Isoflurane induced eNOS signaling and cardioprotection

Baotic, Ines

Chapter 1

General Introduction and Scope of the Thesis

Most acute medical conditions precipitate an increased risk of ischemic cardiovascular complications, including non-cardiac and cardiac operations. The risk progressively increases with age and accounts for 15% to 50% of deaths occurring within 30 days following non-cardiac surgery (Mangano 1990; Smeili and Lotufo 2015). Perioperative myocardial infarction is the primary cardiovascular complication in patients with preexisting cardiovascular disease (CVD) undergoing anesthesia and surgery, and therapeutic interventions to prevent myocardial injury are critically needed to reduce the associated morbidity and mortality (Morgan, Mikhail et al. 2006).

The discovery of a natural cellular protective mechanism against ischemia offered the opportunity to effectively harness organs at risk. In 1986, Murry et al. described ischemic preconditioning (IPC), a cardioprotective intervention in which brief periods of myocardial ischemia and reperfusion subsequently rendered the myocardium resistant to infarction during a subsequent more prolonged period of coronary artery occlusion and reperfusion (Murry, Jennings et al. 1986). Myocardium can tolerate brief periods (generally up to 15 minutes) of myocardial ischemia (Verma, Fedak et al. 2002), and although cardiomyocytes experience ischemic injury, the damage in young and healthy heart is generally reversible upon prompt reperfusion. In contrast, prolonged ischemia produces irreversible injury, including predominantly cardiomyocyte necrosis and endothelial cell injury collectively referred to as reperfusion injury (Verma, Fedak et al. 2002). Similarly, ischemic postconditioning (IPoC), an intervention by serial, brief interruptions of coronary circulation at the onset of reperfusion may reduce myocardial infarct size (Verma, Fedak et al. 2002). However, despite IPC's and IPoCs effectiveness in mitigating reperfusion injury as found in several animal models (Murry, Jennings et al. 1986) and in humans (Yellon, Alkhulaifi et al. 1993), it has the distinct disadvantage of requiring temporary occlusion of a coronary artery. The disadvantage of coronary occlusion is absent in a third form of preconditioning termed remote ischemic preconditioning (RIPC). This noninvasive procedure constitutes a repeated inflating and deflating of a standard blood-pressure cuff placed on the upper arm or thigh to induce transient ischemia and reperfusion, is under clinical investigation (Cheung, Kharbanda et al. 2006; Ali, Callaghan et al. 2007; Hoole, Heck et al. 2009; Botker, Kharbanda et al. 2010; Hausenloy, Candilio et al. 2015; Bulluck, Yellon et al. 2016). The current mechanistic concept is that bloodborne factor is produced in the remote tissue in response to RIPC subsequently conveys protection to the myocardium (Hausenloy and Yellon 2008).

Interestingly, volatile anesthetic agents induce similar protective mechanisms in myocardium as those governing ischemic preconditioning (van Ackern, Vetter et al. 1985; Kikuchi, Dosenovic et al. 2015). Following the identification of this so-called anesthetic preconditioning (APC) in laboratory animals, several investigations in patients during cardiac surgery demonstrated that volatile anesthetics precondition the human myocardium and may improve outcome after cardiac surgery (Kiani, Mirmohammad Sadeghi et al. 2013; Kunst and Klein 2015). However, evidence supporting the use of volatile anesthetics for cardioprotection in patients undergoing cardiac and non-cardiac surgery is conflicting. There are different possible explanations for negative results in clinical trials, including inadequate statistical power, heterogeneity in patient populations, and uncontrolled variables such as concomitant drug treatment and different conditions during coronary artery bypass grafting (CABG) operations, such as e.g. intermitted cross-clamp versus cardioplegic arrest (De Hert, Cromheecke et al. 2003; De Hert, Van der Linden et al. 2004; Flier, Post et al. 2010).

The presence of certain disease states also represents an important confounding factor during clinical investigations. For example, diabetes and hyperglycemia are important independent predictors of increased peri-operative cardiovascular risk (Gerstein, Pais et al. 1999; Ishihara, Inoue et al. 2003), although the mechanisms responsible are incompletely understood. Such may be related to diabetes and hyperglycemia increasing the production of reactive oxygen species (ROS) (Boudina, Sena et al. 2007), decreasing the availability of nitric oxide (NO) (Giugliano, Marfella et al. 1997), impairing endothelial function (Marfella, Verrazzo et al. 1995) and attenuating coronary microcirculatory responses to myocardial ischemia (Kersten, Brooks et al. 1995). Evidence indicates that diabetes markedly attenuates the cardioprotective signal transduction mechanisms activated by preconditioning. Diabetes or acute hyperglycemia affects infarct size reduction in response to APC (Tanaka, Kehl et al. 2002), IPC (Kersten, Schmeling et al. 1998) and pharmacological preconditioning (Hassouna, Loubani et al. 2006; Kim, Kim et al. 2012; Sharma, Mahadevan et al. 2013) with myocardial infarct size being directly related to blood glucose concentration. While diabetes may thus significantly increase perioperative risk, only few studies have evaluated methods to modify this risk.

