±4% ($P<0.05$ *versus* Time Control) whereas SI did not affect passive force. Sevoflurane itself induced negative inotropy and reduced F_{dev} to 50% of $F_{dev,start}$. However, this negative inotropy was completely reversed after the washout of sevoflurane, 15 minutes prior to SI. Preconditioning with sevoflurane improved the ischemic injury-related decrease in $F_{dev,rec}$ to 74±7% ($P<0.05$ *versus* SI), but did not improve $F_{max,rec}$ (56±4%; $P>0.05$ *versus* SI).

Sevoflurane-induced PKC-δ Translocation and Activation

Figures 3.3 and 3.4 represent functional and immunohistochemical data, respectively, on the role of PKC-δ in sevoflurane-induced cardioprotection. Figure 3.3 shows that chelerythrine (a) and rottlerin (b) both completely abolished sevoflurane-induced preservation of $F_{dev,rec}$ (36±4% and 46±4% for chelerythrine and rottlerin respectively; both $P<0.01$ *versus* sevoflurane). Figure 3.4 shows the translocation pattern of PKC-δ in response to sevoflurane in both longitudinal (a and d) and cross-sectional (b, c and e-h) sections. In control sections, a cytosolic localization of the PKC-δ isoform can be distinguished (a-c). In addition, the red sarcolemmal counterstain is visualized in panel c. In contrast, in trabeculae exposed to sevoflurane PKC-δ is present as a striation-like translocation pattern in the longitudinal sections (d, *white arrows*). In cross-sectional sections, PKC-δ is visualized in a patchy distribution following distinct linear membrane structures of the cardiomyocytes (e, *white arrows*). The sarcolemmal counterstain colocalizes with PKC-δ (f), which is indicated by the yellow color. Interestingly, the sevoflurane-induced translocation of PKC-δ to the sarcolemma was blocked by both chelerythrine (g) and rottlerin (h).

Lack of Sarcolemmal PKC-ε Translocation in Sevoflurane-induced Cardioprotection

Figure 3.5 shows the distribution of PKC-ε in control (a and b) and sevoflurane-exposed trabeculae (c and d). In all panels, green represents specific PKC-ε staining, and blue indicates

the nuclear DAPI staining. In panel b and d, the sarcolemma has been counterstained by wheat germ agglutinin (red color). In control trabeculae, PKC-ε is localized in the peri-nuclear area of both cardiomyocytes and interstitial cells, as is shown by the green color surrounding the nuclei of cardiomyocytes. Sevoflurane exposure results in a reduction of peri-nuclear PKC-ε in cardiomyocytes but not in interstitial cells.

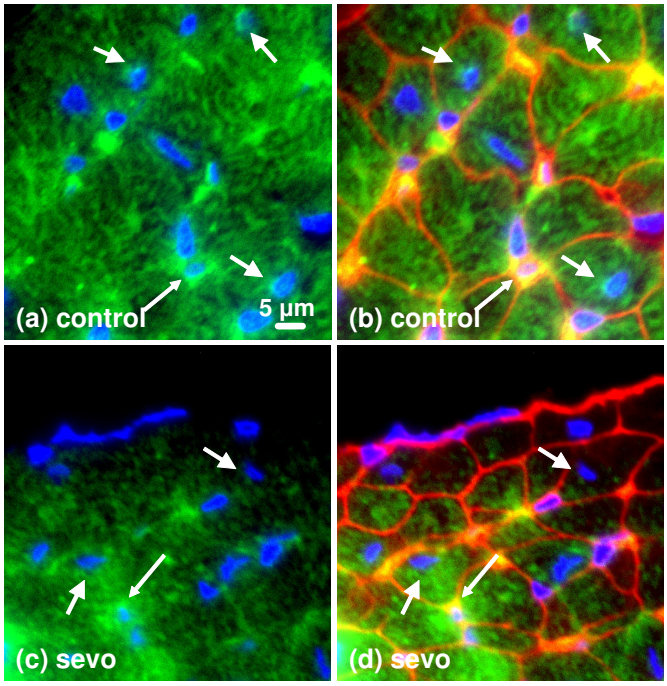

Figure 3.5 Protein kinase C (PKC)-ε distribution in control (*a* and *b*) and sevoflurane (sevo)-exposed trabeculae (*c* and *d*). In all panels, *green* represents specific PKC-ε staining, and *blue* indicates the nuclear 4',6 diamidino-2-phenylindole (DAPI) staining. In *panels b* and *d*, *red* represents the wheat germ agglutinin (WGA) staining of the sarcolemmal glycocalyx. In control trabeculae, PKC-ε is localized peri-nuclear area, as is demonstrated by the *green* surrounding the nuclei of both cardiomyocytes (*bold arrowheads*) and interstitial cells (*thin arrows*). Note that exposure to sevoflurane results in a reduction of peri-nuclear PKC-ε in cardiomyocytes but not in interstitial cells.

The Role of ROS and MitoK^+_{ATP} Channels in Sevoflurane-induced Cardioprotection

The role of mitoK^+_{ATP} channels and ROS production in sevoflurane-induced cardioprotection in recovery of force and PKC-δ translocation is shown in figure 3.6 and 3.7 respectively. Inhibition of mitoK^+_{ATP} channels by 5-HD (figure 3.6a) and ROS scavenging by MPG (figure 3.6b) both abolished sevoflurane-induced cardioprotection. The inhibitors did not establish modulation of F_{dev} or intrinsic cardioprotective properties. Although 5-HD inhibits sevoflurane-induced cardioprotection, figure 3.7 shows that 5-HD does not abolish

sevoflurane-induced PKC-δ translocation to the sarcolemma (a and b). In contrast, ROS scavenging by MPG inhibits sevoflurane-induced translocation of PKC-δ (c and d). Figure 3.8 represents the sevoflurane-induced formation of nitrotyrosine as indicated by green fluorescence. In time controls, nitrotyrosine was undetectable (a and b), whereas sevoflurane clearly induced sarcolemmal nitrosylation (c and d). The involvement of ROS in the peroxynitrite modification of sarcolemmal tyrosine residues was shown by the absence of nitrotyrosine when trabeculae were exposed to sevoflurane in the presence of MPG (e and f).

DISCUSSION

In this study, we demonstrated that sevoflurane-induced cardioprotection in functional cardiac tissue depends on activation of the PKC-δ isoform. Furthermore, exposure of trabeculae to sevoflurane induces translocation of PKC-δ but not of PKC-ε from the cytosol to the cardiomyocyte sarcolemma. PKC-δ translocation acts *via* the production of reactive oxygen species (ROS), but not the opening of mitoK^+_{ATP} channels. Finally, sevoflurane induces formation of sarcolemmal nitrotyrosine, which represents the reaction of peroxynitrite with tyrosine residues. To our knowledge, this is the first study showing the involvement of PKC-δ and its relative order compared with ROS and opening of mitoK^+_{ATP} channels in sevoflurane-induced cardioprotection.

Several studies showed specific translocation patterns of various PKC isoforms in response to ischemic as well as pharmacological preconditioning. Inagaki *et al.* (2000) studied PKC translocation patterns of the cardioprotective agent JTV519, and demonstrated that this compound induced a translocation of PKC-δ but not of PKC-ε to the sarcolemma (27). Recently, Julier *et al.* (2003) demonstrated that sevoflurane itself results in translocation of PKC-δ to the sarcolemma in human atrial tissue, in addition to translocation of PKC-ε to the intercalated discs and mitochondria (23). However, these sevoflurane-treated patients received significantly more phenylephrine compared to control subjects in order to maintain blood pressure. Because α-adrenergic stimulation induces PKC translocation (17) and cardioprotection (28) the sevoflurane-induced effects were biased by the phenylephrine-

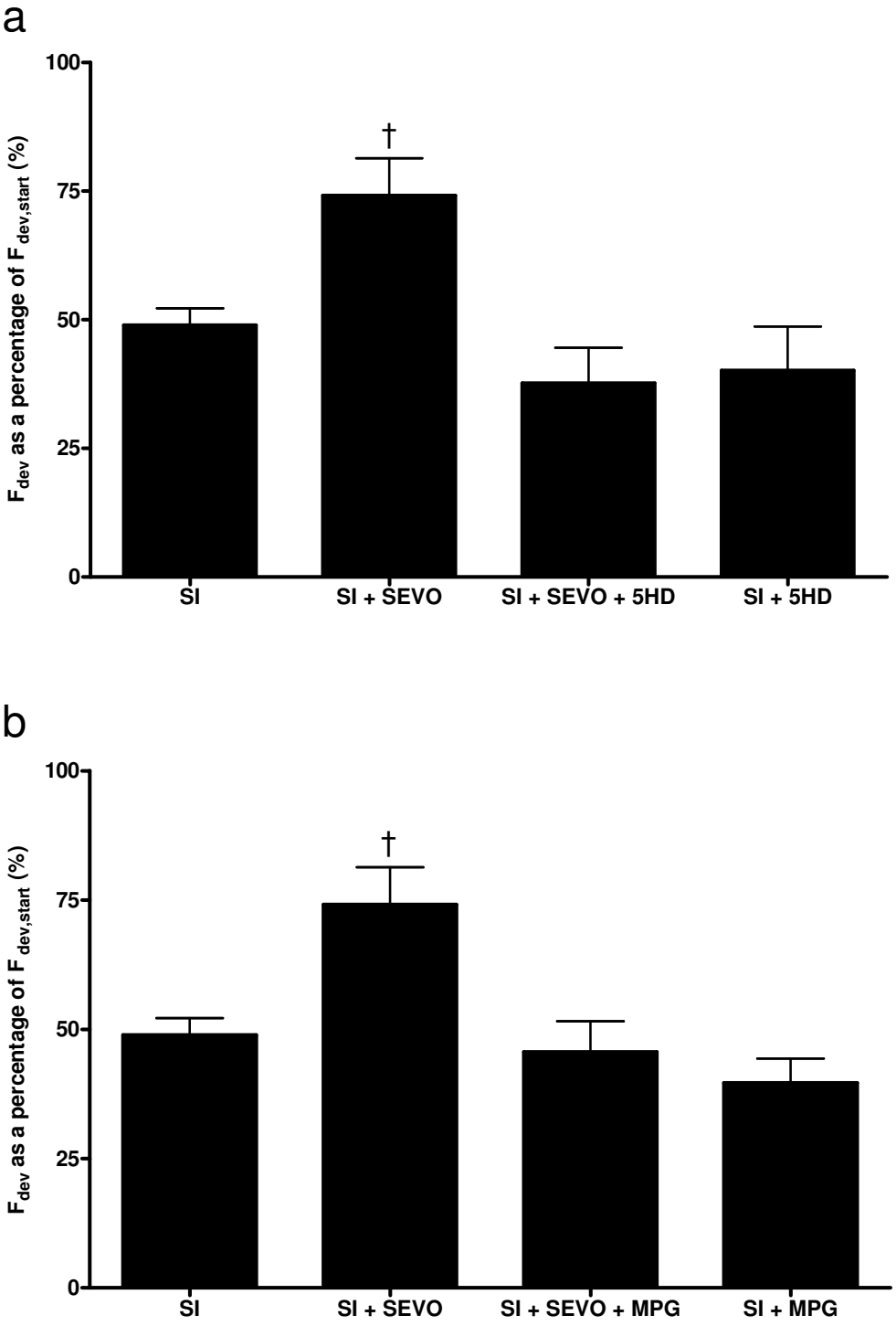

Figure 3.6 The role of adenosine triphosphate-sensitive mitochondrial K^+ (mitoK^+_{ATP}) channels and reactive oxygen species in sevoflurane (SEVO)-induced cardioprotection in functional experiments. Recovery of force ($F_{dev,rec}$) is expressed as percentage of initial force ($F_{dev,start}$) for different groups. Selective inhibition of mitoK^+_{ATP} channels by 5-hydroxydecanoic acid (5-HD) (*a*) and scavenging of reactive oxygen species by n-(2-mercaptopropionyl)-glycine (MPG) (*b*) abolished the cardioprotective effect of sevoflurane. 5-HD and MPG revealed no intrinsic cardioprotective effects. † $P<0.05$ *versus* simulated ischemia (SI), SI+SEVO+inhibitor and SI+inhibitor.

induced changes. We demonstrate with immunofluorescent analysis that sevoflurane itself induces translocation of PKC-δ to the sarcolemma. In addition, our functional experiments show that PKC-δ activation may be important for sevoflurane-induced cardioprotection. This is in agreement with observations showing that PKC-δ is primarily involved in pharmacological preconditioning with opioids (14), adenosine (18) and phenylephrine (17). However, we do not rule out that other PKC isoforms, like PKC-ε, are involved in sevoflurane-induced preconditioning as well.

Interestingly, a functional role for PKC-ε was recently established during sevoflurane-induced cardioprotection in guinea-pig hearts in addition to studies showing that ischemic preconditioning is mediated by PKC-ε (22,29-31). Some studies even indicate that PKC-δ inhibition is cardioprotective, as was demonstrated by the increased resistance against ischemia-reperfusion damage in mice hearts overexpressing a δV1 inhibitor peptide (32). Nevertheless, the latter study also established the necessity of an active form of the PKC-δ because overexpression of the δV1 inhibitor peptide at intermediate or very high doses resulted in cytoskeletal deformations, a lethal phenotype and less protection to I/R, respectively. Liu *et al*. (2001) demonstrated that ischemic preconditioning resulted in a translocation of both PKC-δ and PKC-ε, whereas upon pharmacological preconditioning with opioids only PKC-δ translocated (19). This is in accordance with our observations that sevoflurane itself does not cause sarcolemmal translocation of PKC-ε in trabeculae. In addition, Fryer *et al.* (2001/2002) showed in isolated rat hearts that opioid-induced cardioprotection, but not ischemic preconditioning, was abolished by pharmacological inhibition of PKC-δ (14,15). These observations indicate that not only distinct signal transduction pathways are involved in ischemic preconditioning and pharmacological preconditioning, but also between different pharmacological preconditioning stimuli.

Recently, a profound role for ROS in the signal transduction of volatile anesthetics-induced cardioprotection has been established (3,7,33). Our results indicate that translocation of PKC-δ occurs downstream of ROS, because PKC-δ translocation could be inhibited by MPG. This is in agreement with observations of Zhang *et al.* (2002) (34), who showed that opioid-induced protection was mediated by ROS, which could not be blocked by rottlerin. In addition, Wang *et al.* (2001) showed that diazoxide-induced protection in isolated rat hearts

was abolished by chelerythrine (35). In contrast, other studies showed that ROS-mediated cardioprotection could not be blocked by PKC inhibition (36). This suggests that ROS may

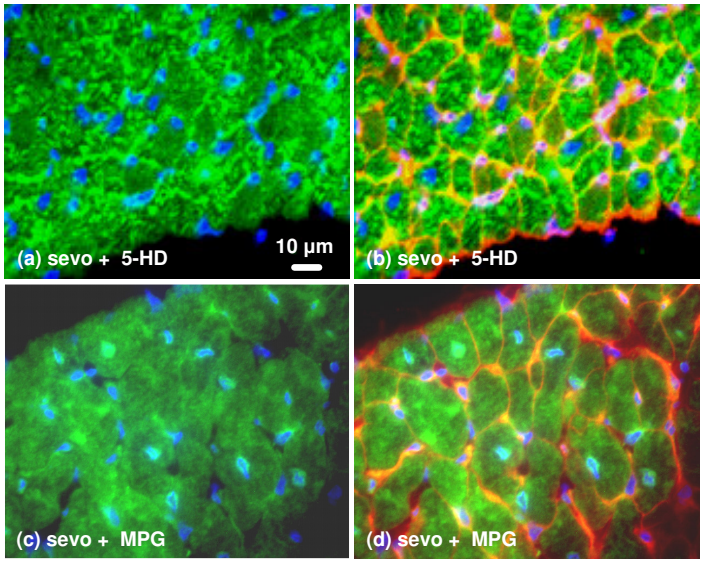

Figure 3.7 Effect of inhibition of adenosine triphosphate-sensitive mitochondrial K^+ ($MitoK^+_{ATP}$) channels and reactive oxygen species on protein kinase C (PKC)-δ translocation in respons to sevoflurane (sevo) in cross-sectional sections of trabeculae. In all panels, *green* indicates specific PKC-δ staining, and *blue* represents the nuclear 4',6 diamidino-2-phenylindole staining (DAPI) staining. *Red* represents the wheat germ agglutinin (WGA) staining of the sarcolemmal glycocalyx. Although 5-hydroxydecanoic acid (5-HD) inhibits sevoflurane-induced cardioprotection (figure 3.6), *a* and *b* show that 5-HD does not abolish sevoflurane-induced PKC-δ translocation to the sarcolemma. In contrast, reactive oxygen species scavenging by n-(2-mercaptopropionyl)-glycine (MPG) inhibits sevoflurane-induced translocation of PKC-δ (*c* and *d*).

also be involved in a signaling pathway independent from PKC. That sevoflurane indeed results in the production of ROS in our model was demonstrated by the positive nitrotyrosine staining of the cardiomyocyte sarcolemma upon exposure to sevoflurane. Nitrotyrosine is induced when nitric oxide and superoxide form peroxynitrite, which can modify tyrosine residues to form nitrotyrosine. Others also demonstrated the involvement of ROS and nitrogen species as triggers of anesthetic preconditioning in isolated guinea pig hearts (37).

It has been hypothesized that ROS production arises from the mitochondria, as volatile anesthetics inhibit complex I of the electron transport chain. Whether opening of the $mitoK^+_{ATP}$ channels is essential for ROS production has not completely been elucidated. However, it was recently demonstrated that 5-HD does not inhibit sevoflurane-induced ROS production (7). In contrast, during isoflurane-induced preconditioning, opening of the

mitoK$^+_{ATP}$ channels was essential for ROS production triggering cardioprotection (33). We demonstrate in functional trabeculae that opening of the mitoK$^+_{ATP}$ channels is essential for inducing the cardioprotective response but that opening of mitoK$^+_{ATP}$ channels is not essential for PKC-δ activation. With respect to the published literature, this supports that mitoK$^+_{ATP}$ channel opening is indeed involved in sevoflurane-induced cardioprotection, but is downstream of PKC-δ translocation. These data suggest that ROS production precedes PKC-δ activation and that PKC-δ activation precedes the opening of mitoK$^+_{ATP}$ channels in the signaling pathway of sevoflurane-induced cardioprotection. Possible target substrates of

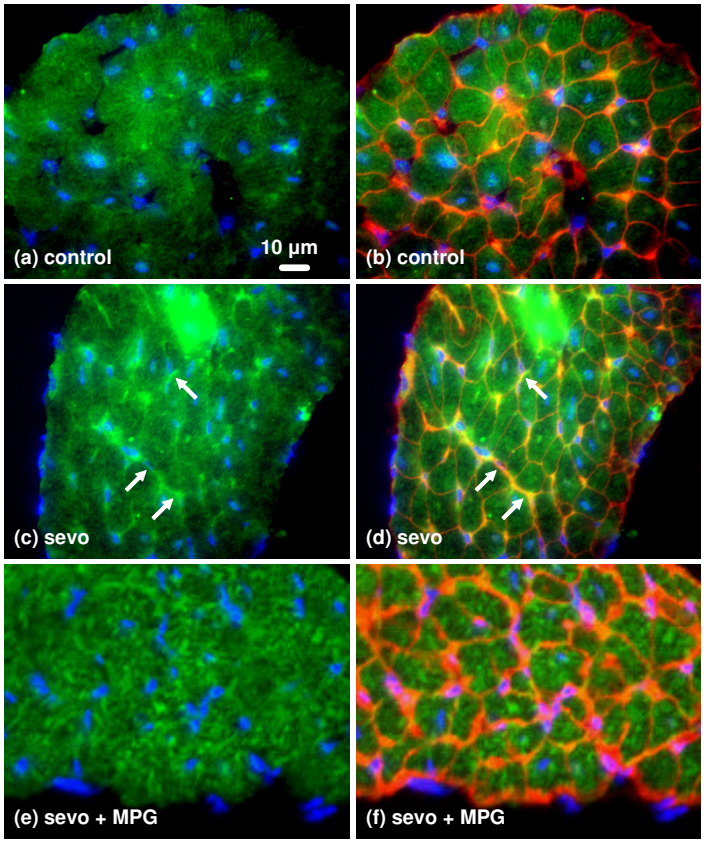

Figure 3.8 Three-dimensional digital imaging microscopy analysis of nitrotyrosine in response to sevoflurane (sevo). In all panels, *green* indicates specific nitrotyrosine staining, and *blue* represents the nuclear 4',6 diamidino-2-phenylindole (DAPI)-staining. *Red* represents the wheat germ agglutinin (WGA) staining of the sarcolemmal glycocalyx. In time controls, nitrotyrosine was undetectable (*a* and *b*), whereas sevoflurane clearly induced sarcolemmal nitrosylation (*c* and *d*; see also *white arrows*). The involvement of ROS in the formation of nitrotyrosine was demonstrated by the absence of nitrotyrosine when trabeculae were exposed to sevoflurane in the presence of n-(2-mercaptopropionyl)-glycine (MPG) (*e* and *f*).

PKC-δ during sevoflurane-induced cardioprotection remain speculative. Targets might include proteins involved in Ca^{2+} homeostasis, as we previously demonstrated by showing that preconditioning with norepinephrine attenuated Ca^{2+} overload and preserved Ca^{2+}-reuptake activity of the sarcoplasmic reticulum (28,38). In addition, PKC has been reported to modify the function of Ca^{2+} handling proteins, such as L-type Ca^{2+} channels (39), the ryanodine receptor (40), sarcoplasmic Ca^{2+} adenosine triphosphatase (41) and the Na^{+}/Ca^{2+} exchanger (42). Furthermore, anesthetic preconditioning may also preserve mitochondrial function (43). Future experiments in our laboratory will be directed toward unraveling the potential end-effector proteins of PKC-δ.

In our laboratory, the *in vitro* model of isolated rat trabecula has been extensively used in previous studies concerning I/R-injury and preconditioning in functional cardiac tissue (3,28,38). Our experiments are performed under relatively hypothermic conditions (24°C) in order to ensure stability of trabeculae over time. This could influence signaling processes, like translocation of PKC isoforms. However, recently it was reported that during cold cardioplegia translocation of PKC isforms still occurred and that the induced cardioprotection is indeed mediated by protein kinase C (44-46). The negative inotropic effect of sevoflurane, when applied before simulated ischemia was completely restored after the washout period. There was no difference in F_{dev} after washout between groups. In studies concerning ischemic and anesthetic preconditioning, chelerythrine, rottlerin, MPG and 5-HD are commonly used inhibitors for studying PKC, PKC-δ, ROS and mitoK^{+}_{ATP} channels, respectively. We used concentrations comparable with data found in literature. Although rottlerin is reported to be a specific inhibitor of the PKC-δ isoform, one should be cautious of unspecific inhibitory effects (47,48). However, it has been demonstrated by Keenan et *al.* (1997) that PKC-δ is more potently inhibited by rottlerin than classical PKC isoforms (49). In addition, we show by immunofluorescent microscopy that rottlerin selectively inhibits sevoflurane-induced translocation of PKC-δ.

In summary, PKC-δ, mitoK^{+}_{ATP} channels and ROS are equally involved in sevoflurane-induced cardioprotection in isolated rat trabeculae. ROS production precedes PKC-δ translocation on stimulation with sevoflurane, while the opening of mitoK^{+}_{ATP} channels occurs downstream of this translocation. Furthermore, sevoflurane exposure induces formation of peroxynitrite, which modifies tyrosine residues in the sarcolemma. We propose

that sevoflurane directly induces ROS and peroxynitrite formation, which may result in recruitment of PKC-δ to the cardiomyocyte sarcolemma. Furthermore, ROS and peroxynitrite may also affect tyrosine residues in the PKC-δ isoform *via* tyrosine kinase, leading to activation of downstream signaling molecules including mitoK^+_{ATP} channels. The downstream localization of mitoK^+_{ATP} channels in the signaling pathway of sevoflurane-induced cardioprotection suggests a role for mitochondria as crucial organelles for cardiac preservation.

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Chapter 4

SEVOFLURANE-INDUCED CARDIOPROTECTION DEPENDS ON PROTEIN KINASE C-α ACTIVATION VIA PRODUCTION OF REACTIVE OXYGEN SPECIES

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ABSTRACT

We previously demonstrated the involvement of the Ca^{2+}-independent protein kinase C (PKC)-δ isoform in sevoflurane-induced protection of cardiomyocytes against ischemia and reperfusion (I/R) injury. Since sevoflurane is also known to directly modulate myocardial Ca^{2+} handling, we investigated in this study the role of the Ca^{2+}-dependent PKC-α isoform in sevoflurane-induced cardioprotective signaling in relation to reactive oxygen species (ROS), adenosine triphosphate-sensitive mitochondrial K^+ ($mitoK^+_{ATP}$) channels and PKC-δ.

Preconditioned (15 minutes 3.8vol% sevoflurane) isolated right ventricular rat trabeculae were subjected to I/R, consisting of 40 minutes superfusion with hypoxic, glucose-free buffer, followed by normoxic glucose containing buffer for 60 minutes. Immunofluorescent microscopy was used to analyze PKC-α translocation in sevoflurane-treated trabeculae. The role of PKC-α, ROS, $mitoK^+_{ATP}$ channels and PKC-δ was established by using the following pharmacological inhibitors: Go6976 (50 nM), n-(2-mercaptopropionyl)-glycine (MPG; 300 μM), 5-hydroxydecanoic acid (5HD; 100 μM) and rottlerin (ROT; 1 μM).

Preconditioning of trabeculae with sevoflurane improved contractile recovery after I/R. This cardioprotective effect was attenuated in trabeculae treated with the PKC-α inhibitor, Go6976. In sevoflurane-treated trabeculae, PKC-α translocated towards mitochondria, as shown by immunofluorescent co-localization with the mitochondrial heat shock protein (GRP75). Sevoflurane-induced translocation of PKC-α was abolished by Go6976 and MPG, but not by 5HD or ROT.

Sevoflurane improves post-ischemic contractile recovery via activation of PKC-α. ROS production, but not opening of $mitoK^+_{ATP}$ channels, precede PKC-α translocation towards the mitochondria. This study shows the involvement of the Ca^{2+}-dependent PKC-α in addition to the well established role of Ca^{2+}-independent PKC isoforms in sevoflurane-induced cardioprotection.

INTRODUCTION

Sevoflurane protects the myocardium from ischemia and reperfusion (I/R)-injury by eliciting a protective signal transduction cascade (1). This cardioprotective signaling cascade relies on the production of reactive oxygen species (ROS), activation of protein kinase C (PKC) and opening of ATP-sensitive mitochondrial K^+ ($mitoK^+_{ATP}$) channels (2-4). However, the exact mechanisms and target end-effector proteins of sevoflurane-induced cardioprotection remain to be elucidated.

In preconditioning related signaling cascades, the Ca^{2+}-independent PKC-isoforms PKC-δ and PKC-ε have been considered most important (4-7), whereas less evidence exists for the involvement of Ca^{2+}-dependent PKC isoforms. Nevertheless, in various cardioprotective signaling cascades, Ca^{2+} has been demonstrated as a central mediator (8-10) and evidence exists that Ca^{2+}-dependent iso-enzymes are activated during ischemia (9,11) and during other preconditioning stimuli, like Ca^{2+}-induced preconditioning (12). Interestingly, since volatile anesthetics profoundly modulate myocardial Ca^{2+} handling (13), activation of Ca^{2+}-dependent PKC isoforms can be expected. However until now the exact relation between Ca^{2+}-dependent PKC-isoforms and volatile anesthetic-induced preconditioning has not been established.

In this study we therefore investigated in isolated right ventricular rat trabeculae whether sevoflurane-induced preconditioning involves activation of the Ca^{2+}-dependent PKC-isoform, PKC-α. We specifically focused on the interaction of PKC-α with more established mediators of volatile anesthetic-induced cardioprotective signaling, i.e. ROS and $mitoK^+_{ATP}$ channels and especially the essential Ca^{2+}-independent PKC isoform PKC-δ (14).

MATERIALS AND METHODS

Animals and Experimental Setup

This study was performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the VU University Medical Center. Our experimental setup has previously been described in detail (14,15). Briefly, male Wistar rats (250-400 gram; Harlan,

Horst, the Netherlands), were anesthetized with sodium pentobarbital (80 mg/kg, intraperitoneally; Ceva Sante Animale BV, Maassluis, Netherlands). Subsequently, the heart was removed and trabeculae were isolated from the right ventricle (length 2-5 mm, diameter<0.2 mm) under contractile arrest conditions. Trabeculae were mounted in an airtight organ bath, superfused with normal Tyrode buffer consisting of 120 mM NaCl, 1.22 mM $MgSO_4$.7H2O, 1.99 mM, NaH_2PO_4, 27.0 mM $NaHCO_3$, 5.0 mM KCl, 1 mM $CaCl_2$ and 10 mM glucose oxygenated with 95% O_2 and 5% CO_2. The muscles were then stretched up to 95% of the optimal length determined by a force-length relation. After stabilization (40 minutes at 27ºC followed by 20 minutes at 24ºC), the initial developed force of contraction ($F_{dev,start}$) and the potentiated force ($F_{pot,start}$), as determined by a post-extrasystolic potentiation protocol (PESP), were recorded. PESP determines the contractile reserve of trabeculae by maximally filling the SR with Ca^{2+} (16). Trabeculae failing to stabilize, spontaneously contracting trabeculae and trabeculae failing to show PESP ($F_{pot,start}$) were excluded.

Experimental Protocol

Figure 4.1 (group A-E) demonstrates the experimental design for all groups exposed to the preconditioning protocol. Ischemia was simulated (except time controls) by superfusion of hypoxic Tyrode without glucose and increasing the stimulation frequency to 1 Hz. 40 minutes after ischemic rigor development, trabeculae were reperfused for 60 minutes by oxygenated normal Tyrode and basal stimulation conditions (0.2 Hz). The recovery of F_{dev} ($F_{dev,rec}$) was expressed as a percentage of the $F_{dev,start}$. Trabeculae were preconditioned for 15 minutes with normal Tyrode saturated with 3.8vol% sevoflurane 30 minutes prior to simulated ischemia and reperfusion (SI/R). After washout of sevoflurane, trabeculae were superfused for 15 minutes with normal Tyrode until SI/R. In a separate experimental group, sevoflurane preconditioning was preceded by addition of Go6976 (12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrollo[3,4-c]carbazole; 50nM; PKC-α inhibitor; Biomol, Heerhugowaard, the Netherlands).

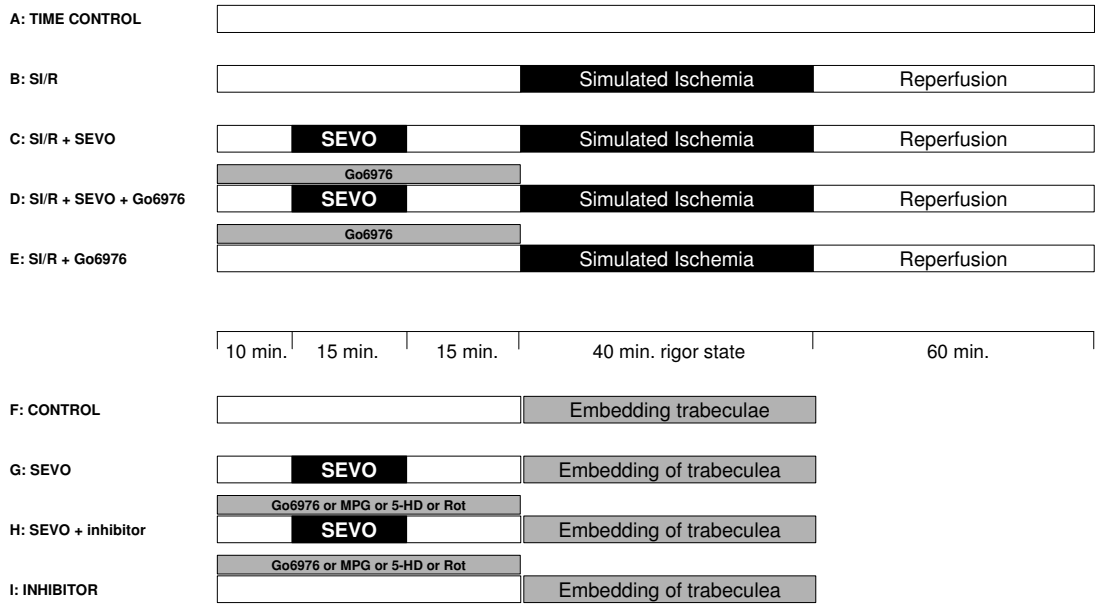

Figure 4.1 Overview of the experimental, randomized design for trabeculae subjected to an ischemia and reperfusion (I/R)-protocol and functional contractile measurements (A-E) or immunofluorescent analysis of PKC-α translocation (F-I). 5-HD=5-hydroxydecanoic acid (100 μM); Go6976=12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrollo[3,4-c]carbazole (50 nM); MPG=n-(2-mercaptopropionyl)-glycine (300 μM); ROT=rottlerin (1 μM); SI/R=simulated ischemia and reperfusion; SEVO=sevoflurane.

Immunohistochemical Analysis

The sevoflurane-induced subcellular redistribution of PKC-α was studied by immunofluorescent staining followed by digital imaging fluorescence microscopy (DIM) in trabeculae subjected to sevoflurane alone, or in combination with Go6976 (50 nM), n-(2-mercaptopropionyl)-glycine (MPG; 300 μM; ROS scavenger; Sigma-Aldrich Chemie BV, Zwijndrecht, Netherlands), 5-hydroxydecanoic acid (5-HD; 100 μM; mitoK^+_{ATP} channel inhibitor; Sigma-Aldrich Chemie BV, Zwijndrecht, Netherlands) or rottlerin (ROT; 1 μM; PKC-δ inhibitor, Sigma-Aldrich Chemie BV, Zwijndrecht, Netherlands), as described previously (14). Briefly, trabeculae subjected to an experimental protocol (figure 4.1, group F-I) were embedded in gelatin and cross-sections were subsequently fixed, stained for PKC-α (Research & Diagnostic Antibodies, Benicia, USA) and counterstained for nuclei 4',6 diamidino-2-phenylindole (DAPI; Vectashield, Vector Laboratories, Burlingame, CA, USA) and mitochondria (anti-GRP75; Stressgen, Ann Arbor, USA) or the sarcolemma (wheat germ agglutinin (WGA); Molecular probes, Invitrogen, Breda, The Netherlands). The sections were analyzed with a ZEISS Axiovert 200 Marianas™ inverted digital imaging microscope using Slidebook™ software (Slidebook™ version 4.1, Denver, CO, USA).

Statistical Analysis

Force experiments were analyzed by analysis of variance (ANOVA) followed by a Tukey post-hoc test analysis. A *P*-value<0.05 was considered to reflect a significant difference. All values are given as means±standard error of the mean (SEM).

RESULTS

General Characteristics

Table 4.1 shows the general characteristics of trabeculae subjected to SI/R (except time controls). In the experimental group I/R+GO, the time to rigor was slightly prolonged when compared to time to rigor of the I/R-group. However, no correlation existed between time to rigor and final contractile recovery and therefore this difference did not bias our observations.

Table 4.1 General Characteristics of Trabeculae Subjected to Simulated Ischemia and Reperfusion.

	CSA, mm^2	$F_{dev,start}$ mN/mm^2	$F_{dev,start}$ / $F_{pot,start}$,%	$F_{pas,start}$, mN/mm^2	Time to rigor, minutes
TIME	0.06 ± 0.02	51 ± 9	62 ± 5	2.1 ± 0.4	
SI/R	0.04 ± 0.01	52 ± 8	47 ± 5	4.2 ± 1.3	28 ± 3
SI/R + SEVO	0.06 ± 0.01	57 ± 10	47 ± 6	4.5 ± 1.1	29 ± 2
SI/R + SEVO + GO	0.05 ± 0.01	43 ± 7	46 ± 6	2.6 ± 0.5	27 ± 2
SI/R + GO	0.02 ± 0.002	60 ± 11	48 ± 7	2.8 ± 0.5	40 ± 4*

* indicates *P*<0.05 compared with the experimental group SI/R and SI/R+SEVO+GO. CSA=cross-sectional area; $F_{dev,start}$=initial developed force before the experiment; $F_{pas,start}$=passive force before the experiment; $F_{pot,start}$=potentiated force development before the experiment; GO=Go6976; SEVO=sevoflurane; SI/R=simulated ischemia and reperfusion; TIME=time control.

Sevoflurane-induced Cardioprotection Depends on PKC-α

After the total experimental protocol (170 minutes) the F_{dev} decreased to 79±7% (n=6; [TIME CONTROL]) whereas SI/R reduced the contractile recovery ($F_{dev,rec}$) to 47±3% ([SI/R]; n=9; *P*<0.05 *versus* [TIME CONTROL]) (figure 4.2). Pretreatment with sevoflurane improved the $F_{dev,rec}$ to 65±3% ([I/R+SEVO]; n=8; *P*<0.05 *versus* [SI/R]). In trabeculae with PKC-α inhibition by Go6976, sevoflurane-induced cardioprotection was completely abolished (42±4% ([I/R+SEVO+GO]; n=10; *P*<0.05 *versus* [SI/R+SEVO]). PKC-α inhibition by Go6976 before SI/R did not show intrinsic effect on the $F_{dev,rec}$ (40±6% [SI/R+GO], n=5, *P*>0.05 *versus* [SI/R]).

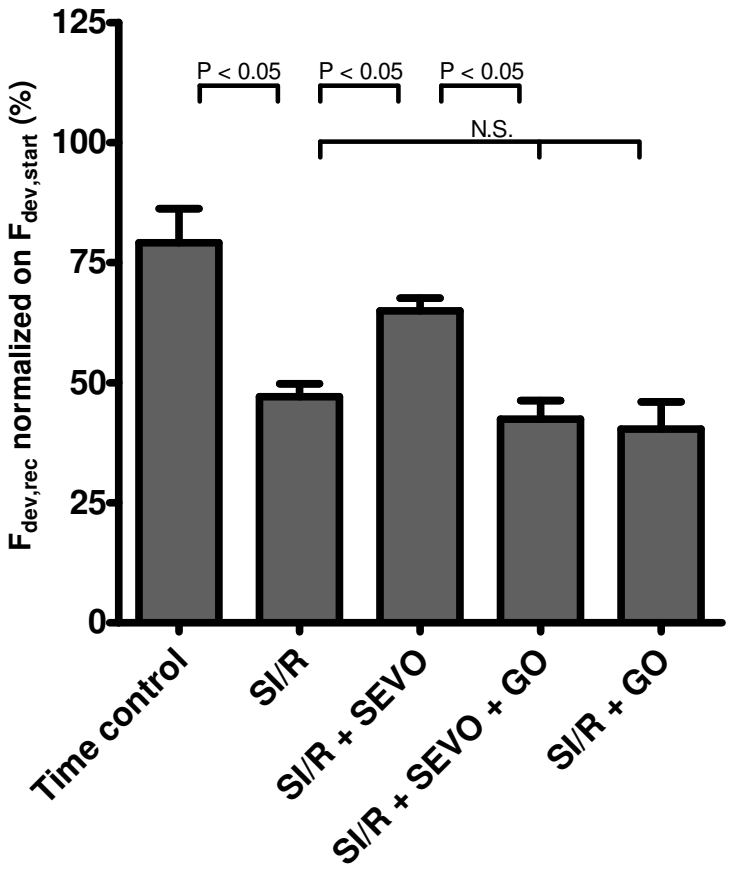

Figure 4.2 The role of PKC-α in sevoflurane-induced cardioprotection. The recovery of the developed force ($F_{dev,rec}$) is expressed as percentage of the initial developed force ($F_{dev,start}$). Pretreatment with sevoflurane (SEVO) increased the post-ischemic contractile force recovery, and this protective effect was attenuated in trabeculae treated with Go6976 (GO) (50 nM). GO=Go6976; SEVO=sevoflurane; SI/R=simulated ischemia and reperfusion. Data are mean±SEM; Time control, n=6; SI/R, n=9; SI/R+SEVO, n=8; SI/R+SEVO+GO, n=10, SI/R+GO, n=5.

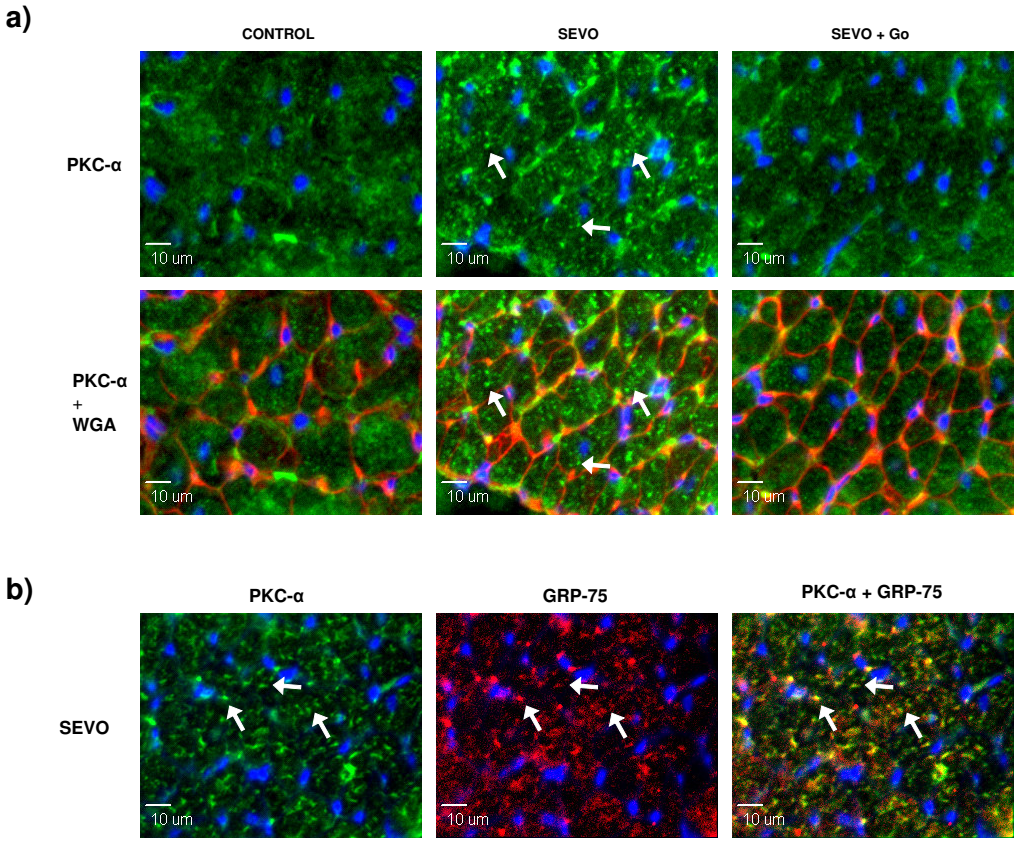

Figure 4.3 PKC-α translocation in response to sevoflurane (SEVO) in cross-sections of isolated rat trabeculae. a) In all panels, *green* represents specific PKC-α staining, *blue* represents 4',6 diamidino-2-phenylindole (DAPI) staining the nuclei and red represents wheat germ agglutinin (WGA) staining the sarcolemma. In control trabeculae PKC-α is predominantly located in the cytosol. After sevoflurane exposure, PKC-α is detected in circumscript dots located within the cytosol (panel *SEVO*, *white arrows*). This sevoflurane-induced PKC-α translocation is effectively attenuated by Go6976 (50 nM) (panel *SEVO+Go*). Go6976 itself had no intrinsic effect on PKC-α translocation. b) PKC-α colocalizes with the mitochondria after sevoflurane-induced preconditioning. In these panels, *green* represents specific PKC-α staining, *blue* represents 4',6 diamidino-2-phenylindole (DAPI) staining the nuclei and *red* a specific antibody against mitochondrial heatshock protein GRP75. After sevoflurane preconditioning, PKC-α (*PKC-α* panel, *white arrows*) translocates towards mitochondria (*GRP75 panel*, *white arrows*) indicated by *yellow* in colocalization analyses (*panel PKC-α+GRP75*, *white arrows*). Go=Go6976; SEVO=sevoflurane.