The signal transduction pathways conferring cardioprotection following APC have been intensely investigated. They display a considerable multi-

level complexity that involves multiple molecular pathways and their complex interactions staged at different intracellular compartments. Among these are e.g. different membrane bound receptors, downstream signaling molecules such as inhibitory G proteins and various intracellular kinases, and sarcolemmal and mitochondrial ion-channels (Zaugg, Lucchinetti et al. 2003). A growing body of evidence implicates endothelial nitric oxide synthase (eNOS)-derived nitric oxide (NO•) as a critical component of APC signal transduction. Further, diabetes is well known to impair different aspects of NO biology, including its synthesis, signaling and availability. Thus, the central theme of this thesis is to address the involvement of eNOS in the impairment of APC by diabetes and hyperglycemia.

1. Anesthetic Preconditioning

1.1. Anesthetic Preconditioning - Pharmacological protection of cardiac ischemia

Myocardial preconditioning describes the experimentally observed phenomenon that an intervention or a trigger, applied prior to prolonged coronary artery occlusion, decreases the extent of a subsequent infarction (Kunst and Klein 2015). The preconditioning trigger can either be an ischemic intervention or a pharmacological stimulus, such as volatile anesthetics (Kunst and Klein 2015). A unique feature of this phenomenon is that myocardium remains protected for a period shortly after withdrawal of the preconditioning stimulus, and this interval is termed the *memory* of preconditioning (Kersten 2011). In addition to this immediate/early window of protection of 1-2 hours after the preconditioning stimulus, preconditioning also induces a delayed phase of protection termed late preconditioning, which persists up to 72 hours (Kunst and Klein 2015).

The first experimental evidence of myocardial protection from ischemia-reperfusion (I/R) injury by volatile anesthetics was obtained using halothane in a dog model, in the 1970s (Bland and Lowenstein 1976; Kunst and Klein 2015). This protective effect was subsequently confirmed by several independent groups using halothane (Davis, DeBoer et al. 1983), enflurane (van Ackern, Vetter et al. 1985) and isoflurane (Davis and Sidi 1989) in different animal models (Kunst and Klein 2015). However, volatile anesthetics had also been shown to cause harmful „coronary steal" in experimental models due to their vasodilatory potential, resulting in shunting of blood flow away from the ischemic myocardium and worsening of myocardial ischemia

(Kunst and Klein 2015). However, the contention that volatile anesthetics induce coronary steal was debunked by several studies in early 1990s (Kersten 2011).

In the clinical setting, APC is a pharmacological strategy whereby exposure to volatile anesthetics before or during cardiac surgery in high risk cardiovascular patients may reduce the risk of peri- and postoperative cardiac complications, thus may improve clinical outcome. For instance, De Hert at al. demonstrated anesthesia supplemented with desflurane and sevoflurane, but not propofol, to preserve left ventricular function with less evidence of myocardial damage in patients undergoing CABG operations (De Hert, Cromheecke et al. 2003). By the early 1990s, the use of volatile anesthetics during cardiac surgery had gained considerable popularity, primarily because it allowed patients to be fast-tracked for early extubation within hours of arrival to the intensive care unit as compared with opioid-based anesthetics (Kersten 2012). Although evidence supports the benefit of volatile anesthetics during cardiac operations, the results of clinical trials in non-cardiac surgery have not supported a salubrious effect. Consequently, recent guidelines have omitted the use of volatile anesthetics in non-cardiac surgery as a cardioprotective strategy because of unclear benefit.

1.2. Mechanisms of Anesthetic Preconditioning

1.2.1. *Modulation of intracellular homeostasis in cardiomyocytes*

The results of numerous investigations have produced substantial insight into the large number of mechanisms underlying the preconditioning effect, ranging from the modulation of intracellular signaling pathways and calcium homeostasis to altering cardiac genes and proteins. Moreover, the mechanisms of early and delayed anesthetic preconditioning differ (Lohr and Kersten 2010). Anesthetics activate various intracellular kinases which phosphorylate and subsequently modify the activity of downstream proteins that are important in mediating cardioprotection (Lohr and Kersten 2010). During early preconditioning, modification of preexisting proteins is responsible for protection, whereas after 24 h, cardioprotection is based on the synthesis of new proteins (Lohr and Kersten 2010).

Two main intracellular signal transduction pathways, directing cardioprotection from cell surface receptors to convergent targets in the mitochondria, have been proposed to explain APC: the reperfusion injury salvage kinases (RISK) pathway acting via G-protein-coupled cell surface receptors and re-

ceptors for growth factors (Hausenloy and Yellon 2004), and the survivor-activating factor enhancement (SAFE) pathway that is activated mainly through the tumor necrosis factor (TNF)-alpha receptor and the signal transducer and activator of transcription (STAT)-3 pathway (Lecour 2009; Kunst and Klein 2015). The RISK pathway contains phosphatidylinositol-3-OH kinase (PI3K)–Akt and p42/p44 extra-cellular signal-regulated kinases (Erk 1/2), both of which have been implicated in cellular survival through their recruitment of anti-apoptotic pathways of protection (Cokkinos 2015; Hausenloy and Yellon 2004; Kunst and Klein 2015). The intracellular signal transduction proteins and molecules in cardiomyocytes that are candidates for interactions with volatile anesthetics (Kunst and Klein 2015) are listed in **Table 1**.