PKC-α Translocation in Response to Sevoflurane

In order to further elucidate the role of PKC-α activation in sevoflurane-induced cardioprotection, the subcellular distribution of PKC-α was evaluated in direct response of sevoflurane. Figure 4.3a shows the translocation pattern of PKC-α in response to sevoflurane and sevoflurane in combination with Go6976 in cross-sections of isolated trabeculae. In figure 4.3a, *green* represents specific PKC-α staining, *blue* represents nuclear DAPI staining and *red* shows wheat germ agglutinin (WGA) staining the sarcolemma. In control trabeculae (figure 4.3a, panel *CONTROL*), PKC-α is distributed homogeneously in the cytosol. In

response to sevoflurane, PKC-α showed a dotted translocation pattern (figure 4.3a, panel *SEVO*, *white arrows*) which co-localized mitochondrial heat shock protein GRP75 (figure 4.3b, *white arrows*). PKC-α translocation was not detected in trabeculae treated with Go7976 (figure 4.3a, panel *SEVO+Go*) and Go6976 treatment itself did not affect PKC-α translocation (results not shown). To identify whether PKC-α translocation occurs upstream or downstream of respectively ROS-production, opening of mitoK$^{+}_{ATP}$ channels or PKC-δ activation, PKC-α translocation was studied in trabeculae treated with MPG, 5-HD and ROT, respectively (figure 4.4). In trabeculae exposed to MPG, sevoflurane-induced translocation of PKC-α was abolished (figure 4.4, panel *SEVO+MPG*). Interestingly, sevoflurane-induced PKC-α translocation was not abolished by 5-HD (figure 4.4, panel *SEVO+5HD*, *white arrows*) or ROT (figure 4.4, panel *SEVO+ROT*, *white arrows*). These data suggest that sevoflurane activates PKC-α *via* ROS-production, while opening of opening of mitoK$^{+}_{ATP}$ channels and activation of PKC-δ are not involved or *act* downstream of PKC-α activation.

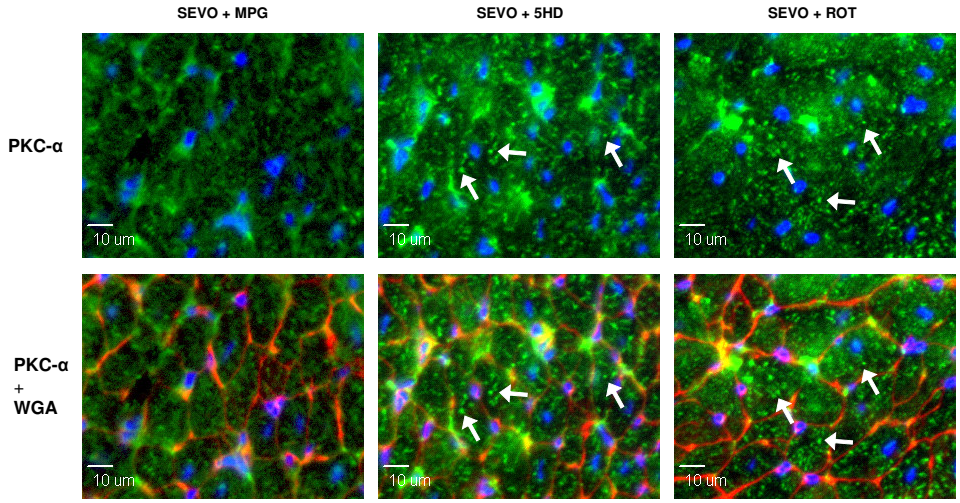

Figure 4.4 Sevoflurane (SEVO)-induced protein kinase C (PKC)-α tranlocation in relation with common mediators of cardioprotective signal transduction pathways. In all panels, *green* represents specific PKC-α staining, *blue* represents 4',6 diamidino-2-phenylindole (DAPI) staining the nuclei and *red* wheat germ agglutinin (WGA) staining the sarcolemma. Sevoflurane induced a translocation of PKC-α from the cytosol towards the mitochondria (see figure 4.3). This translocation was reduced by scavenging reactive oxygen species during preconditioning with n-(2-mercaptopropionyl)-glycine (MPG; 300 μM)(panel *SEVO+MPG*). Interestingly, sevoflurane-induced PKC-α translocation was not attenuated by 5-hydroxydecanoic acid (5HD, 100 μM)(panel *SEVO+5HD, white arrows*) or by rottlerin (ROT, 1 μM) (panel *SEVO+ROT, white arrows*).

DISCUSSION

The present study shows for the first time that, in addition to the previously described role of the Ca^{2+}-independent PKC-δ isoform, sevoflurane-induced cardioprotection involves activation of the Ca^{2+}-dependent isoform PKC-α. In addition, sevoflurane-induced translocation of PKC-α is dependent on the production of ROS, occurs upstream of opening of mitoK^+_{ATP} channels and is independent of PKC-δ activation.

Cardioprotective signaling elicited by preconditioning has been demonstrated to rely on PKC activation. However the sequence of events as well as the specific PKC isoforms involved in this protective cascade have been widely discussed (2,4,17). PKC-δ and PKC-ε have been considered most important in ischemic (18), opioid (19) and volatile anesthetic-induced preconditioning (6,7).

Indeed, we demonstrated that the sevoflurane-induced cardioprotective response in our model depends on PKC-δ activation, whereas PKC-ε is not primarily involved. Furthermore, we showed that PKC-δ translocation in response to sevoflurane depends on ROS-production, but not on opening of mitok$^+_{ATP}$ channels (14). Importantly, the present data show for the first time that the Ca^{2+}-dependent PKC-α isoform is activated by sevoflurane and is involved in sevoflurane-induced cardioprotective signaling as well. It has previously been described that volatile anesthetics are able to increase PKC-α activity in purified rat brain extracts as well as skeletal muscle (20). In addition, PKC-α translocates in response to other preconditioning stimuli, like ischemia (11,21), phenylepinephrine (22), Ca^{2+} (9) and opiods (19), and specific PKC-α inhibition has been reported to abolish cardioprotection induced by ischemic preconditioning (23,24).

Our data suggest that PKC-α is activated by sevoflurane via the formation of ROS, but not opening of mitoK^+_{ATP} channels. This ROS dependent activation of PKC-α has been supported by Vanden Hoek *et al.* (2000) demonstrating that PKC-α inhibition attenuated cardioprotection by ischemic preconditioning, but did not affect ROS production during preconditioning (24). However, in contrast to our findings Hassouna *et al.* (2004) showed that IPC as well as diazoxide-induced preconditioning involved PKC-α acting down-stream of the mitoK^+_{ATP} channels (23). In addition, it is reported that upon preconditioning with the mitoK^+_{ATP} channel-opener diazoxide PKC-α translocates towards the sarcolemma (25).

Diazoxide as well as sevoflurane increase intracellular ROS production and scavenging of ROS with MPG attenuates subsequent preconditioning (14,26). However, sevoflurane-induced ROS production has been reported to be independent of mitoK^+_{ATP} channels (27). Therefore, the explanation underlying these contradictory findings may relate to differences in the mechanism of ROS production.

How sevoflurane-induced PKC-α activation contributes to the improved contractile recovery after I/R-injury remains speculative. PKC-α has been demonstrated to be actively involved in the regulation of cell survival and apoptosis (28,29), in addition to direct involvement in the regulation of contractility of cardiomyocytes (30-33). Although others showed that PKC-α translocates towards the sarcolemma in response to Ca^{2+} and ischemia (9), we found that PKC-α translocated towards mitochondria. This implies that direct modification of contractile -or Ca^{2+} handling proteins in our model may be less likely. Interestingly, mitochondrial PKC-α has been associated with increased phosphorylation of the anti-apoptotic protein Bcl2 (34). Recently, Imahashi *et al.* (2004) showed that modulation of myocardial energy metabolism by Bcl2 overexpression contributed to increased recovery after I/R (35). We therefore speculate that this may be among the potential mitochondrial targets of PKC-α.

Interestingly, serial signaling steps between different PKC-isoforms have been reported to occur in several cardioprotective signaling cascades. Recently, Inagaki and Mochly-Rosen (2005) showed that ethanol-induced cardioprotection relies on both PKC-δ and PKC-ε activation (36). In addition, serial PKC signaling was also suggested in human atrial appendages with respect to PKC-ε and PKC-α (23). In contrast, the present study shows that PKC-α activation is independent of PKC-δ, although others showed that PKC-α increases PKC-δ protein levels via stabilization of PKC-δ mRNA (37). Therefore serial PKC signaling with respect to PKC-α and PKC-δ seems to be less likely in our model, because of the acute and parallel activation of different isoforms by sevoflurane.

Finally, the finding that the Ca^{2+}-dependent PKC-α isoform contributes to sevoflurane-induced protective signaling may be considerably relevant for the clinical induction of cardioprotection pathological myocardium. Alterations in myocardial Ca^{2+} handling as well as expression of PKC-isoforms have been demonstrated in diseased myocardium, i.e. due to heart failure. PKC-α activity is dose-dependently modified by Ca^{2+} (38), and likewise it's activity and expression is increased in failing human myocardium (39). Therefore, in patients

with reduced myocardial function due to heart failure, sevoflurane-induced cardioprotective signaling cascade may be altered.

Study Limitations

Specific comments have to be made in relation to our present results. This study largely depends on the specificity of the indolocarbazole compound Go6976. Go6976 has been described to be able to effectively discriminate between Ca^{2+}-dependent PKC isoforms with no effect on the novel PKC isoforms (40). In the present study we focused on PKC-α, as representative of the Ca^{2+}-dependent PKC isoforms, in the signaling cascade of sevoflurane-induced protective signaling. The specificity of Go6976 was confirmed as PKC-α translocation induced by sevoflurane was abolished by this inhibitor. However, with this approach we cannot entirely exclude the involvement of the other Ca^{2+}-dependent isoform PKC-β in our observed effects.

In summary, in addition to the previously described role of the Ca^{2+}-independent PKC-δ isoform, sevoflurane-induced preconditioning depends on activation of the Ca^{2+}-dependent PKC-α isoform. During sevoflurane-induced preconditioning, PKC-α translocates towards the mitochondria. This mitochondrial translocation of PKC-α is dependent on the production of ROS, but is independent of opening of mitoK^{+}_{ATP} channels and PKC-δ. Although the exact relationship between PKC-α and PKC-δ remains to be established, the co-involvement of the Ca^{2+}-dependent PKC-α isoform in sevoflurane-induced cardioprotection may be of clinical relevance for the development of cardioprotective strategies in the diseased myocardium with altered Ca^{2+} handling.

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Chapter 5

CARDIOPROTECTION VIA ACTIVATION OF PROTEIN KINASE C-δ DEPENDS ON MODULATION OF THE REVERSE MODE OF THE NA^+/CA^{2+}-EXCHANGER

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ABSTRACT

Pre-treatment with the volatile anesthetic sevoflurane protects cardiomyocytes against subsequent ischemic episodes due to a protein kinase C (PKC)-δ mediated preconditioning effect. Sevoflurane directly modulates cardiac Ca^{2+} handling and since Ca^{2+} also serves as a mediator in other cardioprotective signaling pathways, possible involvement of the Na^+/Ca^{2+}-exchanger (NCX) in relation with PKC-δ in sevoflurane-induced cardioprotection was investigated.

Isolated right ventricular rat trabeculae were subjected to simulated ischemia and reperfusion (SI/R), consisting of superfusion with hypoxic, glucose-free buffer for 40 minutes after rigor development, followed by reperfusion with normoxic glucose containing buffer. Preconditioning with sevoflurane prior to SI/R improved isometric force development during contractile recovery at 60 minutes after the end of hypoxic superfusion (83±7% [sevo] *versus* 57±2% [SI/R]; n=8; $P<0.01$). Inhibition of the reverse mode of the NCX by KB-R7943 (10 μM) or SEA0400 (1 μM) during preconditioning attenuated the protective effect of sevoflurane. KB-R7943 and SEA0400 did not have intrinsic effects on the contractile recovery. Furthermore, inhibition of the NCX in trabeculae exposed to sevoflurane reduced sevoflurane-induced PKC-δ translocation towards the sarcolemma, as demonstrated by digital imaging fluorescent microscopy. The degree of PKC-δ phosphorylation at $serine^{643}$ as determined by western blot analysis was not affected by sevoflurane.

Sevoflurane-induced cardioprotection depends on the NCX preceding PKC-δ translocation presumably via increased NCX-mediated Ca^{2+} influx. This may suggest that increased myocardial Ca^{2+}-load triggers the cardioprotective signaling cascade elicited by volatile anesthetic agents similar to other modes of preconditioning.

INTRODUCTION

The myocardium contains intrinsic protective mechanisms against ischemia/reperfusion (I/R) injury, which can be triggered by several stimuli, including volatile anesthetics like sevoflurane. The cardioprotective properties of sevoflurane depend on activating protein kinase C (PKC) and producing reactive oxygen species (ROS) similar to ischemic preconditioning (IPC) and Ca^{2+}-preconditioning (CPC). Cardioprotection induced by IPC and CPC is mediated via Ca^{2+} and reduction of cellular Ca^{2+} influx reduces PKC activation and subsequent protection against I/R-injury (1).

Sevoflurane reduces myocardial Ca^{2+} availability, but paradoxically increases sarcoplasmic reticulum (SR) Ca^{2+} content (2). Changes in cellular SR Ca^{2+}-load are associated with activation of survival and/or death signaling pathways (3), and therefore provide a potential mechanism for cardioprotective signaling. Sevoflurane reduces Ca^{2+} influx via the L-type Ca^{2+} channels (4), and therefore another Ca^{2+} loading mechanism may be involved. One of the key regulation proteins of myocardial Ca^{2+} loading is the Na^{+}/Ca^{2+}-exchanger (NCX) via NCX-dependent Ca^{2+} influx in exchange for Na^{+} (=reverse mode of the NCX) (5). Until now, it is unknown whether there is a particular contribution of the reverse mode of the NCX in sevoflurane-induced alterations in Ca^{2+} homeostasis. Furthermore, the possible role of the NCX in the activation of signaling proteins involved in sevoflurane preconditioning, like PKC, has not been addressed. In this study, we therefore investigated the involvement of the reverse mode of the NCX in sevoflurane-induced cardioprotective signaling and specifically focused on the relation with the PKC-δ isoform, which was previously shown to be essentially involved in the sevoflurane-induced preconditioning (6).

MATERIALS AND METHODS

Animals and Experimental Setup

This study was performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the VU University Medical Center. Our experimental setup has previously been described in detail (6). Briefly, male Wistar rats (250-400 gram; Harlan, Horst, the Netherlands), were anesthetized with sodium pentobarbital (80 mg/kg, i.p., Nembutal®, Sanofi Sante BV) and the heart was subsequently removed. Trabeculae were isolated from the right ventricle (length 2-5 mm, diameter<0.2 mm) under contractile arrest conditions and mounted in an airtight organ bath developed for isometric force measurements. The muscles were superfused with oxygenated (95% O_2 and 5% CO_2) Tyrode buffer with the following composition: 120 mM NaCl, 1.22 mM $MgSO_4.7H_2O$, 1.99 mM NaH_2PO_4, 27.0 mM $NaHCO_3$, 5.0 mM KCl, 1 mM $CaCl_2$ and 10 mM glucose. Subsequently, muscle length was set at 95% of the maximal length as determined by a force-length relation.

Experimental Protocol

After 60 minutes equilibration (40 minutes: 27º C/0.5 Hz and 20 minutes: 24ºC/0.2 Hz), the initial developed force of contraction (F_{dev}) and the potentiated force (F_{pot}), as determined by a post-extrasystolic potentiation protocol (PESP), were recorded. PESP determines the contractile reserve of trabeculae by maximally filling the SR with Ca^{2+}. Trabeculae failing to stabilize, spontaneously contracting trabeculae or failing to show PESP were excluded. Figure 5.1A depicts the design for all groups exposed to different the preconditioning protocols. Ischemia was simulated in trabeculae (except time controls) by superfusion with hypoxic Tyrode without glucose and increasing stimulation frequency to 1 Hz for 40 minutes after rigor development as described previously (6). Trabeculae were preconditioned for 15 minutes with normal Tyrode saturated with 3.8vol% sevoflurane 30 minutes prior to simulated ischemia and reperfusion (SI/R). After washout of sevoflurane, trabeculae were superfused for 15 minutes with normal Tyrode until SI/R. In separate experimental groups, the preconditioning phase was preceded by addition of KB-R7943 (KBR; 10 μM; Tocris Cookson) or SEA0400 (SEA; 1 μM; synthesized in Taisho Pharmaceutical, Saitama, Japan).

After 60 minutes of reperfusion the recovery of F_{dev} ($F_{dev,rec}$) was expressed as a percentage of F_{dev} before SI/R.

Effect of Sevoflurane on NCX-mediated Ca^{2+} loading

The contribution of Ca^{2+} influx via the reverse mode of the NCX during sevoflurane was evaluated by experimental design shown in figure 5.2A. To study transsarcolemmal Ca^{2+} influx via the NCX, electrical stimulation was stopped and trabeculae were superfused with a low Na^+-buffer consisting of normal Tyrode except the replacement of 120 mM NaCl with 120 mM tetramethylammoniumchloride (TMA) (7). After 60 seconds, superfusion was switched back to normal Tyrode and as superfusion with low Na^+-buffer increases the electrochemical potential for Ca^{2+}-uptake via the NCX, the developed force after resuming electrical stimulation was potentiated (F_{TMA}). F_{TMA} was normalized on the F_{dev} during basal stimulation conditions before the Ca^{2+} loading protocol and provides an estimate for the amount of Ca^{2+} loading. Trabeculae, exposed to 3.8vol% sevoflurane in the presence or absence of the NCX inhibitors KB-R7943 (10 μM) or SEA0400 (1 μM) were subjected to this Ca^{2+} loading protocol (figure 5.2B).

Immunofluorescent Microscopy of PKC-δ Distribution

Figure 5.3A demonstrates the experimental design for all groups exposed to analysis of PKC-δ localization. The sevoflurane-induced subcellular redistribution of PKC-δ before SI/R was studied by immunofluorescent staining and by digital imaging fluorescence microscopy (DIM), as described earlier (6). Briefly, trabeculae subjected to an experimental protocol were embedded in gelatin and cross-sections were subsequently fixed, stained for PKC-δ (Research & Diagnostic Antibodies, Benicia, USA), counterstained for the sarcolemma (10% (vol/vol) wheat germ agglutinin (WGA; Molecular Probes, Invitrogen, Breda, The Netherlands) and nuclei 4',6 diamidino-2-phenylindole (DAPI)-containing mounting medium (Vector Laboratories, Burlingame, CA, USA). The sections were analyzed with a ZEISS Axiovert 200 Marianas™ inverted digital imaging microscope workstation using dedicated imaging and analysis software obtained from Intelligent Imaging Innovations (Denver, CO, USA).

Western Blot Analyses for PKC-δ Phosphorylation

For PKC-δ phosphorylation analysis, trabeculae were subjected to different experimental protocols (figure 5.3A). Proteins were separated by gel electrophoresis (10 µg per lane), blotted onto a nitrocellulose membrane and stained for phosphorylated PKC-δ at serine[643] (Cell Signaling Technology) as well as for total PKC-δ (Research & Diagnostic Antibodies, Benicia, USA). The immunoreactive bands were visualized by chemiluminescence (Amersham) and quantified using a charge coupled device camera (Fuji Science Imaging Systems) in combination with AIDA Image Analyzer software (Isotopenmessgeräte; Staubenhardt, Germany).

Statistical Analysis

Data was analyzed by analysis of variance (ANOVA), followed by either Tukey or Dunnett post-hoc test. For all analyses a *P*-value<0.05 was considered to reflect a significant difference. All values are given as means±standard error of the mean (SEM).

Table 5.1 General Characteristics of Trabeculae Subjected to Simulated Ischemia and Reperfusion.

	CSA, mm^2	$F_{dev,ini}$ mN/mm^2	$F_{dev,ini}$ / $F_{pot,ini}$,%	$F_{pas,ini}$, mN/mm^2	Time to rigor, minutes
TIME	0.06 ± 0.02	51 ± 9	62 ± 5	2.6 ± 0.6	
SI/R	0.04 ± 0.009	55 ± 9	48 ± 5	3.5 ± 0.6	28 ± 3
SI/R + SEVO	0.05 ± 0.02	58 ± 10	46 ± 6	3.9 ± 1	31 ± 3
SI/R + SEVO + KB-R7943	0.02 ± 0.005	64 ± 9	62 ± 5	4.5 ± 0.4	20 ± 8
SI/R + SEVO + SEA0400	0.02 ± 0.009	35 ± 12	55 ± 7	1.4 ± 0.08	33 ± 10
SI/R + KB-R7943	0.03 ± 0.01	43 ± 15	43 ± 6	3.2 ± 0.5	25 ± 2
SI/R + SEA0400	0.02 ± 0.0001	49 ± 16	46 ± 11	2.7 ± 0.8	41 ± 1*

* *P*<0.05 compared with the experimental group SI/R+SEA0400 *versus* SI/R+SEVO+KB-R7943. Data presented as mean±SEM. CSA=cross-sectional area; $F_{dev,ini}$=initial developed force before the experiment; $F_{dev,ini}/F_{pot,ini}$=ratio between initial developed force and initial potentiated force as determined by PESP; $F_{pas,ini}$=initial passive force; SEVO=sevoflurane; SI/R=simulated ischemia and reperfusion, TIME=time control.

RESULTS

Inhibition of the Reverse Mode of the NCX Attenuates Sevoflurane-induced Cardioprotection

The initial contractile parameters showed minor variation between the experimental groups (table 5.1). The time to rigor development was nominally prolonged in the SI/R+SEA0400 group, but this did not affect contractile recovery in this experimental group. SI/R reduced the contractile recovery ($F_{dev,rec}$) to 57±2%, whether preconditioning with sevoflurane improved the myocardial force development to 83±7% (P<0.05 *versus* [SI/R]) (figure 5.1B).