There is substantial evidence that mitochondria are not only endpoints but also direct targets of volatile anesthetics and act as triggers of protection following APC. Mitochondria play a critical role in determining whether myocardium recovers upon reperfusion after a period of ischemia. Particularly, opening of the mitochondrial permeability transition pore (mPTP) is crucial in cardiomyocyte death by inducing a collapse of the mitochondrial transmembrane potential, leading to obstruction of oxidative phosphorylation, in turn reducing ATP production and increasing production of ROS (Weiss, Korge et al. 2003). The main trigger for mPTP opening is a calcium (Ca^{2+}) overload of the mitochondrial matrix that occurs during ischemia and is potentiated by oxidative stress that predominates during reperfusion (Halestrap, Clarke et al. 2007). Anesthetic preconditioning affects mitochondria in several ways. First, activation of the RISK and SAFE pathways by APC exert a distinct action on mitochondria that include activation of mitochondrial ATP-dependent potassium channels (mitoK_{ATP}) through protein kinase C-coupled signaling pathways (Zaugg, Lucchinetti et al. 2002). The opening of mitoK_{ATP} and subsequent inhibition of mPTP (Piriou, Chiari et al. 2004; Krolikowski, Bienengraeber et al. 2005) protects the cardiomyocyte by decreasing cytosolic and mitochondrial Ca^{2+} concentrations (Zaugg and Schaub 2003). In addition, APC may exert its protective action on mitochondria independent of RISK and SAFE pathways by direct effects on mitoK_{ATP} that induce flavoprotein oxidation (Kohro, Hogan et al. 2001), as reflected in distinct changes in NADH before, during, and after ischemia (Riess, Camara et al. 2002). Further, APC may either directly or indirectly interact with mitochondrial complex I (Hanley, Ray et al. 2002) and/or complex III (Sedlic, Pravdic et al. 2010; Hirata, Shim et al. 2011) thus promoting a limited formation of ROS ('signaling ROS'). In turn, signaling ROS may induce or promote the activation of intracellular protein mediators that mediate APC induced cardioprotection (Ludwig, Weihrauch et al. 2004).

Table 1. Effects of volatile anesthetic preconditioning on signal transduction proteins in myocardium after I/R injury

Cardiomyocyte	Experimental finding*	Experimental model	Volatile anesthetic
Cytosol			
PKC	PKC-delta activation preceded by ROS release	Rat myocardial trabeculae *in vitro*	Isoflurane (Bouwman, Musters et al. 2004)
	PKC-delta and PKC-epsilon translocation, and Src PTK activation	Rat heart *in vivo*	Isoflurane(Ludwig, Weihrauch et al. 2004)
	PKC-epsilon and ERK1/2	Rat heart *in vivo*	Desflurane (Toma, Weber et al. 2004)
	PKC-delta activation depends on modulation of Na+/Ca2+ exchanger	Right ventricular rat trabeculae *in vitro*	Sevoflurane (Bouwman, Salic et al. 2006)
	PKC-epsilon activation	Rat cardiomyocytes	Isoflurane (Pravdic, Sedlic et al. 2009)
	PKC-alpha and -epsilon translocation and activation	Guinea pig hearts *in vitro*	Sevoflurane(Okusa, Miyamae et al. 2009)
	PKC-delta, and -alpha activation, phosphorylation of Akt and GSK-3 beta, ERK1/2 activation	Human right atrial appendages, 3 cycles of preconditioning *in vivo*	Isoflurane and sevoflurane (Mellidis, Ordodi et al. 2014)
ERK1/2	ERK1/2 triggered HIF1α and VEGF up-regulation	Rat hearts *in vivo*	Isoflurane (Wang, Weihrauch et al. 2006)
PI3K/Akt	PI3K/Akt activation and attenuation of myocardial apoptosis	Rabbit heart *in vivo*	Isoflurane (Raphael, Abedat et al. 2006)
5'AMP PK	5'AMP-activated protein kinase, ROS induced	Rat hearts *in vitro*	Sevoflurane(Lamberts, Onderwater et al. 2009)
Cyclooxygenase	Cyclooxygenase-2: critical mediator	Dog hearts *in vivo*	Isoflurane (Alcindor, Krolikowski et al. 2004)
Cav-3	Cav-3 expression and caveolae are critical mediators	Cav-3 knockout mice, hearts *in vivo* and cardiomyocytes *in vitro*	Isoflurane (Horikawa, Patel et al. 2008)
	Cav-3-dependent cyclooxygenase-2 inhibition	Cav-3 knockout mice *in vivo*	Sevoflurane (Zhao, Wang et al. 2013)
NO	NO release mediated protection	Rabbit hearts *in vivo*	Desflurane (Tsai, Lin et al. 2004)e
NOS	Activation of NOS	Rabbit hearts *in vivo*	Desflurane (Smul, Lange et al. 2006)
ROS	ROS generation from electron transport chain complex III	Rabbit hearts *in vivo*	Isoflurane (Ludwig, Tanaka et al. 2004)

Cardiomyocyte	Experimental finding*	Experimental model	Volatile anesthetic
	ROS mediates attenuation of mitochondrial respiration complex I	Guinea pig myocardial mitochondria	Sevoflurane (Riess, Eells et al. 2004)
	ROS generated PKC-alpha activation	Rat right ventricular trabeculae *in vitro*	Sevoflurane (Bouwman, Musters et al. 2007)
	ROS generation	Human atrial trabeculae	Sevoflurane and desflurane (Hanouz, Zhu et al. 2007)
	ROS generation, and ROS dependent protection	Adult ventricular rat cardiomyocytes	Sevoflurane and desflurane (Sedlic, Pravdic et al. 2009)
	ROS generation	Cardiomyocytes from hESC	Isoflurane (Sepac, Sedlic et al. 2010)
	attenuation of complex I activity and ROS generation	Rat hearts *in vitro*	Isoflurane (Hirata, Shim et al. 2011)
GLUT-4*	GLUT-4 increase and Cav-3/GLUT-4 localization	Cav-3 knockout and wild-type mice *in vivo*	Isoflurane (Tsutsumi, Kawaraguchi et al. 2010)*
Cav-1*	Production and phosphorylation of Cav-1	Wild-type mice and mice adult cardiac myocytes	Isoflurane (Patel, Tsutsumi et al. 2007)*
PKA*	Activation of PKA	Rabbit heart *in vivo*	Desflurane and sevoflurane (Lange, Smul et al. 2006)*
Mitochondrium			
mPTP	Improved resistance of mPTP to Ca2+ induced opening	Rabbit hearts *in vivo*	Desflurane (Piriou, Chiari et al. 2004)
	mitoKATP activation induced mPTP inhibition	Rabbit hearts *in vivo*	Isoflurane (Krolikowski, Bienengraeber et al. 2005)
	Delayed opening of mPTP	Cardiomyocytes from hESC	Isoflurane (Sepac, Sedlic et al. 2010)
	Delayed opening of mPTP	Rat cardiomyocytes	Isoflurane (Pravdic, Sedlic et al. 2009)
	O-GlcNAc modification of mitochondrial voltage-dependent anion channel inhibits opening of mPTP	Mouse myocytes	Isoflurane (Hirose, Tsutsumi et al. 2011)
mitoKATP	Activation of mitoKATP channels	Rabbit hearts *in vivo*	Isoflurane (Krolikowski, Bienengraeber et al. 2005)
	Activation of human cardiac mitoKATP channels	Lipid bilayers	Isoflurane (Jiang, Nakae et al. 2007)

Cardiomyocyte	Experimental finding*	Experimental model	Volatile anesthetic
BKCa	Activation of BKCa (PKA mediated)	Mouse hearts *in vivo*	Desflurane (Redel, Lange et al. 2008)
Cell nucleus			
NF-kappa B	Attenuation of NF-kappa B activation at the end of I/R	Rat hearts *in vitro*	Sevoflurane(Zhong, Zhou et al. 2004)
	Activation of NF-kappa B, up-regulation of autophagy, decreased apoptosis before I/R	Rat hearts *in vitro*	Sevoflurane (Lu, Liu et al. 2009)
	Inhibition of NF-kappa B during I/R	Rat hearts *in vivo*	Sevoflurane(Konia, Schaefer et al. 2009)
	Up-regulation of NF-kappa B and anti-apoptosis factors before I/R	Rat hearts *in vivo*	Sevoflurane(Wang, Xie et al. 2010)
HIF1α	Activation of HIF1α	Rabbit hearts *in vivo*	Isoflurane (Raphael, Zuo et al. 2008)

Akt, protein kinase B; AMP, adenosine monophosphate; BKCa, large-conductance calcium-activated K+ channel; Cav-1, caveolin-1; Cav-3, caveolin-3; ERK, extracellular signal regulated kinase; GLUT-4, Glucose transporter type-4; GSK, glycogen synthase kinase; HIF, hypoxia inducible factor 1 alpha; hESC, human embryonic stem cells; I/R, cardiac ischemia-reperfusion; mitoKATP channel, mitochondrial ATP-sensitive potassium channel; mPTP, mitochondrial permeability transition pore; NF, nuclear factor; NO, nitric oxide; NOS, nitric oxide synthase; O-GlcNAc, O-linked beta-N-acetylglucosamine; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase C; ROS, reactive oxygen species; Src PTK, sarcoma protein tyrosine kinase; VEGF, vascular endothelial growth factor. Table adapted from (Kunst and Klein 2015) with modifications.*

2.1.2. *Anesthetic preconditioning and NO signaling in endothelial cells*

In addition to protection in cardiomyocytes, volatile anesthetics exert endothelial protection, which may be of relevance for myocardial protection (Kunst and Klein 2015). The actions of volatile anesthetics to modulate various signal transduction proteins in endothelial cells (Kunst and Klein 2015) are listed in **Table 2**. A growing body of evidence implicates eNOS derived NO as a critical mediator of APC (Amour, Brzezinska et al. 2009) and also suggests that a NO biosynthetic pathway is importantly modulated by disease states (Baotic, Weihrauch et al. 2015). Three distinct nitric oxide synthase (NOS) isoforms, neuronal NOS (nNOS), inducible NOS (iNOS), and eNOS, contribute to NO production in the heart; however, eNOS, but not nNOS or iNOS, seems to play a major role during APC (Baotic, Weihrauch et al. 2015).