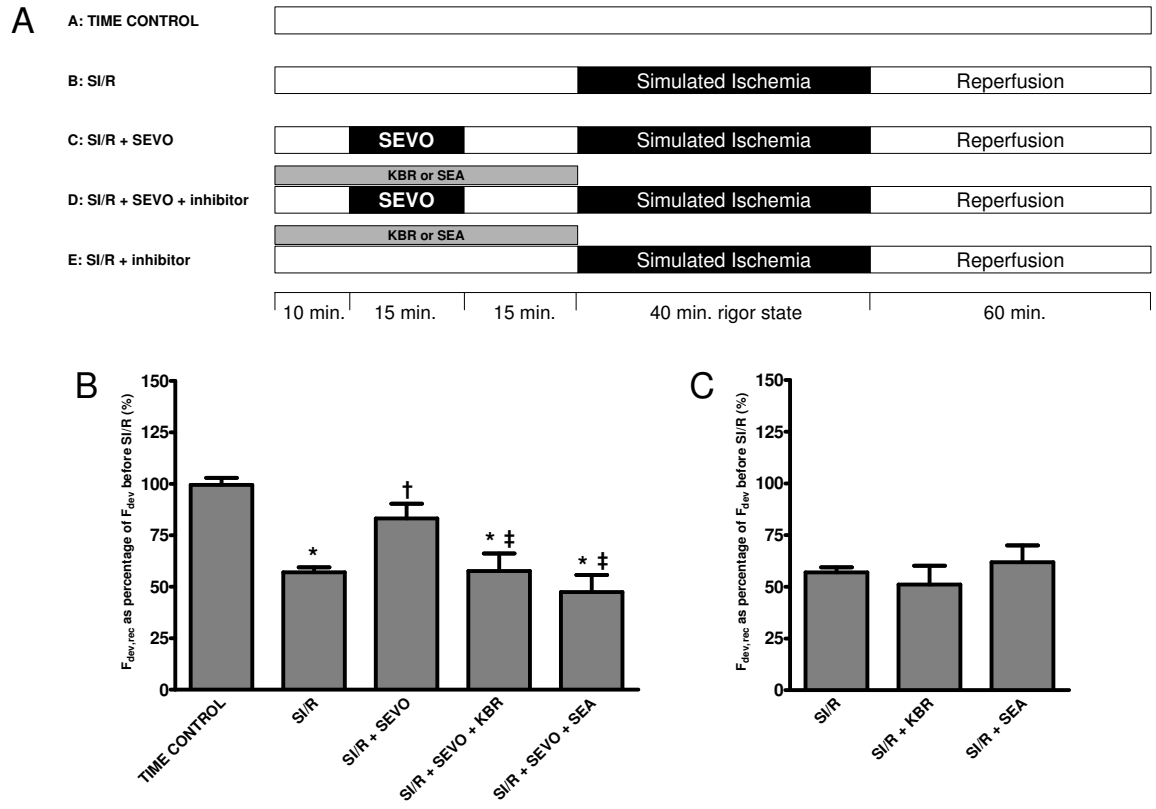

Figure 5.1 The role of the reverse mode of the NCX in sevoflurane-induced cardioprotection. (A) Experimental design for trabeculae subjected to simulated ischemia and reperfusion (SI/R). (B) The recovery of the developed force ($F_{dev,rec}$) after SI/R is expressed as percentage of the developed force just before SI/R. Pretreatment with sevoflurane improved the post-ischemic contractile force recovery and this protective effect was attenuated when the NCX was inhibited during preconditioning with either KB-R7943 (KBR; 10μM) or SEA0400 (SEA; 1μM). (C) KB-R7943 and SEA0400 did not affect the $F_{dev,rec}$ after SI/R. Data are mean±SEM and represent 8 experiments, except for TIME CONTROL (n=6), SI/R+KBR (n=6), SI/R+SEVO+SEA (n=3) and SI/R+SEA (n=3). * P<0.05 *versus* TIME CONTROL, † P<0.05 *versus* SI/R, ‡ P<0.05 *versus* SI/R+SEVO.

Inhibition of the reverse mode of the NCX during preconditioning by either KB-R7943 or SEA0400 completely reversed the protective effect of sevoflurane on contractile recovery. Both KB-R7943 and SEA0400 did not show an intrinsic effect on the contractile recovery (figure 5.1C). Although both inhibitors slightly induced positive inotropy, as was previously reported by others, this did not interfere with our study objectives (8,9).

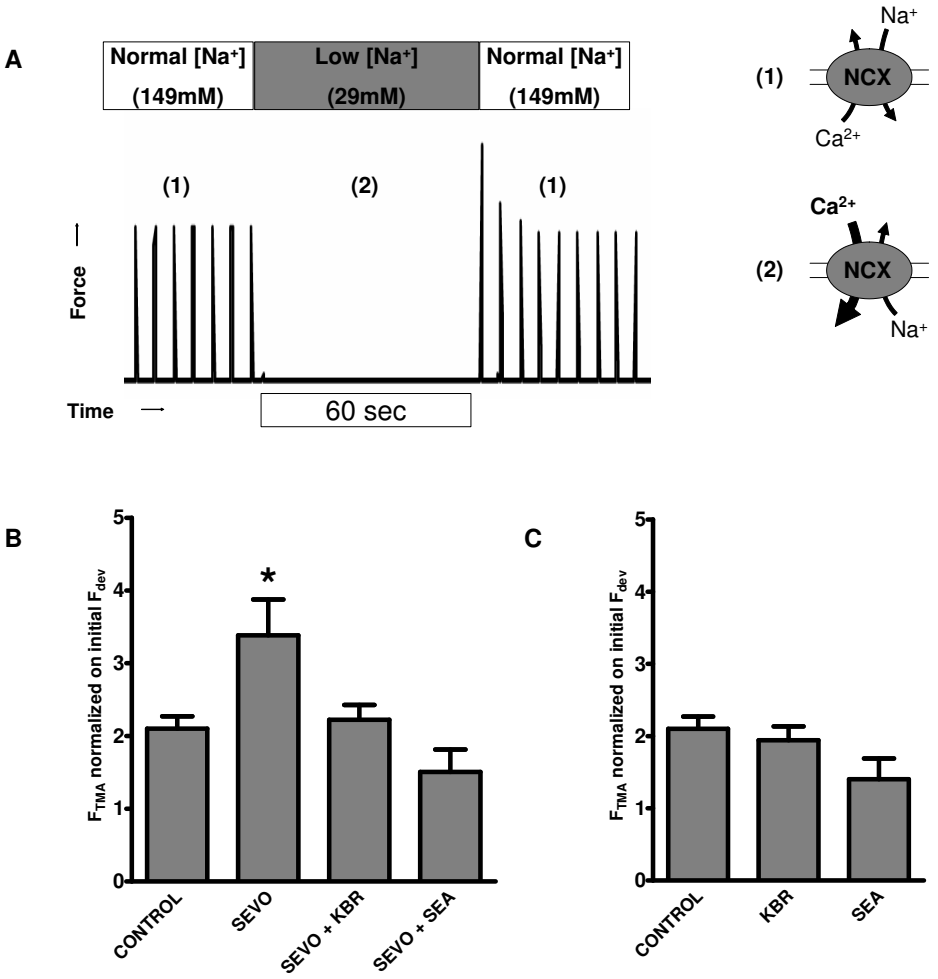

Figure 5.2 Sevoflurane can increase Ca^{2+} influx via the reverse mode of the NCX. (A) Overview of the experimental protocol studying the effects of sevoflurane on NCX-mediated Ca^{2+} loading. (B) Developed force after low Na^+ superfusion (F_{TMA}) is normalized on the developed force during basal stimulation conditions (initial F_{dev}) before the Ca^{2+} loading protocol. During sevoflurane F_{TMA} is increased compared to control trabeculae. This effect of sevoflurane was attenuated in trabeculae with inhibition of the NCX by KB-R7943 (10 μM) as well as by SEA0400 (1 μM). Effect of KB-R7943 (KBR) and SEA0400 (SEA) itself on low Na^+-induced Ca^{2+} loading protocol (C). Data are expressed as mean±SEM (n=5, except SEVO+SEA and SEA, n=3). * $P<0.05$ *versus* CONTROL.

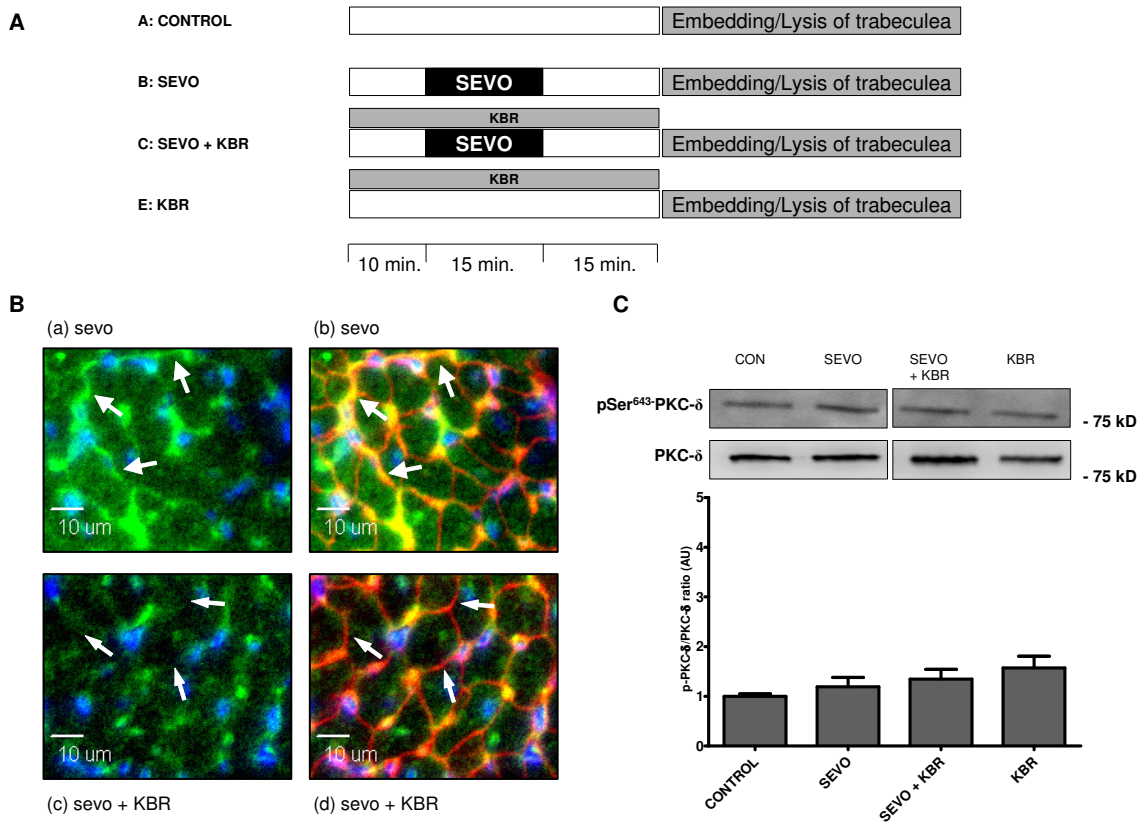

Figure 5.3 PKC-δ activation in response to sevoflurane. (A) Schematic overview of the experimental protocol studying sevoflurane-induced PKC-δ activation. (B) Digital imaging microscopy analyses of protein kinase C (PKC)-δ translocation: in all panels, *green* represents specific PKC-δ staining and *blue* the nuclear DAPI staining. In panel b and d, *red* represents the wheat germ agglutinin (WGA) staining of the sarcolemmal glycocalyx. In response to sevoflurane (sevo), PKC-δ translocated towards the sarcolemma (panel *a* and *b*, *white arrows*) as previously shown (6). Inhibition of the reverse mode of the NCX abolished this specific translocation of PKC-δ (panel *c* and *d*, *white arrows* indicate sarcolemma lacking PKC-δ staining). (C) Sevoflurane (SEVO) did not increase PKC-δ phosphorylation on serine^{643}. The ratio of serine^{643}-phosphorylated PKC-δ and total PKC-δ is expressed as relative change compared to control in arbitrary units (AU). Data are mean±SEM, n=5. SEVO=sevoflurane, KBR=KB-R7943.

Sevoflurane Increases NCX-mediated Ca^{2+} loading

Low Na^+-induced Ca^{2+} loading was used to study the effect of sevoflurane on Ca^{2+} influx via the reverse mode of the NCX (figure 5.2). Sevoflurane increased force development (F_{TMA}) after 60 seconds of low Na^+ superfusion, suggesting increased intracellular Ca^{2+} loading. This increased force development due to sevoflurane was abolished in trabeculae treated with KB-R7943 or SEA0400.

Differential Involvement of the Reverse Mode of the NCX in Sevoflurane-induced Translocation and Phosphorylation of PKC-δ

Figure 5.3B shows the translocation pattern of PKC-δ in response to sevoflurane and sevoflurane in combination with KB-R7943 in cross-sections of isolated trabeculae. In all panels, *green* represents specific PKC-δ staining, *blue* represents nuclear DAPI staining and *red* shows sarcolemmal staining with wheat germ agglutinin (WGA). As previous reported, sevoflurane induced translocation of PKC-δ from the cytosolic compartment to the sarcolemma (figure 5.3B, panel *a* and *b*) (6). This sevoflurane-induced translocation of PKC-δ was effectively reduced in trabeculae treated with KB-R7943 (figure 5.3B, panel *c* and *d*). This translocation most likely does not depend on phosphorylation of PKC-δ at serine^{643}, as sevoflurane treatment did not affect the ratio of phosphorylated PKC-δ to total PKC-δ, as shown by western blot analyses (figure 5.3C).

DISCUSSION

Sevoflurane-induced preconditioning is mediated via activation and translocation of PKC-δ, as was earlier shown in isolated rat trabeculae (6). The present study shows that sevoflurane-induced cardioprotection, and particularly the involvement of PKC-δ in this protective mechanism, depends on the activity of the reverse mode of the NCX. Several lines of evidence were provided for this role of the NCX in sevoflurane-induced protective signaling: 1) sevoflurane-induced cardioprotection was abolished by KB-R7943 and SEA0400 possibly via reduction of Ca^{2+}-uptake via the NCX, 2) sevoflurane increased NCX-mediated Ca^{2+} loading indicating that sevoflurane can facilitate Ca^{2+} influx via the NCX, 3) pharmacological blockade of the reverse mode of the NCX attenuated sevoflurane-induced translocation of PKC-δ.

In various protective signaling cascades, an important role for Ca^{2+} for has been demonstrated (1). Indeed, cardioprotection induced by IPC and CPC can be abolished by Ca^{2+} influx inhibition, especially Ca^{2+} influx via the L-type Ca^{2+} channels or the NCX (1,8). Interestingly, inhibition of the L-type channels as well as indirect inhibition of Na^+/Ca^{2+}-exchange coincided with both a reduction of PKC translocation and PKC-activity (10). Our present data

show that inhibition of the reverse mode of the NCX during sevoflurane preconditioning attenuates cardioprotection and reduces PKC-δ translocation. This therefore implies that increased NCX mediated Ca^{2+} influx is upstream in sevoflurane-induced cardioprotective signaling, in parallel to other preconditioning stimuli.

Our data seem to be in contrast to previous literature, showing that volatile anesthetics reduce Ca^{2+} availability and inhibit the NCX (11,12). However, sevoflurane can increase intracellular (SR) Ca^{2+}-load (2,13), and in this study we specifically demonstrate that sevoflurane indeed augments force development after low Na^+ superfusion, confirming that sevoflurane can facilitate NCX-mediated Ca^{2+} influx. Several explanations may account for these contradictory results. In the majority of studies, myocardial NCX function is evaluated in isolated cells, and is not confirmed in functional experiments as in our investigation. Furthermore, volatile anesthetic-induced alterations on myocardial Ca^{2+} handling and action potential depend both on concentration and duration of exposure to the anesthetic (14,15). The mechanism of sevoflurane-induced facilitation of reverse Na^+/Ca^{2+} exchange may involve an increased Na^+-load due to inhibition of the Na^+/K^+-ATPase, and the unequal effect of sevoflurane on Na^+- and Ca^{2+}-entry during depolarization (4,13,15). This changes the electrochemical driving force in favor of the Na^+-dependent Ca^{2+} influx. In addition, Na^+/Ca^{2+}-exchange is under ionic control of Ca^{2+}, Na^+ and H^+ via catalytic regulation of the intracellular loop of the NCX (5). In isolated mouse papillary muscles devoid of regulation by Ca^{2+} and Na^+ the contribution of reverse Na^+/Ca^{2+}-exchange is increased, as shown by increased rest potentiation (16). H^+-ions inhibit concentration-dependent ion transport via the NCX, but in cells devoid of ionic control by H^+, inhibition of NCX ion transport is reduced even at physiological pH (5). Therefore we speculate that sevoflurane may influence NCX activity by altering the ionic regulation of the NCX by Na^+, Ca^{2+} and H^+, and thereby increasing NCX-induced myocardial Ca^{2+} loading. Another potential mechanism may involve a volatile anesthetic-induced increase in intracellular pH by direct stimulation of Na^+/H^+-exchange and thereby increase NCX ion transport (17). However, in our experimental model alterations in hydrogen exchange may only minimally affect intracellular pH due to the buffer-capacity of the bicarbonate-containing superfusion solution (18).

The present data implicate that modulation of Ca^{2+} handling via the reverse mode of the NCX precede PKC-δ activation in the cardioprotective signal transduction pathway elicited by

sevoflurane. However, it is unknown how PKC-δ is modulated by alterations in myocardial Ca^{2+} handling. Interestingly, PKC-δ is a Ca^{2+}-independent PKC isoform, in contrast to the Ca^{2+}-dependent isoforms PKC-α and PKC-β_1. However, it is well described that several preconditioning stimuli that depend on Ca^{2+} induce translocation of both Ca^{2+}-dependent and -independent isoforms, suggesting isoform crosstalk (1,10). The link between Ca^{2+} handling and PKC-δ modulation may involve ROS-dependent PKC-δ translocation as well as phosphorylation. We previously demonstrated in isolated rat trabeculae that sevoflurane-induced PKC-δ translocation depends on the production of ROS (6), which could be a result of altered Ca^{2+} handling in the myocardium. In particular, mitochondrial Ca^{2+} loading may induce ROS production. Increased Ca^{2+} influx via the reverse mode of the NCX may contribute to this mitochondrial ROS production, with PKC activation as result.

We showed that sevoflurane induces translocation, but not increased serine^{643} phosphorylation of PKC-δ. Interestingly, sevoflurane-induced translocation of PKC-δ was attenuated by NCX inhibition. The discrepancy between sevoflurane-induced PKC-δ translocation and phosphorylation may be due to the presence of several PKC-δ (auto-) phosphorylation sites, and serine^{643} has been demonstrated to be an auto-phosphorylation site involved in the regulation of its enzymatic activity (19). Therefore, it may be that translocation of PKC-δ involves phosphorylation of a different site than serine^{643}, which was not investigated in the present study.

Several limitations must be considered in the interpretation of the present results. Stable and reliable contractile performance was ensured by conducting the experiments at hypothermic conditions, which may affect ion-exchange. However, we previously demonstrated in isolated trabeculae that results of similar experiments on NCX function were unaffected by temperature (7). Secondly, KB-R7943 was developed as a selective NCX inhibitor, and up to 10 μM it is reported to be specific for Ca^{2+}-entry via NCX with little influence on Ca^{2+} efflux rate (20). To exclude aspecific effects of KB-R7943, experiments to inhibit the reverse mode of the NCX were repeated in the presence of SEA0400, a novel NCX inhibitor (21). In our trabeculae, consistent with previous publications KB-R7943 as well as SEA0400 exerted a slight positive inotropic effect, suggesting some inhibitory effect on the forward mode of the NCX, and were not easily washout out from the preparation (8,22). However, the relative alteration in basal force development of trabeculae exposed to NCX inhibition did not induce

cardioprotection, and did not alter rigor development or final recovery characteristics. Finally, we have not directly shown activation of the NCX by sevoflurane by a direct measurement of intracellular Ca^{2+}. However, our results do show that pharmacological inhibition of the reverse mode of the NCX attenuates sevoflurane-induced PKC-δ activation and subsequent cardioprotection.

In summary, we demonstrate that sevoflurane-induced cardioprotection is dependent on the reverse mode of the NCX preceding PKC-δ translocation presumably due to facilitated Ca^{2+}-uptake via the NCX. Our results imply that sevoflurane-induced facilitation of NCX dependent Ca^{2+} influx, serves as trigger for myocardial protection against ischemic episodes similar to other modes of preconditioning. This essential, but novel, role of the NCX in cardioprotective signaling may provide possible clues for the clinical induction of cardioprotective strategies.

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Chapter 6

PILOT-STUDIES OF LONG-TERM HIGH-FAT DIET-INDUCED MYOCARDIAL CONTRACTILE DYSFUNCTION AND THE EFFICACY OF SEVOFLURANE-INDUCED CARDIOPROTECTION

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ABSTRACT

A considerable number of patients undergoing anesthesia suffer from type 2 diabetes and in these patients silent myocardial ischemia may be present. Since anesthesia and surgery may precipitate myocardial ischemia, cardioprotective strategies prior to surgery may be of great importance in this high-risk patient group. In this study, using a rat model of high-fat diet-induced insulin-resistance/type 2 diabetes, in vivo cardiac function was related to in vitro contractile function of isolated trabeculae and the efficacy of the sevoflurane-induced cardioprotective response was evaluated in this model.

Male Wistar rats received a diet high in saturated fat (HFD) for 20 weeks and in vivo cardiac function was assessed by transthoracic echocardiography. In vitro contractile function was evaluated in isolated right ventricular trabeculae by analyzing isometric force development (F_{dev}) at 95% of the maximal muscle length. Trabeculae were subjected to post-extrasystolic- and post-rest potentiation in order to determine the maximal force development (F_{max}), contractile reserve (1 - F_{dev}/F_{max}), the Ca^{2+}-recirculation fraction and the contribution of transsarcolemmal Ca^{2+} fluxes. In addition, preconditioned (3.8vol% sevoflurane for 15 min) trabeculae were subjected to ischemia and reperfusion, consisting of 40 min superfusion with hypoxic, glucose-free buffer, followed by normoxic glucose containing buffer for 60 min.