Although many studies indicate that endogenous NO is not required for IPC-induced early preconditioning, exogenous or pharmacologically increased endogenous NO production elicits an early preconditioning effect (i.e. NO is sufficient but not necessary for early preconditioning) (Zaugg, Lucchinetti et al. 2003). Conversely, NO has an obligatory role in late preconditioning (Zaugg, Lucchinetti et al. 2003). It has been previously demonstrated that the trigger and mediator phases of delayed preconditioning with isoflurane were blocked by the nonselective NOS inhibitor, l-NG-nitroarginine methyl ester (L-NAME), whereas selective inhibitors of nNOS or iNOS had no effect (Chiari, Bienengraeber et al. 2005; Baotic, Weihrauch et al. 2015). Isoflurane increases the phosphorylation of serine 1177 on eNOS and stimulates NO production in human coronary artery endothelial cells and preconditions myocardium against infarction through an eNOS-sensitive pathway (Toda, Toda et al. 2007; Baotic, Weihrauch et al. 2015). However, the precise mechanisms whereby isoflurane modulates NO biosynthesis are incompletely understood (Baotic, Weihrauch et al. 2015). eNOS activity is regulated by intracellular localization, posttranslational modifications, protein–protein interactions, and tetrahydrobiopterin (BH4) cofactor availability (Baotic, Weihrauch et al. 2015). Because of the dominant role of NO, late preconditioning is viewed as a state of enhanced NO synthesis (Zaugg, Lucchinetti et al. 2003). The most likely cardioprotective effects of NO in late preconditioning are: (i) inhibition of Ca^{2+}influx; (ii) antagonism of β-adrenergic stimulation; (iii) opening of K_{ATP} channels; (iv) antioxidant actions; (v) activation of COX-2 with the synthesis of prostanoids; and (vi) reduced contractility and myocardial oxygen consumption (Zaugg, Lucchinetti et al. 2003).

Table 2. Effects of volatile anesthetic preconditioning on signal transduction proteins in endothelium

Experimental finding*	Experimental model	Volatile anaesthetic
Inhibition of endothelial NF-kappa B activation	Human umbilical vein, endothelial cells	Desflurane (Li, Zhang et al. 2008)
Inhibition of TNF-alpha-stimulated expression of adhesion molecules ICAM-1, VCAM-1 and E-selectin	Human umbilical vein, endothelial cells	Desflurane (Biao, Zhanggang et al. 2005)
Prevention of TNF-alpha-induced adhesion molecule expression	Human umbilical vein, endothelial cells	Isoflurane (Weber, Kandler et al. 2008)
Inhibition of endothelial / leucocyte adhesion	Human volunteers	Sevoflurane (Lucchinetti, Ambrosio et al. 2007)
Preservation of glycocalix from I/R-induced degradation by attenuation of lysosomal cathepsin B release	Guinea pig hearts *in vitro*	Isoflurane (Annecke, Chappell et al. 2010)
Endothelial protection against ischaemia mediated by PKCs and mitoKATP channels	Bovine pulmonary arterial endothelial cells	Isoflurane (Feng and Zuo 2011)
NOSs (eNOS and iNOS)	NOSs knockout mice	Desflurane (Redel, Stumpner et al. 2013)
Increase in HSP 90 (HSP 90-eNOS interaction)*	HCAEC	Isoflurane (Amour, Brzezinska et al. 2009)*
Enhanced production of BH4*	HCAEC	Isoflurane (Amour, Brzezinska et al. 2010)*
Increased GTPCH-1 protein synthesis *	HCAEC	Isoflurane (Baotic, Weihrauch et al. 2015)*

BH4, tetrahydrobiopterin; eNOS, endothelial nitric oxide synthase; GTPCH-1, guanosine triphosphate cyclohydrolase-1; HCAEC, human coronary artery endothelial cells; HSP 90, heat shock protein 90; ICAM-1, intercellular adhesion molecule-1; iNOS, inducible nitric oxide synthase; I/R, cardiac ischemia–reperfusion; mitoKATP channel, mitochondrial ATP-sensitive potassium channel; NF, nuclear factor; NOSs, nitric oxide synthases; PKC, protein kinase C; TNF, tumor necrosis factor; VCAM-1, vascular adhesion molecule-1 Table adapted from (Kunst and Klein 2015) with modifications.*

2.1.3. *APC Cardioprotection Enhanced by Endothelial - Cardiomyocyte Crosstalk*

Endothelial cells can affect cardiac function in various ways depending on their cardiac localization, of wich the anatomy is reviewed thoroughly by Brutsaert (Brutsaert 2003). It is indeed important to distinguish between the contribution of the cardiac endothelial cells in the myocardial capillaries and at the endocardium, and the contribution of the coronary vascular endothelium in the major epicardial and smaller intramyocardial coronary arteries and veins (**Fig. 1**) (Brutsaert 2003). The latter, i.e. vascular endothelium in the coronary conduit and resistance vessels, merely controls coronary artery function as in any other vascular bed in the body, thus indirectly contributing to cardiac function by controlling coronary blood supply to the myocardium (Brutsaert 2003). Cardiac endothelial cells in the myocardial capillaries (MyoCapE) and in the endocardial endothelium (EE), in contrast, are in close proximity to adjacent cardiomyocytes, allowing for direct cellular communication and signaling between both cell types (Brutsaert 2003).