Echocardiographic analyses of cardiac function showed the development of a dilated cardiomyopathy with a slight reduction of fractional shortening. Trabeculae of HFD-rats developed higher forces, but passive force development was unaffected. Interestingly, the Ca^{2+}-recirculation fraction was not altered (0.67±0.03 [LFD] *versus* 0.69±0.02 [HFD], $P>0.05$), but contractile reserve of HFD-trabeculae was reduced (0.53±0.04 [LFD] *versus* 0.40±0.05 [HFD], $P<0.05$). Finally, HFD-trabeculae showed an increased resistance to ischemia and reperfusion compared to LFD-trabeculae, and preconditioning with sevoflurane was not beneficial for the contractile recovery of HFD-trabeculae.

In conclusion, we demonstrated in these pilot-experiments that long-term high-fat feeding in rats is associated with cardiac alterations, which may be especially maladaptive during increased workloads. Secondly, our data demonstrate that contractile function of HFD-hearts after ischemia and reperfusion injury is better preserved, but in these hearts sevoflurane-induced preconditioning may not be beneficial to contractile recovery after I/R.

INTRODUCTION

The prevalence of type 2 diabetes mellitus (DM2) is expected to increase since the combination a of western type diet and physical inactivity predisposes to develop obesity. Anesthesiologists will encounter more frequently patients with diabetes undergoing anesthesia and surgery. Importantly, this group of patients is prone to the (perioperative) development of cardiovascular complications, most notably myocardial ischemia and reduced myocardial function leading to (postoperative) congestive heart failure (1). Development of perioperative cardioprotective strategies against ischemic injury, like preconditioning, could therefore reduce postoperative cardiovascular complications in this patient population.

Cardiovascular complications of the diabetic myocardium may occur due to alterations in contractile function, which is characterized in insulin-deficient laboratory animals by decreased contractile force, elevated end-diastolic pressures and reduced contraction and relaxation rates (2,3). Modulations of myocardial Ca^{2+} handling due to depressed function of the Na^{+}/Ca^{2+}-exchanger (NCX) and the sarcoplasmic reticulum (SR) seem to underlie this diabetes-induced dysfunction (4). The pathophysiology of myocardial dysfunction in insulin-resistant states, including obesity and DM2, has not been elucidated and may be due to other mechanisms compared to insulin-deficient diabetes. Isolated papillary muscles of rats exposed to an 8 week high-fat diet intervention showed higher force development during basal conditions, but a reduced recovery after increased workloads (5). Protein expression levels of the Ca^{2+} handling proteins, sarcoplasmic reticulum ATPase (SERCA) and the ryanodine receptor (RyR) were not altered, but phospholamban (PLB) hypophosphorylation was observed.

Another factor contributing to the development of cardiovascular comorbidity in hearts of diabetic patients may relate to alterations in signaling pathways relevant to cardioprotective cascades, like protein kinase B (Akt/PKB), protein kinase C (PKC), reactive oxygen species (ROS) and adenosine triphosphate (ATP)-sensitive mitochondrial K^{+} ($mitoK^{+}_{ATP}$) channels (5-7). Several studies investigating common preconditioning stimuli in the diabetic myocardium, like ischemic preconditioning (IPC) and anesthetic preconditioning (APC), showed conflicting results (8,9-14) Therefore, it is still unclear how the efficacy of preconditioning of diabetic cardiomyocytes is affected by these alterations in the

cardioprotective cascades. Ghosh *et al*. (2001) showed a blunted cardioprotective response in cardiomyocytes from diabetic patients (8). This reduced efficacy of preconditioning was confirmed in several experimental studies using insulin-deficient animals (9,10,13,14). Interestingly, Tsang *et al*. (2005) recently showed that the myocardium of type 2 diabetic rats could be protected against ischemia and reperfusion (I/R)-injury by increasing the ischemic preconditioning stimulus with multiple cycles of ischemia (11). In addition, Kehl *et al*. (2003) reported that impaired isoflurane-induced preconditioning during hyperglycemia could be restored by N-acetylcysteine, suggesting a role for oxidative stress in the disturbance of the cardioprotective response (12). Furthermore, we recently provided evidence that sevoflurane-induced cardioprotection could be abolished by inhibition of the (NCX) in isolated trabeculae from normal rats suggesting that NCX-mediated modulation of Ca^{2+} homeostasis is important in mediating sevoflurane-induced cardioprotective signaling (15). This may provide another possible mechanism for the observed alterations of the cardioprotective capacity of diabetic hearts, as NCX-function is reported to be depressed in diabetic hearts (4).

In this study, we therefore aimed to relate in more detail diabetes-induced myocardial dysfunction to alterations of the intrinsic cardioprotective capacity of diabetic myocardium in a clinical relevant animal model for insulin resistance. We first investigated in vivo cardiac function of normal rats exposed a high-fat diet (HFD) for 20 weeks in relation with in vitro contractile parameters of isolated trabeculae in order to further characterize cardiac dysfunction induced by this high-fat diet. Secondly, we studied the sensitivity of trabeculae of HFD-rats to I/R-injury and explored the cardioprotective capacity of the volatile anesthetic sevoflurane against ischemic contractile dysfunction in this model.

MATERIALS AND METHODS

Animals and Diets

This study was performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the VU University Medical Center. Adult male Wistar rats (n=13; body weight=350g, Harlan, Horst, The Netherlands) were fed a high-fat diet (HFD) rich in saturated fatty acids or a low-fat diet (LFD) for 20 weeks as described previously (5). The

experimental diets were obtained from Hope Farms (Woerden, The Netherlands). HFD (cat#4148.02) consisted of 25 wt% fat (of which 92.12 g/kg was palmitate and 100.24g/kg was oleate 100.24 g/kg), 32 wt% protein and 25 wt% carbohydrate. The control LFD was isocaloric and contained 8 wt% total fat (of which 29.12 g/kg was palmitate and 32.08 g/kg was oleate), 22 wt% protein and 60 wt% carbohydrate.

In Vivo Myocardial Function Measured by Echocardiography

After 16 weeks diet exposure transthoracic echocardiography was performed in anesthetized rats (2% isoflurane and 0.3L/min O_2) by using an ALOKA ultrasound (Prosound SSD-4000 Plus, Aloka, Biomedic, Almere, Netherlands) equipped with a 13 MHz linear interfaced array transducer. Motion mode (M-Mode) and 2D echo images were obtained in the parasternal long- and short axis views of the left ventricle (LV) at mid-papillary level. LV diameters and wall-thickness at end-diastole (EDD) and end-systole (ESD) were measured from the M-Mode images from three cardiac cycles and averaged. LV fractional shortening (FS) was calculated as: [(EDD-ESD)/EDD] x 100%.

In Vitro Contractile Function

Experimental Setup

Myocardial contractile function and susceptibility to I/R-injury was evaluated in vitro after 20 weeks of diet by performing force measurements in isolated right ventricular trabeculae. Our experimental setup and applied buffer solutions have previously been described in detail (16,17). Briefly, the animals were anesthetized with sodium pentobarbital (80 mg/kg, i.p., Nembutal®, Sanofi Sante BV). Subsequently, the heart was removed and perfused through the aorta with Tyrode buffer (120 mM NaCl, 1.22 mM $MgSO_4$.7H2O, 1.99 mM NaH_2PO_4, 27.0 mM $NaHCO_3$, 5.0 mM KCl, 1 mM $CaCl_2$, 10 mM glucose) containing 30 mM 2,3-butanedione monoxime and 15 mM KCl to protect the myocardium during dissection (18). Isolated trabeculae were mounted in an airtight organbath developed for isometric force measurements, and superfused with normal Tyrode buffer (27ºC; 2 ml/min). Trabeculae were isometrically stimulated (0.5 Hz) and stretched until the developed force (F_{dev}) was maximal

(=L_{max}). Thereafter the length of the trabeculae was set at 95% of L_{max} followed by 40 minutes of stabilization at 27ºC and 20 minutes at 24ºC with a stimulation frequency of 0.2Hz.

Contractile Parameters of Trabeculae

Parameters of contractile function (developed force (F_{dev}), passive force (F_{pas}), maximal contraction rate (+dF/dt), maximal relaxation rate (–dF/dt), time to peak contraction (T_p) and time to half relaxation (tHR)) were recorded at 95% of L_{max} unless indicated otherwise. In order to study systolic and diastolic function, standardized force-length relations were determined by increasing the muscle length from 80% to 100% of L_{max} with 2% increments in length. To evaluate contractile function in more detail, trabeculae were subjected to a post-rest potentiation protocol (PRP) to estimate net transsarcolemmal Ca^{2+} fluxes (19) and a post-extrasystolic potentiation protocol (PESP) to determine maximal force development (F_{max}), estimate contractile reserve (CR) as well as the Ca^{2+}-recirculation fraction (20-22). During PRP, electrical stimulation was paused for 10, 15, 20, 40, 60, 120 and 240 seconds, respectively. Rest potentiation was quantified by normalizing the potentiated contraction to F_{dev} during basal stimulation conditions. This provides an estimate of the amount of transsarcolemmal Ca^{2+}-flux into the cardiomyocytes (23). The PESP protocol consisted of applying 1, 5, 10 or 15 extra systolic electrical stimulations with a frequency of 5 Hz followed by 5 seconds rest. The first contraction after resuming the electrical stimulation (0.2 Hz) is considered F_{max} (24,25). Contractile reserve was defined as 1 – F_{dev}/F_{max}. The Ca^{2+}-recirculation fraction was estimated from the decay of force after PESP (20-22).

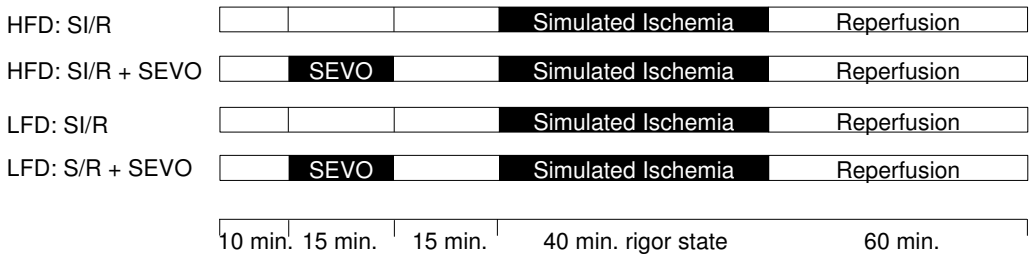

Figure 6.1 Overview of the randomized experimental design for trabeculae subjected to an SI/R- and preconditioning protocol. SEVO=sevoflurane; SI/R=simulated ischemia and reperfusion.

Sensitivity to I/R-injury and Sevoflurane-induced Cardioprotection in Trabeculae of HFD-Rats

Figure 6.1 displays the experimental design for trabeculae exposed I/R and the preconditioning protocol. Ischemia was simulated in trabeculae as described earlier (16). Trabeculae were preconditioned for 15 minutes with normal Tyrode saturated with 3.8vol% sevoflurane 30 minutes prior to I/R. After washout of sevoflurane, trabeculae were superfused for 15 minutes with normal Tyrode until the onset of simulated ischemia (SI). F_{dev} as well as F_{pas} after SI and 60 minutes of reperfusion were evaluated by performing a standardized force-length relation. As baseline contractile parameters of the HFD-group and the LFD-control group were significantly different (see results), forces after SI and reperfusion were expressed as percentage of force development at L_{max} as determined before the SI protocol.

Statistical Analysis

Data were analyzed by a (two-way) analysis of variance (ANOVA) followed by post-hoc tests or an unpaired t-test. For all analyses a P-value<0.05 was considered to reflect a significant difference. All values are given as means ± standard error of the mean (SEM).

RESULTS

Characteristics of Animals and Isolated Trabeculae

Body weight and fasting bloodglucose were significantly higher in HFD-rats *versus* LFD-control rats (Table 6.1). After 5 weeks, HFD-rats gained more body weight compared to the LFD-controls (see figure 6.2). Although heart weights of the HFD-animals were higher, there was no difference when normalized to body weight. Trabeculae of HFD-rats tended to slightly larger in cross-sectional areas and length, but this increase did not reach statistical significance. The increased body weight and fasting blood glucose are determinants of the HFD-induced metabolic changes.

Table 6.1 General Characteristics of Rats and Isolated Trabeculae after 20 weeks of High-fat or Low-fat Diet.

	LFD	HFD
Body weight (g)	565 ± 7	632 ± 31*
Heart weight (g)	1.82 ± 0.05	1.95 ± 0.08
Heart weight / body weight / 100g	0.32 ± 0.01	0.31 ± 0.01
Fasting bloodglucose (mmol/L)	5.36 ± 0.12	5.98 ± 0.16*
Isolated trabeculae		
CSA (mm^2)	0.049 ± 0.01	0.066 ± 0.02
Muscle length (mm)	3.961 ± 0.2	4.7 ± 0.4

* $P<0.05$ High-fat diet (HFD) versus Low-fat diet (LFD), CSA=cross-sectional area. HFD, n=12; LFD, n=14.

In Vivo Cardiac Function

Echocardiographic analysis of in vivo cardiac function is shown in figure 6.3. Representative examples of 2D and M-Mode echocardiographic images are shown in figure 6.3a. The left ventricular end-diastolic diameter (LVEDD, $P<0.01$) as well as the left ventricular end-systolic diameter (LVESD, $P<0.01$) was increased in HFD-rats, indicating cardiac dilatation. In addition, left ventricular end-systolic wall thickness (LVESW) was also increased in HFD-rats, which is suggestive for a hypertrophied myocardium. Finally, myocardial systolic function, measured by fractional shortening (FS), showed a slight reduction in hearts of HFD-rats.

HFD Alters Cardiac Contractile Function in Vitro

Force-length relationships of trabeculae isolated from HFD- and LFD-hearts are shown in figure 6.4a. With increase in length both the passive and developed force increased. Although F_{pas} was not different between HFD- and LFD-trabeculae, the F_{dev} of HFD-trabeculae was higher for every length of the force-length relation. Figure 6.4b and 6.4c show a more detailed analysis of contractile parameters of the isolated trabeculae at 95% of L_{max}. During basal stimulation conditions, the F_{dev} as well as the +dF/dt are increased in HFD-trabeculae (F_{dev}: 38±5 mN/mm^2 [LFD] *versus* 79±17 mN/mm^2 [HFD], $P<0.05$; +dF/dt: 310±38 $mN.mm^{-2}.s^{-1}$

[LFD] *versus* 759±161 $mN.mm^{-2}.s^{-1}$ [HFD], $P<0.05$), whereas the relaxation parameters tHR and –dF/dt were not significantly altered (tHR: 333±6 ms [LFD] *versus* 351±10 ms [HFD], $P>0.05$; -dF/dt: 402±45 $mN.mm^{-2}.s^{-1}$ [LFD] *versus* -539±113 $mN.mm^{-2}.s^{-1}$ [HFD], $P>0.05$). This may suggest that altered Ca^{2+} availability, and not Ca^{2+} sensitivity, underlies the increased F_{dev} in HFD-trabeculae. To further characterize diet-induced contractile changes, PESP was used to determine maximal force development (F_{max}) and contractile reserve by maximal loading of the SR with Ca^{2+} and to estimate the Ca^{2+}-recirculation fraction (figure 6.5). Although HFD-trabeculae showed an increased F_{dev} as compared to LFD-trabeculae, no significant difference was detected in F_{max} (78±6 mN/mm^2 [LFD] *versus* 94±15 mN/mm^2 [HFD], $P>0.05$). Interestingly, although the contractile reserve of HFD-trabeculae was reduced (0.53±0.04 [LFD] *versus* 0.40±0.05 [HFD], $P<0.05$), the estimated Ca^{2+}-recirculation fraction was not altered (0.67±0.03 [LFD] *versus* 0.69±0.02 [HFD], $P>0.05$). In addition, transsarcolemmal Ca^{2+}-fluxes were estimated by PRP and HFD-trabeculae showed a reduced PRP, suggesting a reduced Ca^{2+}-flux over the sarcolemma (see figure 6.6).

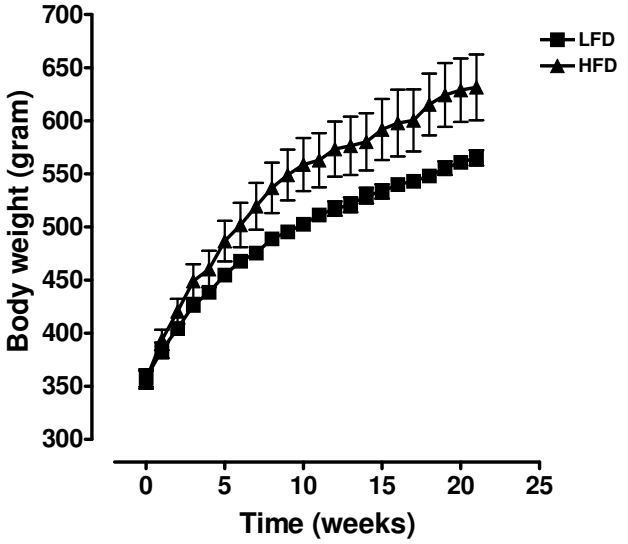

Figure 6.2 Body weight gain of rats subjected to 20 weeks of high-fat diet compared to rats subjected to a low-fat diet. HFD=high-fat diet, LFD=low-fat diet.

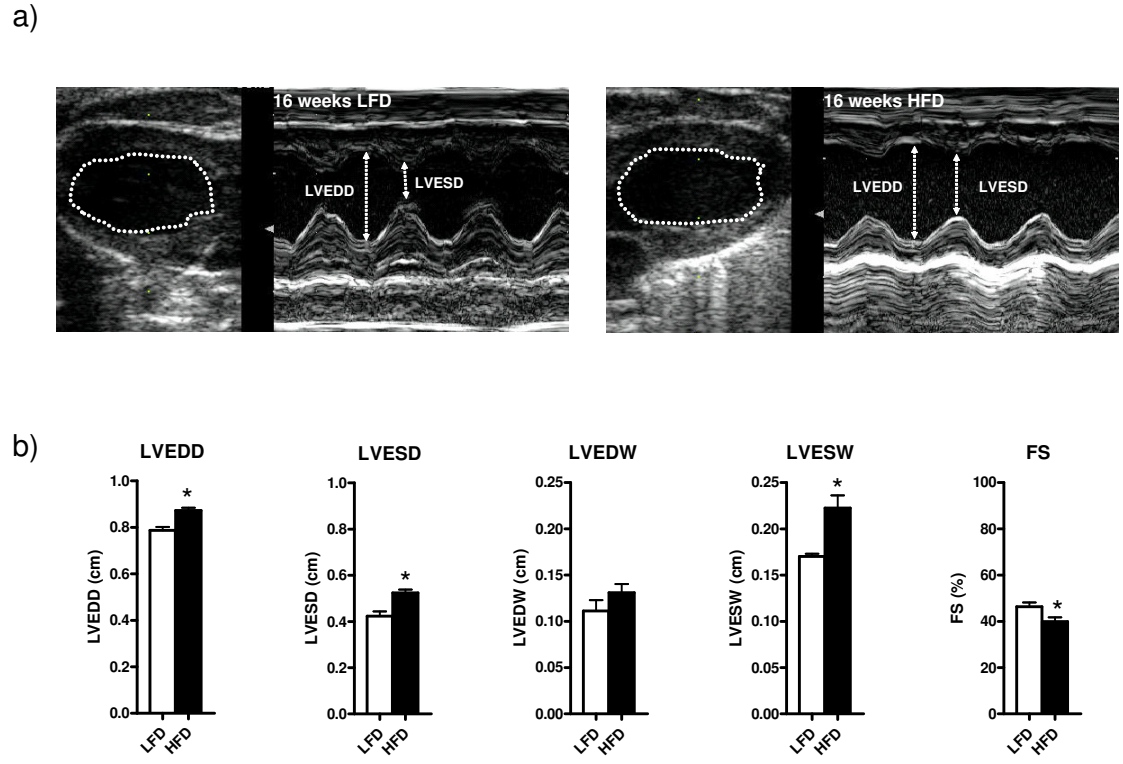

Figure 6.3 In vivo myocardial function determined by echocardiography at 16 weeks of high-fat diet feeding. a) Representative examples of 2D ultrasound images and M-Mode recordings of the left ventricle. b) At 16 weeks of HFD the left ventricular end-diastolic as well as the end-systolic diameter is increased. In addition, the left ventricular end-systolic wall thickness of HFD-hearts is also increased, whereas the increase in end-diastolic wall thickness did not reach statistical significance. Interestingly, the fractional shortening, indicative for myocardial function, is slightly reduced after 16 weeks of HFD. FS=fractional shortening; HFD=high-fat diet; LFD=low-fat diet; LVEDD=left ventricular end-diastolic diameter; LVESD=left ventricular end-systolic diameter; LVEDW=left ventricular end-diastolic wall thickness; LVESW=left ventricular end-systolic wall thickness. * indicates $P<0.05$ *versus* LFD; Data are expressed as mean±SEM. LFD, n=14; HFD, n=12.

High-fat Diet Increases Resistance to I/R-injury

Figure 6.7 shows force-length relationships of HFD- and LFD-trabeculae after SI and 60 minutes of reperfusion (for intervention protocols see figure 6.1). In trabeculae of LFD-rats I/R severely increased passive force while reducing developed force. Interestingly, HFD-trabeculae showed lower passive forces and increased developed forces after I/R (figure 6.7a), thereby suggesting an increased resistance to I/R-injury. In addition, preconditioning of LFD-trabeculae with sevoflurane improves post-ischemic contractile dysfunction as shown by reduced passive forces and increased developed forces (figure 6.7b). In contrast, the F_{dev} of preconditioned HFD-trabeculae was reduced, whereas the F_{pas} was not significantly altered

(figure 6.7c). This suggests that sevoflurane induced preconditioning may not be beneficial to contractile function after I/R in trabeculae from rats subjected to the HFD.

DISCUSSION

In this pilot-study we investigated in vivo and in vitro cardiac function in a rat model of chronic high-fat diet-induced insulin resistance/type 2 diabetes, and demonstrated that long-term high-fat diet induces profound changes in myocardial contractile properties. Several lines of evidence were provided: (1) in vivo echocardiographic evaluation showed in HFD-animals the development of a dilated cardiomyopathy with a slightly reduced fractional shortening, (2) isolated trabeculae of HFD-rats had higher basal force development, but a reduced contractile reserve after maximal potentiation, (3) the Ca^{2+}-recirculation fraction of HFD-trabeculae was unaffected and potentiated force after post-rest potentiation was reduced. Interestingly, isolated HFD-trabeculae show an increased resistance against I/R-injury, but preconditioning of these trabeculae with sevoflurane may not be beneficial in order to improve post-ischemic contractility.

Diabetes-induced contractile dysfunction has been characterized by decreased contraction and relaxation rates, reduced peak force and increased passive force development associated with defects in myocardial Ca^{2+} handling (4). Most of these observations are based on experimental studies performed on chemically-induced insulin-deficient hyperglycemic models (2,3), and have recently been confirmed in insulin-resistant diabetic animals (26-28). However, experimental insulin-resistance animal models mostly rely on genetically manipulated rodents. Ouwens *et al*. (2005) developed an animal model of lipotoxic induced insulin-resistance by subjecting normal rats to HFD during 7 weeks (5). After 7 weeks of HFD, animals were insulin-resistant as indicated by higher plasma levels of glucose and insulin after an oral glucose tolerance test as well as reduced activation of cardiac IRS1/phosphatidylinositol 3'-kinase (PI3-kinase/protein kinase B (PKB/Akt)) mediated signaling in response to insulin. In addition, hearts of HFD-rats were hypertrophied and cardiomyocytes of HFD-rats showed extensive mitochondrial degeneration. In contrast to

previous studies, papillary muscles of HFD-rat hearts showed higher developed forces, but decreased recovery after increased workload.

In the present study, isolated trabeculae of rats subjected to HFD during 20 weeks also developed higher contractile forces without altered passive force development, confirming our previous findings after 7 weeks of HFD-feeding. Although the underlying mechanism remains speculative, the present data may provide some clues for the mechanism that is involved in

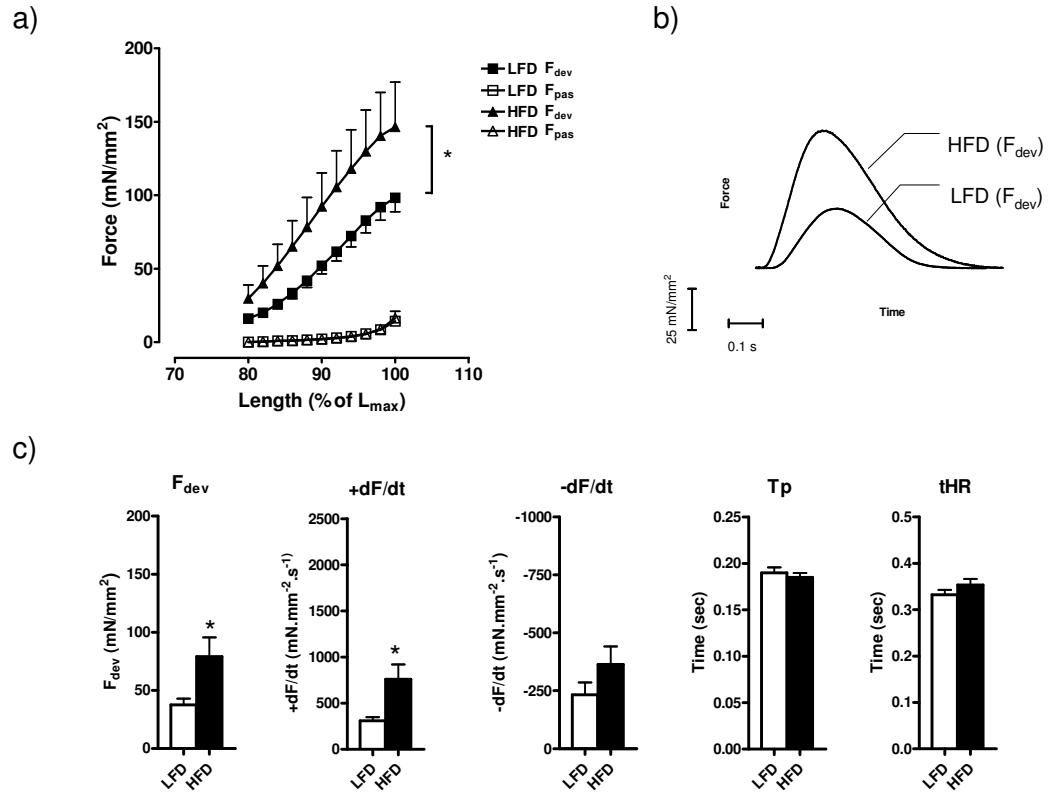

Figure 6.4 In vitro measurements of contractile function in isolated right heart trabeculae from rats fed a high-fat diet (HFD) or a low-fat diet (LFD). a) Force-length relation of trabeculae from LFD- and HFD-rats. The passive force development was not different between LFD- (open squares) and HFD-rats (open triangles). However, trabeculae from HFD-rats (solid triangles) had higher developed forces for every length of the force-length relation. * indicates $P<0.05$ (two-way ANOVA), diet effect; b) Representative tracing of a trabecular contraction at 95% of the L_{max} of LFD- and HFD-rats. c) Analysis of contractile parameters (F_{dev}, +dF/dt, -dF/dt, T_p, tHR) of trabeculae at 95% L_{max} from rats fed with LFD (*white bars*) or HFD (*black bars*). Both the amount and the maximal positive rate of force development were higher in trabeculae of HFD-rats. The relaxation parameters (-dF/dt, and tHR) were not different between LFD- and HFD-rat trabeculae. * indicates $P<0.05$ *versus* LFD (T-test). Data are expressed as mean±SEM; LFD, n=14; HFD, n=12. +dF/dt=maximal positive force development rate; -dF/dt=maximal negative force development rate; F_{dev}=developed force; F_{pas}=passive force; L_{max}=maximal length; tHR=time to half relaxation; T_p=time to peak contraction.

lipotoxicity-induced contractile dysfunction. Both reduced Ca^{2+} release from the sarcoplasmic reticulum (SR) and depressed SR Ca^{2+}-re-uptake, contributing to reduced developed forced and slowed relaxation respectively, have been reported during diabetes (2-4,27,29-32). Therefore, the underlying mechanism for contractile dysfunction during diabetes may be a depressed SR function. Indeed, overexpression of the sarcoplasmic Ca^{2+}-ATPase (SERCA) has been associated with the reversal of diabetes-induced contractile changes (31,33). Our data suggest impairment of SR function in cardiomyocytes of HFD-rats as the post-rest potentiation as well as the contractile reserve was reduced. In our previous study, these alterations were associated with a slight increase in PLB expression in addition to a reduction of phosphorylation of PLB at $Serine^{16}$ (5). Expression levels of other Ca^{2+}-cycling proteins, like SERCA and the ryanodine receptor were not altered. This would suggest an increased inhibitory action of PLB on SERCA. However, the Ca^{2+}-recirculation fraction, a measure of the contribution of the SR to Ca^{2+}- handling, as well as the relaxation times, a measure of the SR Ca^{2+}-re-uptake kinetics, were unaffected in these trabeculae. Howarth *et al*. (2005) recently showed increased cardiomyocyte shortening after 5 and 7 months of HFD-feeding in mice (34). Ca^{2+} transients were unaffected in these cardiomyocytes, and it was therefore suggested that this altered contractile function was due to alterations in Ca^{2+} sensitivity of the myofilaments. Our present results may be related to alterations in the sensitivity of the contractile proteins for Ca^{2+}. Preliminary measurements in isolated - triton-skinned - cardiomyocytes, suggested a slightly higher Ca^{2+} sensitivity in cardiomyocytes from HFD-rats (data not shown). Interestingly, this increase in Ca^{2+} sensitivity was not reduced by incubation with PKA. In analogy, this PKA independent increase in Ca^{2+} sensitivity was found by Knott *et al*. (2002) in cardiomyocytes from failing human hearts (35). Aspecific, complete dephosphorylation of the myocardial contractile system, abolished differences between failing and non-failing cardiomyocytes, and therefore, contractile dysfunction was attributed to differential phosphorylation of contractile system by other kinases, like PKC. It is well established that in diabetic hearts PKC activity is increased (36,37). Recently, Pi *et al*. (2003) showed that PKC activation increases Ca^{2+} sensitivity by phosphorylation of TnI on specific PKC-sites ($serine^{43}$, $serine^{45}$ and $serine^{144}$) and thereby opposing PKA-dependent phosphorylation (38). Another mechanism may be an increased accumulation of

diacylglycerol (DAG). In various models insulin-resistance, increased PKC activity has been associated with increased DAG levels (39-41). Interestingly, Pi *et al*. (1995) showed that

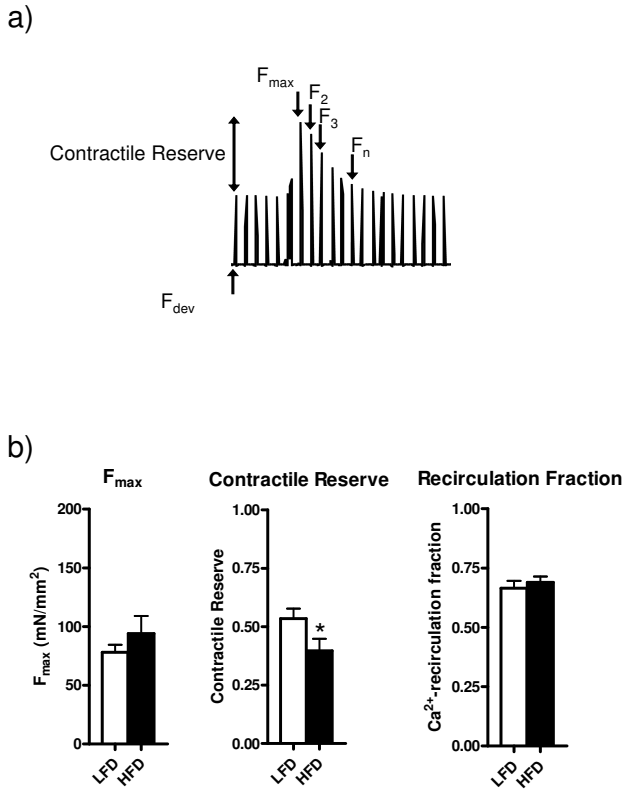

Figure 6.5 Contractile function after post-extrasystolic potentiation (PESP) in isolated trabeculae of low-fat diet (LFD)- and high-fat diet (HFD)- rats. a) Representative tracing of a PESP-stimulus protocol. Stimulation at steady state was 0.2 Hz. Maximal force development (F_{max}) was determined by stimulating the trabecula with a frequency of 5 Hz for 1, 5, 10 or 15 beats. Steady state stimulation (0.2 Hz) was resumed after a 5 seconds pause. The first contraction after high frequency stimulation was potentiated and the highest potentiated force development equals F_{max}. From this PESP contractile reserve could be derived and was defined as $1 - F_{dev}/F_{max}$. The exponential decay of force after PESP is described by F_{n+1}=D x F_n+Constant. D is the slope of the linear regression when F_{nth} contraction is plotted against contraction F_{n+1}, and estimates the fraction of Ca^{2+}-recirculating into the sarcoplasmic reticulum. b) Maximal force development, contractile reserve and the Ca^{2+}-recirculation fraction of trabeculae from LFD- (*white bars*) and HFD-rats (*black bars*). Maximal force development in trabeculae of HFD-rats shows wide variation and therefore no significant difference could be detected. However, the contractile reserve of HFD-trabeculae was lower compared to LFD-trabeculae. The HFD-induced changes in cardiac contractility were not accompanied by alterations in the Ca^{2+}-recirculation fraction. * indicates $P<0.05$ *versus* LFD (T-test); Data are expressed as mean±SEM. LFD, n=14; HFD, n=12.

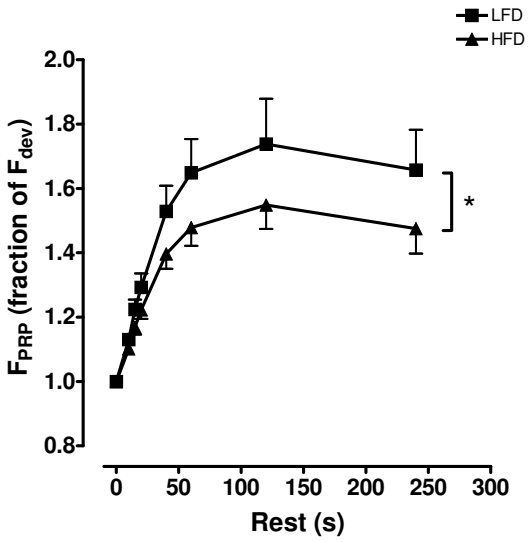

Figure 6.6 Post-rest potentiation (PRP) of trabeculae from low-fat diet (LFD)- and high-fat diet (HFD)-rats. After a variable rest period, the first contraction is potentiated (F_{prp}) and this force is expressed as fraction of developed force (F_{dev}) during steady state conditions. HFD (*solid triangles*) compared to LFD (*solid squares*) reduced the potentiation of force after rest in isolated trabeculae. PRP is an estimate for net transsarcolemmal Ca^{2+} fluxes, and therefore it indicates that in trabeculae of HFD-rats the Ca^{2+} influx is reduced. * indicates $P<0.05$ (two-way ANOVA), diet effect; Data are expressed as mean±SEM. LFD, n=14; HFD, n=12.

DAG itself can induce a positive inotropy in rat cardiomyocytes (42). Interestingly, it is subsequently demonstrated that this DAG-induced increase in contractility could be enhanced by fatty acids and is PKC-dependent and related to increased Ca^{2+} transients (43).

HFD-trabeculae subjected to I/R-injury showed lower passive forces and higher developed forces after 60 minutes of reperfusion, suggesting a decreased sensitivity to I/R-injury of insulin-resistant cardiomyocytes. This is in contrast with clinical observations and various experimental studies showing increased susceptibility to I/R-injury in diabetic patients and animals (44). In contrast, decreased sensitivity to I/R in insulin-deficient animal-models (45-48) as well as in insulin resistance experimental models (49) has been reported (50). In a comprehensive review Paulson (1997), summarized the existing literature on insulin-deficient hyperglycemic animal models of diabetes and concluded that the sensitivity to I/R-injury of diabetic hearts may depend on the presence of fatty acids during I/R, the severity and the type of ischemia (low flow *versus* no flow ischemia), and the duration and the severity of the hyperglycemic state (44). Whether these factors also determine the susceptibility of insulin-resistant myocardium to I/R is not clear from the experimental data. Wang *et al.* (2003) showed that an increased resistance to I/R-injury coincided with the development of

hyperglycemia in insulin-resistant Zucker rats, independent from the presence of fatty acids, severity of the I/R-stimulus and the duration of diabetes (50). In contrast, Maddaford *et al*. (1997) reported that JCR:LA-cp rats, which are insulin-resistant due to a gene mutation in the leptin receptor, were also resistant to mild and severe I/R-injury at 3 months of age, while this resistance disappeared with increasing age (6 and 9 months) (49). In insulin-deficient rats, Tosaki *et al*. (1996) also demonstrated increased resistance to I/R-injury early during the progression the diabetes (51).

During ischemia, oxidative phosphorylation ceases and metabolism switches to anaerobic production of ATP via glycolysis, producing lactate and protons. Intracellular acidosis, due to intracellular proton accumulation, is counteracted via exchange of protons via the Na^+/H^+-exchanger (NHE) leading to Na^+-overload. Via the NCX, Ca^{2+} will accumulate, which initiates irreversible cell damage. The increased resistance to I/R-injury in our model may be explained by the following mechanisms. In the diabetic myocardium, the glycolysis rate is reduced, which may be beneficial during ischemia as fewer protons will accumulate (52). Secondly, sarcolemmal ion transport of diabetic cardiomyocytes has been reported to be reduced (44). Specifically, NHE and NCX are less active in diabetic myocardium, leading to a reduction of Ca^{2+} overload (53,54). In addition, increased reactive oxygen radical stress has been demonstrated in diabetic hearts and therefore the free radical scavenging enzymes are up regulated (55). This could protect cardiomyocytes against oxygen radical induced stress during I/R. Finally, PKC activity is increased in cardiomyocytes from diabetic hearts. PKC is involved in various cardioprotective signaling pathways and indeed it has been recently demonstrated by Malhotra *et al*. (2005) that during diabetes specifically PKC-ε protects against apoptosis (48). Interestingly, this isoform has been often reported to be involved in protective signaling pathways against I/R-injury.

In this respect, our finding that sevoflurane-induced preconditioning of HFD-trabeculae before I/R may not be beneficial for the protection against I/R-injury is remarkable. At this moment we are unable to explain the underlying mechanism. In previous studies, we demonstrated that in our model the sevoflurane-induced cardioprotection is mediated via the NCX, ROS, PKC-δ and PKC-α and mitoK^+_{ATP} channels (15,16,56). Alterations in each of these mediating signaling steps in the diabetic heart have already been discussed and could

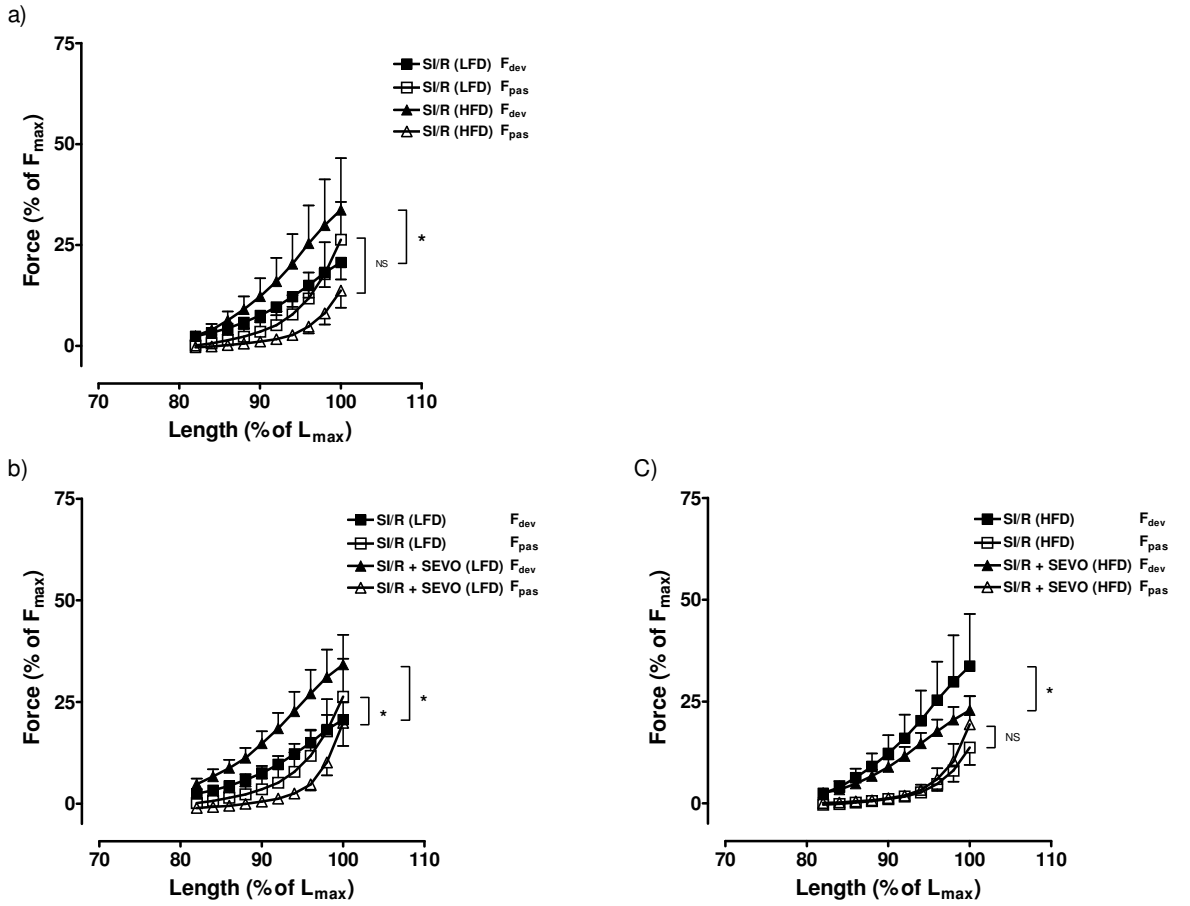

Figure 6.7 Force-length relations of (preconditioned) trabeculae from low-fat diet (LFD)- and high-fat diet (HFD)-rats after simulated ischemia and reperfusion (SI/R). In all panels forces are expressed as percentage of the force development at L_{max} of the force-length relation before the experimental protocol. a) The developed force (F_{dev}) of trabeculae from HFD-rats (*solid triangles*) recovers better than the F_{dev} of trabeculae from LFD-fed rats (solid squares). The difference in F_{pas} after SI/R is not statistically different. b) Preconditioning with 3.8 volume% sevoflurane protects trabeculae from LFD-fed rats from SI/R-injury. The F_{dev} of preconditioned LFD-trabeculae (*solid triangles*) is higher and the F_{pas} of LFD-trabeculae (*open triangles*) is lower compared to non-preconditioned trabeculae. c) However, preconditioning of HFD-trabeculae with sevoflurane seems not to increase the resistance to I/R-injury. The F_{dev} of preconditioned HFD-trabeculae (*solid triangles*) is lower than F_{dev} of non-preconditioned HFD-trabeculae (*solid squares*). The recovery of F_{pas} is not significantly altered by pretreatment with sevoflurane in HFD-trabeculae (*open triangles*). * indicates $P<0.05$ (two-way ANOVA), diet-effect; Data are expressed as mean±SEM; SI/R (LFD) and SI/R+SEVO (LFD), n=7; SI/R (HFD), n=3; SI/R+SEVO, n=6.

contribute to the reduced efficacy of sevoflurane-induced preconditioning in diabetic trabeculae.

The data presented in this study should be interpreted with some caution. The number of trabeculae included in the experiments on I/R and preconditioning is relatively small and, therefore the data should be considered as preliminary. We are however confident about the in vivo and in vitro findings regarding HFD-induced contractile changes, as these confirm our

previous results (5). However, in order to be able to define an underlying mechanism, these experiments should be extended with direct measurements of Ca^{2+} transients, Ca^{2+} sensitivity of the contractile system, expression levels and phosphorylation status of Ca^{2+} handling proteins (RyR, SERCA, PLB and NCX) and activity of the specific proteins involved in the regulation of Ca^{2+} homeostasis and contractility.