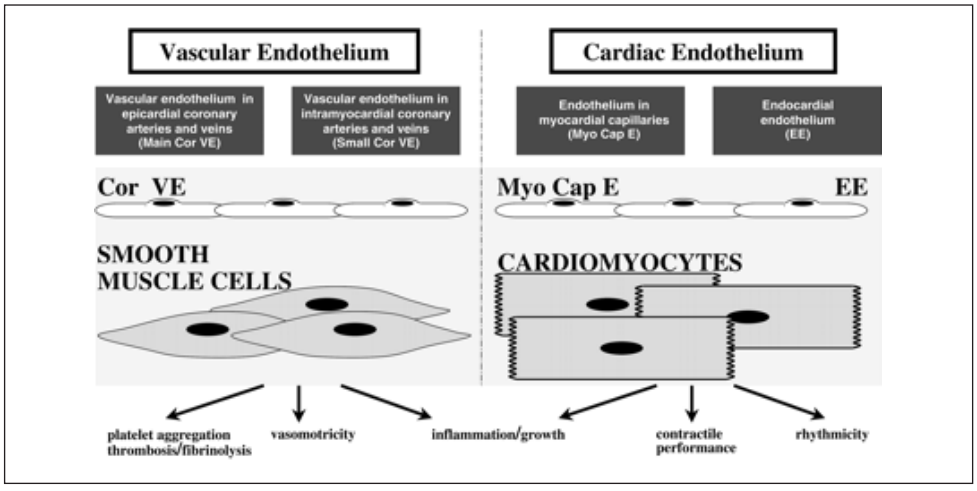

Figure 1. Organization of coronary vascular (CorVE) and cardiac (MyoCapE, EE) endothelial cells in the heart (Brutsaert 2003).

Vascular endothelium from epicardial and intramyocardial coronary arteries (left; CorVE) produce factors mainly influencing vascular related responses, comprising of the regulation of clotting (thrombosis/fibrinolysis), vasomotor tone (coronary vasomotricity) and inflammation; CorVE thus affects myocardium only indirectly through changes in myocardial blood supply. In contrast, cardiac endothelium of capillaries (right; MyoCapE) or epicardium (right; EE) signal directly to the immediate subjacent cardiomyocytes with effects on cardiac growth, contractile performance, and rhythmicity.

Endothelial-cardiomyocyte crosstalk depends critically on their intercellular distance and cell number ratio. The latter depends on the capillary-to-cardiomyocyte ratio and intercapillary distance, which will vary between species and cardiac sampling site (Brutsaert 2003). In left ventricular wall and papillary muscle of adult rat and neonatal mice, capillary-to-cardiomyocyte number ratio on cross-sectional views varies from 0.91 to 1.12 (Brutsaert 2003). The lower figure of 0.5 observed in human „endomyocardial" biopsies from right ventricular trabeculae can be ascribed to the close proximity of endocardial endothelial cells that are the dominant endothelial cells in this zone (Brutsaert 2003). In fact, cardiac endothelial cells outnumber cardiomyocytes by 3:1, although cardiac endothelial cell-to-cardiomyocyte volume (or mass) ratio is of the order of only 0.04–0.05 (Anversa, Olivetti et al. 1980; Brutsaert 2003). The intercapillary distance was reported to be 20.2 µm in the ventricular wall and 15.6 µm in papillary muscle of normal rat heart and 6 µm in normal neonatal mice heart (Brutsaert 2003). With a distance of ~1 µm between the capillary endothelial cell and the nearest cardiomyocyte, this provides for an action (diffusion) radius of ~3–12 µm for each capillary endothelial cell into the neighboring cardiomyocytes (Brutsaert 2003). This distance is well within reach for the highly liposoluble endothelium-derived NO to act as an efficient endothelial-myocardial signaling agent, despite is short biological half-life of 20 s (Brutsaert 2003).

While evidence both indicates that NO is a likely paracrine factor capable of relaying signals between endothelial cells and cardiomyocytes and that eNOS derived NO is a critical component of APC induced signal transduction (Amour, Brzezinska et al. 2009), the distinct contribution of endothelial cells versus cardiomyocytes to NO signaling has been poorly evaluated. The non-selective NOS-inhibitor L-NAME blocked early APC (Amour, Brzezinska et al. 2009) and isoflurane failed to protect against myocardial infarction or mPTP opening in $eNOS^{-/-}$ mice (Ge, Pravdic et al. 2010). Additionally, the trigger and mediator phases of delayed APC were also blocked by L-NAME. The mechanisms responsible for isoflurane-induced NO production in endothelial cells are incompletely defined. One possible candidate protein for activating preconditioning-related pathways is hypoxia-inducible factor 1 alpha (HIF1α) (Li, Wang et al. 2006; Wang, Weihrauch et al. 2006).