In summary, using in vivo echocardiography and in vitro contractile measurements in isolated trabeculae we demonstrated in this pilot-study that cardiac function in rats subjected to HFD-feeding is specifically altered. The findings suggest that cardiac function is preserved under basal conditions presumably by myocardial adaptive changes. However, increased workloads are less well tolerated due to a reduced contractile reserve. Interestingly, preliminary results on contractile function during I/R-injury suggest that the function of diabetic cardiomyocytes is better preserved. However, sevoflurane-induced preconditioning may not be beneficial to contractile recovery after I/R. These findings may be of considerable clinical relevance, as it shows the intimate relation between insulin-resistance and the development of cardiac dysfunction. In addition, these data provide possible clues and show the relevance for the development of clinical cardioprotective strategies for the diseased myocardium.

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Chapter 7

MAIN CONCLUSIONS AND GENERAL DISCUSSION

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MAIN CONCLUSIONS

In this thesis, we studied sevoflurane-induced preconditioning in isolated right ventricular rat trabeculae and specifically focused on the sequence of events of previously established essential key elements (1). The main conclusions are briefly summarized and figure 7.1 schematically illustrates the mechanism we propose based on the findings as described in this thesis.

Cardioprotective Signaling is Independent of the Applied Ischemic Stimulus

In experimental conditions a variety of ischemic stimuli are employed in order to study the adverse sequelae of ischemia and reperfusion (I/R)-injury. However, variations in the ischemic stimulus may result in variations in involved signal transduction molecules in the cardioprotective signaling cascade. We compared sevoflurane-induced cardioprotection during hypoxia and metabolic inhibition with cyanide and showed that sevoflurane-induced cardioprotective signaling involves reactive oxygen species (ROS), protein kinase C (PKC) and adenosine triphosphate-sensitive mitochondrial K^+ (mitoK^+_{ATP}) channels in both ischemic protocols. We therefore concluded that the relative contribution of signal transduction is independent of the applied ischemic stimulus **(chapter 2)**.

Protein Kinase C-δ and Protein Kinase C-α are Both Essential in Sevoflurane-induced Cardioprotection

In **chapter 2** we showed that inhibition of PKC during preconditioning with sevoflurane abolished the cardioprotective effect of sevoflurane. We subsequently demonstrated that specific inhibition of the Ca^{2+}-independent PKC-δ isoform as well as inhibition of the Ca^{2+}-dependent PKC-α isoform during preconditioning attenuates sevoflurane-induced cardioprotection **(chapter 3, 4)**. Upon preconditioning with sevoflurane, PKC-δ translocates towards the sarcolemma and PKC-α to the mitochondria as shown by immunofluorescent co-localization studies, whereas translocation of PKC-ε was not observed. We therefore conclude that sevoflurane-induced cardioprotective signaling depends on activation of PKC-δ as well as PKC-α. Both PKC-isoforms seem to target on different end-effector proteins.

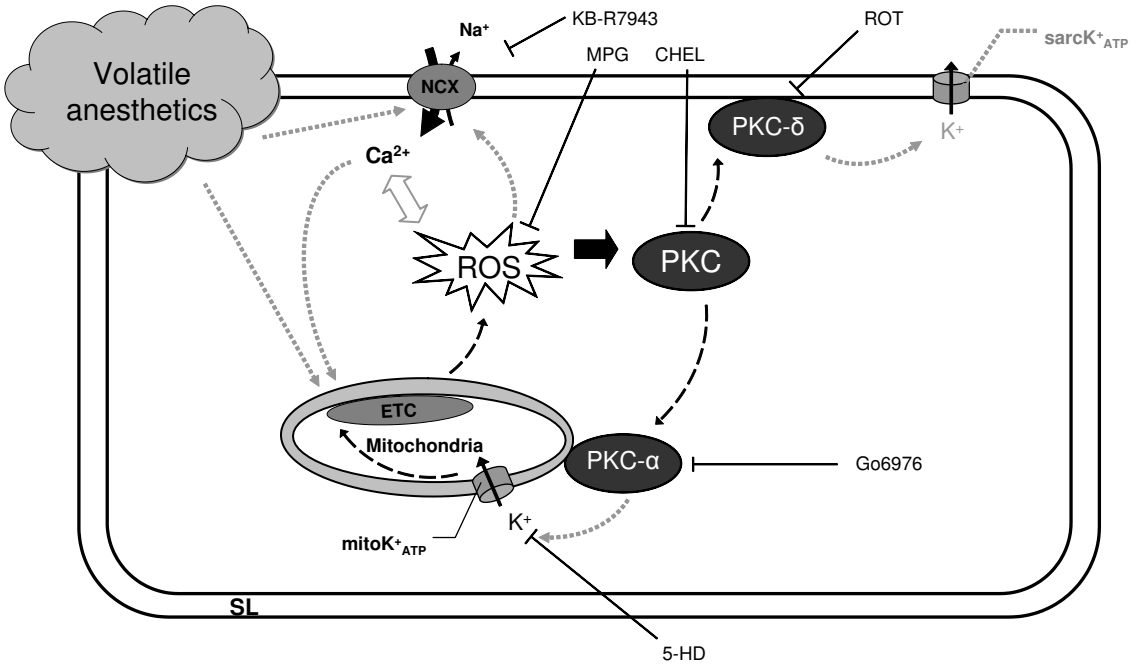

Figure 7.1 Overview of the studied signal transduction pathways involved in sevoflurane-induced preconditioning. In isolated rat trabeculae sevoflurane activates PKC, and subsequently PKC-δ translocates to the sarcolemma and PKC-α translocates to the mitochondria. Activation of both PKC isoforms is essential, as selective inhibition attenuates the protective effect of sevoflurane. Possible (end-effector) targets of PKC are indicated by the gray arrows. PKC activation requires sevoflurane-induced ROS production as well as an active reverse mode of the NCX. Sevoflurane is able to facilitate Ca^{2+} influx via the reverse mode of the NCX and we therefore presume that sevoflurane-induced protective signaling is mediated via Ca^{2+} influx via the NCX. The exact sequence of events upstream of PKC activation remains unclear as the interaction of ROS and NCX was not addressed in our experiments. Potential signaling steps are indicated by the dashed gray arrows and may provide a possible basis for future experiments. 5-HD=5-hydroxydecanoic acid (inhibitor of $mitoK^+_{ATP}$ channels); CHEL=chelerythrine (general PKC inhibitor); ETC=electron transport chain; KB-R7943=inhibitor of the reverse mode of the NCX; Go6976=12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrollo[3,4-c]carbazole (inhibitor of PKC-α); $mitoK^+_{ATP}$=ATP-sensitive mitochondrial K^+_{ATP} channel; MPG=n-(2-mercaptopropionyl)-glycine (ROS-scavenger); NCX=Na^+/Ca^{2+}-exchanger, PKC=protein kinase C; ROS=reactive oxygen species; ROT=rottlerin (PKC-δ inhibitor); $sarcK^+_{ATP}$=ATP-sensitive sarcolemmal K^+-channel.

Reactive Oxygen species Precede Protein Kinase C Activation in Sevoflurane-induced Cardioprotection

After sevoflurane exposure nitrotyrosine residues were present in the sarcolemma indicating sevoflurane-induced ROS production **(chapter 3)**. Scavenging of ROS as well as inhibition of the $mitoK^+_{ATP}$ channels during preconditioning with sevoflurane abolished the preservation of contractile recovery after I/R-injury **(chapter 2, 3)**. We demonstrated with immunofluorescent analysis that sevoflurane failed to induce translocation of PKC-δ and PKC-α when ROS were scavenged during preconditioning, whereas during $mitoK^+_{ATP}$ channel inhibition translocation of PKC-δ and PKC-α in response to sevoflurane was still

observed. We therefore conclude that upstream signaling of PKC in the sevoflurane-induced cardioprotective signaling cascade depends on ROS production and not on mitoK^+_{ATP} channel opening **(chapter 3, 4)**.

Sevoflurane-induced Cardioprotection Depends on the Reverse Mode of the Na^+/Ca^{2+}-exchanger

Volatile anesthetics profoundly modulate myocardial Ca^{2+} handling (**chapter 1**), but sevoflurane-induced alterations in Ca^{2+} handling have not been linked to its cardioprotective signaling cascade. In **chapter 5** we demonstrate that pharmacological inhibition of the reverse mode of the Na^+/Ca^{2+}-exchanger (NCX) (=NCX-dependent Ca^{2+} influx mode) completely attenuated sevoflurane-induced cardioprotection. Furthermore, we demonstrated that sevoflurane increased low Na^+-induced Ca^{2+} loading, which was repressed by inhibition of the reverse mode of the NCX. We therefore conclude that there is a profound role for the reverse mode of the NCX in sevoflurane-induced cardioprotection, suggesting a central role for Ca^{2+} in the protective signaling cascade.

Modulation of the Reverse Mode of the Na^+/Ca^{2+}-exchanger Precedes PKC-δ Translocation

In order to relate the role of the NCX during preconditioning with signaling molecules involved in sevoflurane-induced cardioprotection, we investigated whether trabeculae subjected to NCX inhibition show sevoflurane-induced translocation of PKC-δ. We found that PKC-δ activation in response to sevoflurane is absent when the reverse mode of the NCX is inhibited in trabeculae. This implies that the NCX is essential for the involvement of PKC-δ in the cardioprotective signaling cascade (**chapter 4**). Based on these observations we speculate that the mechanistic link between the NCX and PKC-δ activation may involve Ca^{2+} as mediator.

Long-term High-fat Diet Feeding is Associated with Profound Alterations in Cardiac Function

By feeding normal rats a high-fat diet (HFD), diabetic cardiomyopathy was developed, characterized by dilated cardiomyopathy with a slight reduction of fractional shortening. Isolated trabeculae of HFD rats developed higher forces during basal stimulation conditions,

but had a reduced contractile reserve during augmented workloads. We therefore conclude that high-fat diet feeding alters cardiac dimensions and function in rats. The data suggest that during basal conditions adaptive changes preserve cardiac function, while these alterations become maladaptive during increased workloads **(chapter 6)**.

The Resistance to I/R-injury and the Efficacy of Sevoflurane-induced Cardioprotection of HFD-hearts is Altered Compared to Control Hearts

Isolated rat trabeculae from HFD-rats were subjected to I/R, and after I/R HFD-trabeculae developed higher post-ischemic contractile forces and lower passive forces compared to control hearts. However, the cardioprotective effect as induced by sevoflurane was more profound in control trabeculae than in HFD-trabeculae. These findings implicate that the diseased myocardium may be more resistant to ischemic stimuli, whereas the cardioprotective effects of sevoflurane may not add a beneficial effect to the recovery from I/R-injury **(chapter 6)**.

GENERAL DISCUSSION

Multiple PKC-isoforms are Involved in Sevoflurane-induced Cardioprotective Signaling

In our experimental model of isolated right ventricular rat trabeculae, sevoflurane preconditioning activates PKC-δ as well as PKC-α, as shown by translocation towards respectively the sarcolemma and the mitochondria. Activation of both PKC-isoforms is required to improve contractile recovery after I/R. These findings are of considerable importance for several reasons.

Activation of PKC-δ is often considered to be detrimental as it has been associated with pro-apoptotic/cell death signaling pathways (2), and this is even more prevalent under ischemic conditions (3). In addition, several studies showed that the beneficial effects of preconditioning, including anesthetic preconditioning, are mediated via PKC-ε (4-6). Therefore, it has been suggested that PKC-ε activation in combination with PKC-δ inhibition provides additive protection against I/R (7). However, this approach may not be a beneficial cardioprotective strategy at all times, as we and others now clearly demonstrate that PKC-δ activation is associated with reduced cellular damage after I/R-injury (8). Second, our data show that more than one PKC-isoform is involved in the sevoflurane-induced cardioprotective signaling cascade. The exact mechanism of interaction of these activated PKC isoforms in cardioprotective signaling cascades remains to be elucidated. Interestingly, Inagaki and Mochly-Rosen (2005) showed that ethanol-induced cardioprotection relied on both PKC-δ and PKC-ε activation and suggested PKC-δ mediated PKC-ε activation as a mechanism (9). Similar PKC serial signaling was reported by Hassouna *et al*. (2004) regarding PKC-ε and PKC-α in human atrial appendages (10). Interestingly, analysis of translocation patterns in PKC-δ inhibited trabeculae suggested that PKC-α activation was not PKC-δ-dependent. Therefore, serial signaling steps with regard to PKC isoform activation seems less likely in our model, and we therefore hypothesize that activation of different PKC-isoforms is a parallel signaling event. Whether PKC-ε is among the PKC isoforms involved in our model can neither be confirmed nor excluded from the present data.

Upstream Signaling of Sevoflurane-induced PKC Activation Involves the Reverse Mode of the Na^+/Ca^{2+}-exchanger and Reactive Oxygen Species

An important implication of the present findings is that both conventional PKCs (PKC-α) and novel PKCs (PKC-δ) are involved in sevoflurane-induced protective signaling. This suggests that PKC activation during sevoflurane preconditioning also involves Ca^{2+}, as PKC-α is a Ca^{2+}-dependent PKC isoform. Interestingly, we found that sevoflurane facilitates Ca^{2+} influx into the cardiomyocyte via the reverse mode of the NCX, and that this modulation of the NCX precedes PKC-δ activation during sevoflurane-induced preconditioning. We therefore hypothesize that NCX-mediated Ca^{2+} influx leads to sevoflurane-induced PKC activation, but direct Ca^{2+}-measurements need to be performed to confirm this hypothesis. The question remains how NCX-mediated Ca^{2+} influx finally activates the Ca^{2+}-independent PKC isoforms as well.

In addition to an active reverse mode of the NCX, upstream signaling of PKC activation involves reactive oxygen species. The ROS-dependency of the cardioprotective response is intriguing, as sevoflurane-induced cardioprotective signaling relies on the production of mediators, that are cytotoxic during I/R-injury. Whether there is an interaction between ROS and the NCX cannot be concluded from the present data. From the literature, there is evidence that ROS are required for modulation of NCX function (11). However, it has also been reported that NCX overexpression predisposes cells to oxidative stress (12). Interestingly, preliminary immunofluorescent analysis of ROS production after sevoflurane exposure during NCX inhibition showed that the amount of sevoflurane-induced sarcolemmal nitrotyrosine was reduced. This may suggest that modulation of NCX function is upstream of ROS production in sevoflurane-induced cardioprotective signaling cascade, but this needs further exploration.

Cardioprotective Signaling in the Diseased Heart

The preconditioning response in the heart has been reported to be reduced in diseased myocardium (13) and therefore coexisting cardiovascular pathology may be an important determinant of the efficacy of cardioprotection induced by preconditioning. Obesity is a frequently encountered comorbidity in patients undergoing surgery, and is associated with increased risk for the development of (anesthesiologic) complications. In a previous study of

our laboratory, alterations in cardiac function in normal rats subjected to long-term HFD were investigated. This study showed alterations in myocardial function and insulin signaling as suggestive for the development of a diabetic cardiomyopathy (14). We were therefore interested whether alterations in cardiac function may modulate the response of the heart to I/R-injury and sevoflurane preconditioning as well.

Remarkably, HFD-trabeculae better resisted I/R-injury, whereas sevoflurane preconditioning did not enhance contractile recovery in HFD-trabeculae subjected to I/R. Therefore, in HFD-trabeculae pretreatment with sevoflurane does not exert beneficial effects after I/R. At this moment, our data do not provide an underlying mechanism, as no interventional study was performed.

However, we hypothesize that myocardial adaptation to compensate for diet-induced alterations in myocardial function may interfere with signaling pathways involved in cardioprotection. Indeed, it was previously demonstrated that HFD-hearts are characterized by altered activity of Ca^{2+} handling proteins, extensive mitochondrial degenerative changes and a reduced activation of the phosphatidylinositol 3'-kinase (PI3-kinase)/protein kinase B (PKB/Akt) pathway (14). In addition, other adaptations that interfere with cardioprotective signaling may include alterations in energy metabolism (15), PKC activity (16), reactive oxygen radical scavenging enzymes (17) and mitochondrial function (18). To elucidate an underlying mechanism in more detail, additional experiments should be performed. However the present data demonstrate the possible impact of myocardial disease to cardioprotective signaling. We speculate that myocardial adaptation to other cardiovascular pathology states, like coronary artery disease and myocardial hypertrophy, may importantly affect protective signaling cascades as well.

Mechanism of Protection

One of the most intriguing questions about the phenomenon of preconditioning concerns the mechanism of protection. It has been well observed that preconditioning reduces Ca^{2+} overload and oxidative stress, thereby preventing cardiomyocyte cell death by necrosis or apoptosis (19-24). Despite almost two decades of extensive research since the introduction of the concept of preconditioning (25), the cardioprotective mechanism seems to vary between the type of stimulus, the kind of tissue and species.

This thesis provides evidence that in sevoflurane-induced cardioprotection the mitochondria are among the target end-effector proteins (targeted by PKC-α) in addition to end-effector proteins in the sarcolemma (targeted by PKC-δ). The mitochondria are often implicated as end-effector of the preconditioning response. Details of the proposed mechanism have been described in **chapter 1** (see: *Mechanisms of protection: end-effector proteins*), and involve preservation of mitochondrial bioenergetics and mitochondrial matrix volume regulation, reduced mitochondrial Ca^{2+} loading and prevention of mitochondrial permeability transition with subsequent release of cellular death signals. In this thesis mitochondria were demonstrated to be essential in mediating the cardioprotective effect.

Another mechanism of protection may be improvement of post-ischemic myocardial Ca^{2+} handling. Decreased contractile function after I/R is due to loss of cardiomyocytes through necrosis, but defects in myocardial Ca^{2+} handling are also involved (26). I/R is associated with reduced SR-function (27), a reduced Ca^{2+} transient (28), a reduction of the Ca^{2+} -induced Ca^{2+} release due reduced Ca^{2+} influx via the L-type Ca^{2+} channels (29) and decreased Ca^{2+} sensitivity of the myofilaments (30). We and others demonstrated that preconditioning preserves Ca^{2+}-uptake activity of the sarcoplasmic reticulum (31,32). This protection was shown to be PKC-dependent (31). Furthermore, improved contractile Ca^{2+} responsiveness during preconditioning has also been suggested to contribute to improved post-ischemic contractile function (21,30).

How PKC-α and PKC-δ are involved in the protective effect of sevoflurane remains speculative. Interaction of PKC-α with pro-survival pathways, like ERK, MAPK and Bcl2 has previously been established and may be important (33-35). In addition, PKCs target mitoK^+_{ATP} channels and therefore the target of PKC-α may also be mitoK^+_{ATP} channels (36). We therefore speculate that PKC-α is either involved in the preservation of mitochondrial function as previously mentioned, or possibly mediates amplification of the preconditioning stimulus by inducing increased (mitochondrial) ROS production via a positive feedback mechanism.

Furthermore, several possible target end-effector proteins reside in the sarcolemma. PKC dependent modification of sarcolemmal Ca^{2+} handling proteins may contribute to a reduction of Ca^{2+} overload due to ischemia, like L-type Ca^{2+} channels (37), NCX (38), and the sarcolemmal Ca^{2+}-ATPase (39). In addition, Marinovic *et al*. (2005) recently showed that the

protective effect of sarcK^+_{ATP} channel opening was PKC-δ-dependent providing another target of PKC-δ (40). Finally, protective preconditioning effects have also been associated with PKC-dependent phosphorylation of connexin43 present in intercalated disks. However, Schulz *et al*. (2003) showed that this effect was primarily mediated via PKC-α (41). This illustrates that different PKC-isoforms have different cross-links with end-effector proteins. In order to establish target proteins of PKCs in our model, crucial experiments would include extension of our immunofluorescent co-localization studies in combination with immunoprecipitation analyses.

Clinical Implications

One of the most intriguing aspects of cardioprotection is whether animal studies can be translated into a clinical relevant cardioprotective strategy. Several clinical studies showed that volatile anesthetics provide cardioprotection in patients undergoing coronary artery surgery (42-45), whereas others failed to demonstrate significant protection (46,47). Cardioprotection consisted of a reduction of plasma troponin I levels (as clinical marker for infarct size), improved myocardial function with a reduction of postoperative inotropic support and a reduced length of stay in the intensive care unit and at the hospital. Furthermore, the use of a cardioprotective anesthetic regimen may reduce the mortality rate at one year after coronary artery surgery (48). Therefore, the clinical potential of cardioprotection by volatile anesthetics seems promising and an anesthesia technique based on inhalational anesthetics in patients with cardiovascular comorbidity scheduled for surgical procedures may be preferred.

It should be mentioned that most inhalational anesthetics are reported to exert beneficial effects during I/R-injury as well as other commonly used pharmacological agents like adenosine, norepinephrine, adenosine, opioids, ethanol, Ca^{2+} and others. Therefore, it seems an attractive option to combine several cardioprotective agents to achieve optimal cardioprotection. However, the results of the present thesis may predict some problems which can be encountered in the development of an optimal cardioprotective strategy. First, we showed the ROS-dependency of sevoflurane-induced cardioprotection and therefore cardioprotective treatments that rely on direct anti-oxidant therapy to reduce cellular I/R-injury may interfere with sevoflurane preconditioning. In the literature, evidence exists that

interaction of cardioprotective measures may indeed be clinically important. For instance, Ca^{2+} channel blockers protect against Ca^{2+} overload during reperfusion (49), but the efficacy of preconditioning in patients with Ca^{2+} channel blockers is reduced (50,51). In this respect, our establishment of a role for the reverse mode of the NCX in cardioprotective signaling may also have implications. Several studies show that inhibition of the reverse NCX during reperfusion limits the amount of cellular Ca^{2+} overload and is therefore protective against I/R-injury (52,53). Recently, Yoshitomi *et al*. (2005) showed that even reverse NCX inhibition before I/R-injury improved contractile recovery in dogs (54). In relation to our data, this may implicate that although inhibition of NCX protects against I/R-injury, it may reduce the efficacy of other cardioprotective strategies. We therefore hypothesize that, due to possible interaction the timing of the applied protective strategies may be critical for optimal cardioprotection.

In addition to adequate timing of the protective stimulus, an optimal protective protocol has been not been established yet and remains controversial. De Hert *et al*. (2004) showed that cardioprotection was only present when patients were continuously exposed to sevoflurane until they entered the intensive care unit (43). Pretreatment with sevoflurane or sevoflurane during reperfusion alone was not protective. This is in contradiction with Fraβdorff *et al*. (2005), showing that a classical preconditioning protocol reduced troponin I plasma levels in patients undergoing coronary artery bypass grafting (45).

Furthermore, we previously mentioned that myocardial adaptation to cardiovascular pathology interferes with protective signaling pathways (see **chapter 6**). This suggests that optimalization of cardioprotective treatment for the individual patient may have to include selection based on coexisting cardiovascular comorbidity.

Finally, it may be argued that cardioprotection by preconditioning may be of limited clinical relevance: the heart can only be protected when an ischemic period is predicted. This is in most cases not feasible in daily clinical practice, with exception of patients scheduled for cardiac surgery. Recently, the phenomenon of “postconditioning” was introduced by Zhao *et al*. (2003), which is instituted during reperfusion by applying several brief ischemic periods (55). Postconditioning was demonstrated to be equally effective as preconditioning and may circumvent the need for prediction of the ischemic period like preconditioning. Volatile anesthetics also provide protection when used in a postconditioning protocol and this may

provide a clinical relevant method to induce cardioprotection for unexpected ischemic periods.

Study Limitations

Extrapolation

It may be questioned whether the present data can be extrapolated to human cardiac tissue. Interestingly, Julier *et al*. (2004) showed that in response to sevoflurane-preconditioning, PKC-δ translocated towards the sarcolemma in atrial appendages of patients undergoing coronary artery surgery (44). This supports the fact that in case of sevoflurane preconditioning similar signaling pathways in rats and humans are involved. In addition, several other studies show that mitoK^+_{ATP} channels and ROS are involved in cardioprotective signaling cascades in human cardiomyocytes (56-58). However, the involvement of the NCX in cardioprotection need to be reproduced in human tissue, as the contribution of the NCX to contraction is larger in human cardiomyocytes (59).

Experimental Model

In our experimental model, the ischemic stimulus consisted of superfusion of hypoxic buffer without glucose and an increase in the stimulation frequency. During simulated ischemia, the flow was not stopped, and therefore accumulation of metabolic waste products may be limited. Therefore, our ischemic stimulus may not entirely mimic ischemic conditions. However, during the simulated ischemia protocol, contractile force reduces and a contracture develops, suggesting that ischemic conditions are present. Although we could not demonstrate evidence of necrosis or apoptosis after 1 hour **(see chapter 2)**, the post-ischemic developed force is reduced suggesting that our model mimics stunning rather than ischemic necrotic cell death (26).

Future Research

To our opinion, several aspects for future research may be distilled from the present dissertation. As we found translocation of PKC-α and PKC-δ to specific subcellular structures, we would propose that future studies include more extensive immunofluorescent

co-localization studies in combination with immunoprecipation analyses. This contributes to detailed analysis of potential target end-effector proteins, thereby providing more insight in the mechanisms of afforded protection. Furthermore, we found that the Na^+/Ca^{2+}-exchanger was involved in protective signaling presumably via alterations in ion balances. We therefore advocate that future studies concerning volatile anesthetic-induced cardioprotection focus on volatile anesthetic-induced alterations in Ca^{2+} and Na^+ balance. Fluorescent measurements of these ions in isolated cardiomyocytes or in functional isolated trabeculae could be of considerable value to study. As previously mentioned, it would be of clinical relevance to study efficacy of cardioprotection in experimental models for heart failure, diabetes and sepsis, as cardioprotective signaling cascades may be altered due to these cardiac pathologies. In this thesis, we also demonstrate that promising protective strategies (NCX-inhibition) may abolish other cardioprotective stimuli. Therefore, cardioprotective stimuli should be evaluated for interaction with other protective stimuli. Finally, preconditioning stimuli may be of limited clinical relevance, as protection can only be afforded when an ischemic period is predicted. Therefore, it may be more clinical relevant to focus on the concept of "postconditioning", which is instituted during reperfusion and is demonstrated to be as effective as the classical preconditioning phenomenon.

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SUMMARY

SUMMARY

Patients undergoing anesthesia and surgery are prone to the development of myocardial ischemia, especially when cardiovascular comorbidity like ischemic heart disease, diabetic cardiomyopathy or heart failure, is present. Perioperative myocardial infarction is associated with a high incidence in mortality and therefore the development of perioperative cardioprotective strategies may importantly reduce the incidence of postoperative mortality and morbidity due to cardiovascular complications. Interestingly, commonly used inhalational anesthetics, like sevoflurane, are able to protect the myocardium against ischemia and reperfusion (I/R)-injury. In particular, pretreatment of cardiomyocytes with sevoflurane before I/R effectively reduces infarct size and improves post-ischemic contractility. This phenomenon is called preconditioning-induced cardioprotection. The cardioprotective effect of inhalational anesthetics relies on complex interactions of various signaling molecules, including protein kinase C (PKC), ATP-sensitive mitochondrial K^+ (mitoK^+_{ATP}) channels and reactive oxygen species (ROS). In this thesis, using right ventricular isolated rat trabeculae we specifically focused on the interaction between the Ca^{2+}-independent PKC isoforms (PKC-δ and PKC-ε), the Ca^{2+}-dependent PKC-α isoform, mitoK^+_{ATP} channels and ROS in the sevoflurane-induced cardioprotective signaling cascade. In addition, we identified a new role for the Na^+/Ca^{2+}-exchanger (NCX) in sevoflurane-induced cardioprotective signaling. Finally we investigated contractile function and the efficacy of sevoflurane-induced cardioprotection in isolated trabeculae of rats fed a high-fat diet (HFD), as a model for insulin-resistance.

In **chapter 1**, we provide general information on the regulation of cardiac contractile function via excitation-contraction coupling and the effects of inhalational anesthetics on the myocardial function. Secondly, the effects of I/R-injury on cardiomyocyte function are described. Finally, we describe the involved signal transduction pathways in anesthetic-induced preconditioning.

Previously, I/R-injury was frequently studied using metabolic inhibition of cardiomyocytes by cyanide. **Chapter 2** describes whether hypoxia, as induced by a low oxygen buffer, induced similar functional and intracellular changes as compared to metabolic ischemia. After preconditioning with sevoflurane, trabeculae were exposed to either hypoxia (I/R) or to metabolic inhibition (MI) by using cyanide, which differentially inhibits the mitochondrial

electron transport chain (ETC). For both ischemic stimuli, we focused on the relative contribution of PKC, ROS and mitoK$^+_{ATP}$ channels by pharmacological inhibition of these molecules during preconditioning with sevoflurane. In both ischemic models sevoflurane improved post-ischemic contractility and in both ischemic models inhibition of either PKC, ROS or mitoK$^+_{ATP}$ channels completely abolished sevoflurane-induced cardioprotection. We therefore conclude that the sevoflurane-induced cardioprotective signaling cascade is not different between both applied ischemic stimuli. This implicates that the mode of inhibition of the mitochondrial electron transport chain does not determine which signal transduction molecules are involved in cardioprotective signaling.

Chapter 3 specifically focuses on the relative order of the Ca^{2+}-independent PKC-δ isoform, production of ROS and the mitoK$^+_{ATP}$ channels in sevoflurane-induced cardioprotective signaling. In this study, we demonstrated that sevoflurane-induced cardioprotection is abolished when specifically PKC-δ is inhibited during preconditioning. Furthermore, by immunofluorescent analysis, it was demonstrated that upon acute stimulation by sevoflurane PKC-δ and not PKC-ε translocated towards the sarcolemma. Interestingly, both direct PKC inhibition and ROS scavenging, but not inhibition of the mitoK$^+_{ATP}$ channels, repressed this sevoflurane-induced PKC-δ activation. Finally, in sevoflurane treated trabeculae nitrosylated proteins were identified in the sarcolemma, indicating the formation of peroxynitrite. We therefore conclude that sevoflurane-induced cardioprotective signaling relies on activation of PKC-δ acting via the production of ROS. Opening of the mitoK$^+_{ATP}$ channels is essential for sevoflurane-induced cardioprotection, but this acts downstream of PKC-δ activation.

We subsequently investigated whether the Ca^{2+}-dependent PKC isoforms are involved in the sevoflurane-induced cardioprotective signaling cascade (**chapter 4**). PKC-α inhibition during preconditioning abolished improvement of contractile recovery after I/R due to sevoflurane-induced cardioprotection. Upon exposure to sevoflurane, immunofluorescent co-localization studies showed that PKC-α was translocated to the mitochondria. This PKC-α translocation proved to be dependent on the production of ROS, but was independent from opening of mitoK$^+_{ATP}$ channels and PKC-δ activation. These data underline the importance of Ca^{2+}-dependent PKC isoforms in addition to the well-established role of Ca^{2+}-independent PKC isoforms in sevoflurane-induced cardioprotective signaling.

In **chapter 5**, we studied sevoflurane-induced alterations in myocardial Ca^{2+} handling in relation to cardioprotective signaling and focused on the possible involvement of the NCX in sevoflurane-induced cardioprotective signal transduction. We demonstrate in this part of the study that sevoflurane is able to enhance intracellular cardiomyocyte Ca^{2+} loading via the reverse mode of the NCX (=Ca^{2+} influx mode). Interestingly, specific inhibition of the reverse mode of the NCX abolished PKC-δ activation towards the sarcolemma and subsequent sevoflurane-induced cardioprotection. Importantly, these data point to the reverse mode of the NCX as an essential new element in sevoflurane-induced cardioprotective signaling. The mechanistic link between the NCX and downstream signaling may involve (localized) alterations in intracellular myocardial Ca^{2+} handling.

Expression and activation of signal transduction molecules, like PKC, ROS and mitoK^{+}_{ATP} channels, as well as cardiac Ca^{2+} handling proteins are altered in cardiomyocytes of diseased myocardium. Therefore, the efficacy of preconditioning-induced cardioprotection may be reduced in the patients with heart disease, while cardioprotective strategies may be most beneficial for these patients. Therefore, in **chapter 6,** we investigated the efficacy of sevoflurane-induced cardioprotection in isolated trabeculae of rats subjected to HFD. In vivo cardiac function was assessed by transthoracic echocardiography and HFD-hearts showed evidence of a dilated cardiomyopathy with a reduced fractional shortening compared to control LFD-hearts. Interestingly, HFD-trabeculae showed increased developed force, whereas contractile reserve after increased workload was reduced. This implicates that in diabetic hearts cardiac function is preserved by myocardial adaptive changes, which become maladaptive during increased workloads. Furthermore, after I/R, HFD-trabeculae showed better recovery of contractile force compared to control trabeculae, suggesting an increased resistance to I/R. Finally, pilot-experiments implicate that in HFD-trabeculae sevoflurane-induced preconditioning may not add beneficial effects to contractile recovery after I/R-injury. These findings may be of considerable clinical relevance, as these data show that perioperative protection of the heart by sevoflurane against I/R-injury may not be effective in the diseased myocardium. This suggests that other therapeutic targets should be defined to maximize clinical cardioprotective strategies in the diseased myocardium during anesthesia and surgery.

Chapter 7 summarizes the main conclusions and (possible) implications of the present findings are discussed from both a mechanistic and clinical perspective. Furthermore, the discussion includes study limitations and suggestions for possible future research projects are proposed.

SAMENVATTING

SAMENVATTING

Patienten die een chirurgische ingreep ondergaan lopen het risico om peri-operatieve cardiale ischemie te ontwikkelen tijdens de peri-operatieve periode. Voor patienten met cardiovaculaire comorbiditeit, zoals ischemisch coronairlijden, diabetische cardiomyopathie en hartfalen, is het risico hierop aanzienlijk verhoogd. Het doormaken van een peri-operatief hartinfarct is geassocieerd met een hoge postoperatieve mortaliteit. Door het hart in de peri-operatieve periode te beschermen tegen de gevolgen van ischemie en reperfusie schade kan de anesthesioloog de kans op cardiovasculaire mortaliteit en morbiditeit verminderen. Daarom is het interressant dat veel gebruikte inhalatie anesthetica, zoals sevofluraan, het myocard kunnen beschermen tegen de schadelijke gevolgen van ischemie en reperfusie (I/R)-schade. Het is gebleken dat voorbehandeling van harten met sevofluraan de infarctgrootte na I/R reduceert en de post-ischemische contractiliteit verbetert in vergelijking met onbehandelde harten. Dit fenomeen wordt preconditioneren genoemd.

Het cardioprotectieve effect van inhalatie-anesthetica wordt intracellulair gemedieerd door een complexe interactie tussen verschillende signaaltransductie moleculen, zoals protein kinase C (PKC), adenosine trifosfaat-gevoelige mitochondriale K^+ (mitoK^+_{ATP}) kanalen en vrije zuurstof radicalen (ROS). In dit proefschrift is in trabekels uit de rechterventrikel van het rattenhart specifiek de interactie onderzocht tussen de Ca^{2+}-onafhankelijke PKC isovormen (PKC-δ en PKC-ε) en de Ca^{2+}-afhankelijke PKC-α isovorm, mitoK^+_{ATP} kanalen en ROS. Daarnaast hebben we de rol van het ion kanaal dat Na^+ uitwisselt tegen Ca^{2+} (NCX) in de cardioprotectieve signaaltransductie route van sevofluraan bestudeerd. Tenslotte hebben we de contractiele functie en de effectiviteit van sevofluraan-geïnduceerde cardioprotectie onderzocht in trabekels van ratten die voor een lange periode waren blootgesteld aan een hoog-vet dieet (HFD), als model voor insuline-resistentie.

Hoofdstuk 1 geeft algemene informatie over de regulatie van cardiale contractie via excitatie-contractie koppeling en de effecten van inhalatie anesthetica op de hartfunctie. Daarnaast worden de effecten van I/R-schade op cardiomyocyten en de huidige kennis over betrokken signaaltransductie routes van anesthetica-geïnduceerde cardioprotectie beschreven.

Het was eerder onduidelijk of de manier waarop ischemie in de hartspiercel gesimuleerd wordt bepaalt welke signaalmoleculen betrokken zijn in de cardioprotectieve

signaaltransductie cascades. Om het effect van de ischemische stimulus op de signaal transductie route te onderzoeken, worden in **hoofdstuk 2** trabekels gepreconditioneerd met sevofluraan en vervolgends blootgesteld aan twee verschillende ischemische stimuli: hypoxie of metabole inhibitie door cyanide. Hypoxie en cyanide remmen op een andere manier de mitochondriale electronen transport keten (ETC). De bijdrage van PKC, ROS en mitoK^+_{ATP} kanalen aan de signaalcascade werd bestudeerd door middel van farmacologische inhibitie van deze signaal moleculen tijdens preconditioneren met sevofluraan. In beide modellen voor ischemie verbeterde preconditioneren met sevofluraan de post-ischemische contractiliteit en door blokkade van zowel PKC, ROS als de mitoK^+_{ATP} kanalen trad het beschermende effect van sevofluraan niet meer op. Hieruit concluderen wij dat de sevofluraan-geïnduceerde cardioprotectieve signaaltransductie route niet afhankelijk is van door de manier waarop de ETC geblokkeerd wordt.

Hoofdstuk 3 is specifiek gericht op de relatieve volgorde van betrokkenheid van de Ca^{2+}-onafhankelijke PKC-δ isovorm, de productie van ROS en de mitoK^+_{ATP}-kanalen in sevofluraan-geïnduceerde cardioprotectie. In deze studie wordt aangetoond dat sevofluraan niet beschermend meer is wanneer specifiek PKC-δ wordt geblokkeerd tijdens het preconditioneren. Met behulp van immunofluorescentie studies, werd aangetoond dat er door acute stimulatie met sevofluraan een translocatie van PKC-δ, en niet PKC-ε, naar de celmembraan van de hartspiercel plaatsvindt. Deze activatie van PKC-δ wordt geremd wanneer ROS worden weggevangen tijdens preconditioneren. Echter, tijdens blokkade van de mitoK^+_{ATP} kanalen is wel activatie van PKC-δ mogelijk. Verder konden in sevofluraan behandelde trabekels genitrosyleerde eiwitten in het sarcolemma gedetecteerd worden, wat de aanwezigheid van peroxynitriet in de plasmamembraan van de hartspiercel aantoont. De conclusie van deze gegevens is dat sevofluraan-geïnduceerde cardioprotectie gemedieerd wordt door PKC-δ activatie via ROS productie. Het openen van de mitoK^+_{ATP} kanalen is essentieel voor cardioprotectie, maar komt in de signaal transductie cascade pas na PKC-δ activatie.

Vervolgens waren we geïnteresseerd of Ca^{2+}-afhankelijke PKC isovormen ook van belang waren in sevofluraan geïnduceerde cardioprotectie en hebben we ons gericht op de rol van PKC-α. **Hoofdstuk 4** laat zien dat inhibitie van PKC-α tijdens het preconditioneren het beschermende effect van sevofluraan blokkeert. Immunofluorescentie co-lokalisatie studies

toonden aan dat na acute blootstelling aan sevofluraan PKC-α naar de mitochondria transloceert. De translocatie van PKC-α was afhankelijk van ROS productie, maar onafhankelijk van mitoK^+_{ATP} kanalen en PKC-δ activatie. Deze bevindingen tonen aan dat het cardioprotectieve effect van sevofluraan door Ca^{2+}-onafhankelijke PKC isovormen en Ca^{2+}-afhankelijke PKC-isovormen wordt gemedieerd. Dit suggereert tevens een rol voor Ca^{2+} in sevofluraan-geïnduceerde cardioprotectie.

In **hoofdstuk 5** hebben we daarom sevofluraan-geïnduceerde veranderingen in myocardiale Ca^{2+}-homeostase in relatie tot cardioprotectieve signaaltransductie bestudeerd. In deze studie laten we zien dat sevofluraan de hoeveelheid intracellulair Ca^{2+} kan verhogen via de omgekeerde modus van de Na^+/Ca^{2+}-exchanger (NCX; Ca^{2+} influx modus). Specifieke blokkade van de NCX tijdens preconditioneren reduceerde PKC-δ translocatie naar de celmembraan aanzienlijk en sevofluraan-geïnduceerde bescherming van de post-ischemische contractiliteit trad niet meer op. Deze data voegen de NCX (in de omgekeerde modus) toe aan de cardioprotectieve signaaltranductie route van sevofluraan. Ca^{2+}, en met name (lokale) veranderingen in de Ca^{2+}-concentratie, vormt mogelijk de schakel als signaal molecuul tussen de NCX en activatie van andere intracellulaire signaal moleculen.

De expressie en activatie van signaaltransductie moleculen, zowel PKC, ROS en mitoK^+_{ATP} kanalen en belangrijke eiwitten voor de cardiale Ca^{2+}-homeostase zijn veranderd in het zieke hart. Hierdoor kan de effectiviteit van cardioprotectie door preconditioneren verminderd zijn in patienten met cardiovasculaire comorbiditeit, terwijl voor deze patienten bescherming van het hart tegen ischemie essentieel is voor het behoud van de hartfunctie. In **hoofdstuk 6** hebben we de contractiele functie en de effectiviteit van sevofluraan-geïnduceerde cardioprotectie in geisoleerde trabekels onderzocht van ratten die door een hoog-vet dieet (HFD) insuline-resistentie en een cardiomyopathie ontwikkelden. In vivo hartfunctie werd gemeten met behulp van transthoracale echocardiografie en de harten van HFD-ratten hadden de kenmerken van een gedilateerde cardiomyopathie met een afname van de ejectiefractie. Ten opzichte van trabekels van controle-dieren, was de in vitro contractiekracht van HFD-trabekels hoger, maar de contractiele reserve van HFD-trabekels was afgenomen. Dit impliceert dat de functie van HFD-harten behouden blijft door adaptieve veranderingen, maar dat de reserve capaciteit van deze harten lager is wanneer de harten worden blootgesteld aan een hogere inspanning. Na I/R was het herstel van de contractiekracht hoger in HFD-

trabekels, wat suggereert dat deze trabekels beter bestand zijn tegen ischemische periodes. De eerste experimenten naar de effectiviteit van sevofluraan-geïnduceerde cardioprotectie in HFD-trabekels laten geen duidelijk voordeel zien van het preconditioneren met sevofluraan op het herstel van de contractiekracht tijdens reperfusie. Deze data laten zien dat in harten die zijn blootgesteld aan een pathofysiologische stimulus, zoals een hoog-vet dieet, de weerstand tegen ischemie en de cardioprotectieve eigenschappen van sevofluraan kunnen zijn veranderd. Mogelijk betekent dit dat voor het zieke hart specifieke peri-operatieve cardioprotectieve maatregelen moeten worden ontwikkeld.

In **hoofdstuk 7** worden de belangrijkste conclusies en implicaties van dit proefschrift samengevat vanuit een mechanistisch en klinisch perspectief. Aanbevelingen voor toekomstig onderzoek zijn in deze discussie opgenomen.

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DANKWOORD

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ABOUT THE AUTHOR

Robert Arthur Bouwman was born on January 28th 1976 in Harderwijk. From 1988 to 1994 he followed his undergraduate education at the Eindhovens Protestants Lyceum. He subsequently started his medical education at the Rijksuniversiteit Groningen and graduated in 2001. During medical education he followed a traineeship in the Robert Jones and Agnes Hunt Orthopaedic Hospital (Oswestry, UK) and performed a biomechanical analysis of K-wire fixation of unstable Colles' fractures under supervision of prof. dr. J.B. Richardson. A second research traineeship he performed in the laboratory of prof. dr. C.S. Deutschmann (Department of Anesthesiology, University of Pennsylvania, Philadelphia, USA). With dr. Y.G. Weiss as mentor, Bouwman was involved in a laboratory study investigating gene therapy as treatment modality for the adult respiratory distress syndrome and participated in a clinical study investigating the hemodynamic response of patients undergoing intraperitoneal photodynamic therapy. After an internship at the intensive care unit of the Leids Universitair Medisch Centrum, he started in 2002 as an MD-clinical research trainee at the Department of Anesthesiology of the VU University Medical Center in Amsterdam. He performed his PhD study presented in this dissertation and started the anesthesiology residency under supervision of prof. dr. J.J. de Lange. Currently he is working as a resident at the Department of Anesthesiology in Het Spaarne Ziekenhuis in Hoofddorp and will subsequently continue his anesthesiology resident training at the Department of Anesthesiology of the VU University Medical Center.