Collectively, current data support the notion that APC protection against cardiac ischemia is rooted in their action on both cardiomyocytes and endothelial cells. Main mechanisms involved in both cell types are schematically depicted in **Fig. 2.**

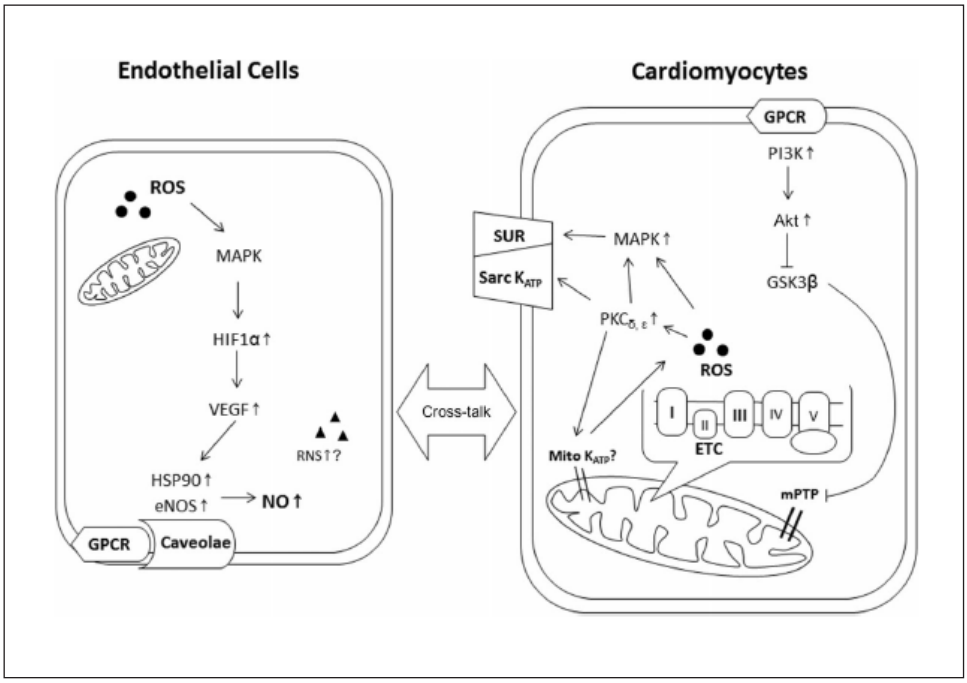

Figure 2. Scheme depicting key elements of the pathways activated in anesthetic-induced protection (Kikuchi, Dosenovic et al. 2015).

The APC exerts its actions both on endothelial cells (left) and cardiomyocytes (right), influencing endothelial cell-to-cardiomyocyte interactions. In endothelial cells, APC induces a limited increase in production of ROS, ultimately resulting in activation of eNOS and increased NO production. In cardiomyocytes, APC activates GPCR and multiple protective signaling pathways towards mitochondria, furthermore directly or indirectly interacting with mitochondrial ETC complex I and/or complex III thus promoting a limited formation of ROS ('signaling ROS'). ETC, electron transport chain; GPCR, G protein-coupled receptors; HIF1α, hypoxia-inducible factor 1α; HSP90, heat shock protein 90; MAPK, mitogen-activated protein kinases; Mito KATP, mitochondrial ATP-sensitive potassium channels; mPTP, mitochondrial permeability transition pore; RNS, reactive nitrogen species; Sarc KATP, sarcolemmal ATP-sensitive potassium channels; SUR, sulfonylurea receptor, VEGF, vascular endothelial growth factor.

3.1. Hyperglycemic Metabolic State and Loss of Cardioprotective APC Signaling

3.1.1. Diabetes Mellitus and APC

Diabetic patients typically represent a large proportion of patients undergoing cardiac surgery, amounting around 30% (Brown, Edwards et al. 2006). Previous research has documented that diabetic patients show excess complications following cardiac surgery, resulting in higher rates of 30 days mortality, stroke, and prolonged ICU stay (Brown, Edwards et al. 2006). Intraoperative blood glucose (BG) control has been evaluated in patients undergoing cardiac surgery to determine if elevated BG during surgery affects mortality and if tight BG control during surgery allows for improved glucose control postoperatively (Gandhi, Nuttall et al. 2005). In a retrospective study, intraoperative BG measurements and outcomes analyses from 409 cardiac surgery patients revealed that intraoperative hyperglycemia was an independent risk factor for perioperative complications, including death. Increase in mean intraoperative glucose concentration of 1.1 mmol/L (20 mg/dL) greater than 5.6 mmol/L (>100 mg/dL) was associated with a 30% increase of an adverse event (Gandhi, Nuttall et al. 2005; Reddy, Duggar et al. 2014). However, the same group performed a randomized trial evaluating the perioperative complications in 400 diabetic patients comparing intensive intraoperative insulin therapy with conventional glucose management during cardiac surgery that did not confirm initial findings from the retrospective study. On the contrary, there was increased incidence of death and stroke identified in the intensive treatment group (Gandhi, Nuttall et al. 2007). Furthermore, when glucose-insulin-potassium (GIK) infusion during surgery and postoperatively for tight control of BG (125–200 mg/dL; 6.9-11.1 mmol/L) was compared with standard therapy without tight control (BG <250 mg/dL; <13.9 mmol/L) in 141 diabetic patients, no difference on 30 days mortality was found, as both amounted 0% (Lazar, Chipkin et al. 2004). However, the GIK infusion arm showed a significant decrease in infection rates, mechanical ventilator time, length of stay, and incidence of atrial fibrillation (Lazar, Chipkin et al. 2004). In a prospective trial of more than 2,000 patients with diabetes undergoing CABG surgery, BG averaged for the first 2 postoperative days was an independent predictor of mortality (Furnary, Zerr et al. 1999; Gu, Pagel et al. 2003). Also, poor intraoperative control of BG concentrations in diabetic

patients undergoing cardiac surgery is associated with a worsened composite outcome measure after surgery (Ouattara, Lecomte et al. 2005).

Evidence indicates that diabetes markedly attenuates the cardioprotective signal transduction mechanisms activated by preconditioning in the experimental setting. Diabetes attenuates infarct size reduction in response to APC with low concentrations of isoflurane in dogs. Moreover, the BG concentrations were related to the infarct size, but the relationship was abolished with higher concentration of isoflurane (Tanaka, Kehl et al. 2002). As mitoK_{ATP} channels have great importance in underlying mechanisms of APC elicited cardioprotection (Nakae, Kwok et al. 2003; Tanaka, Weihrauch et al. 2003; O'Rourke 2004), the influence of diabetes on mitoK_{ATP} activation has been thoroughly investigated. It has been shown that the decrease in myocardial infarct size produced by the mitoK_{ATP} channel agonist diazoxide was abolished in a canine model of diabetes, which is confirming the detrimental role of hyperglycemia on mitoK_{ATP} channel activation (Kersten, Montgomery et al. 2001). More recently, this has been confirmed in diabetic human myocardium (Hassouna, Loubani et al. 2006). However, the translation of experimental evidence into clinical practice has not produced equivocal results to date, nor in cardioprotective effects of APC in patients undergoing cardiac surgery nor in diabetes abolishing that effect.

3.1.2. *Acute Hyperglycemia and APC*

Increased risk on perioperative complications in diabetic patients seems partly related to hyperglycemia *per se.* Acute hyperglycemia (AHG) alone is a major predictor of peri-operative cardiovascular morbidity and mortality. Recent evidence strongly implicates perioperative hyperglycemia, in the absence of diabetes, as an independent predictor of death after noncardiac surgery (Frisch, Chandra et al. 2010). Further, hyperglycemia in the first postoperative day was associated with subsequent adverse outcomes (nonfatal stroke, MI, septic complication, or death): for each 1-mmol/l increase above 6.1 mmol/l, risk increased by 17% (McAlister, Man et al. 2003). To date, the mechanisms that confer this AHG related increased risk are poorly understood. In addition, AHG has also been observed to negatively affect the APC-induced myocardial protection in dogs in severity dependent manner (Kehl, Krolikowski et al. 2002). Evidence also indicates that AHG markedly attenuates cardioprotective signal transduction produced by volatile anesthetics in rabbits (Amour, Brzezinska et al. 2010).

3.2. Mechanisms by which hyperglycaemia and diabetes affect anesthetic preconditioning

Experimental studies evidenced that AHG increases the production of ROS, decreases the availability of NO, impairs endothelial function, and attenuates coronary microcirculatory responses to myocardial ischemia. The way diabetes and/or AHG are blocking the cardioprotective action of APC seems primarily rooted in changes of mitochondrial metabolism. The overall changes in mitochondrial bioenergetics in AHG/diabetes favors an excessive production of ROS. The rapid metabolism of excess glucose likely provides extra substrates for mitochondria, increasing activity of the respiratory chain and increasing $\Delta\Psi m$ (Sedlic, Muravyeva et al. 2017). The resulting mitochondrial hyperpolarization leads to excess ROS production, which is known to steeply increase with the increase in $\Delta\Psi m$ (Starkov and Fiskum, 2003; Lambert and Brand, 2004). High glucose stimulation of ROS production presumably occurs due to impeded proton pumping and obstructed electron flow, which favors electron „leak" and incomplete oxygen reduction (Sedlic, Muravyeva et al. 2017). Therefore, even subtle elevation of oxygen consumption (electron flow along respiratory chain) combined with more pronounced increase in $\Delta\Psi m$ by AHG may have substantial effects on ROS production, especially in stressed cells after I/R injury (Sedlic, Muravyeva et al. 2017). In keeping with this view, attenuation of high glucose induced ROS production by specific elimination of ROS generated by mitochondrial hyperpolarization, decreases cell injury (Baotic, Ge et al. 2013; Sedlic, Muravyeva et al. 2017). Likewise, in the presence of antimycin A, a blocker of complex III, high glucose failed to increase oxygen consumption by cardiomyocytes (Sedlic, Muravyeva et al. 2017). The results from Sedlic et al. support these observations and demonstrate that the normalization of $\Delta\Psi m$ by 2,4 dinitrophenol (DNP) acutely reduced ROS production by high glucose (Sedlic, Muravyeva et al. 2017). Also, their results show that high glucose rapidly increases NAD(P)H fluorescence intensity and increases the rate of oxygen consumption in cardiomyocytes (Sedlic, Muravyeva et al. 2017). Collectively, these data support the notion that excess glucose accelerates oxidative phosphorylation at the cost of excess mitochondrial ROS production because of the induction of mitochondrial hyperpolarization.

In addition to its action on mitochondria, AHG/diabetes also influences affects the bioavailability of NO. By favoring ROS production, AHG/diabetes inactivates NO to form peroxynitrite that induces substrate nitration

(Creager, Luscher et al. 2003). Further, AHG/diabetes decreases the NO bioavailability by influencing eNOS activation through inadequate phosphorylation of Serine 1177 and eNOS compartmentalization (Baotic, Ge et al. 2013). Also, hyperglycemia affects BH4 and heat shock protein (Hsp) 90, a physiologic binding partner of eNOS, which regulates eNOS phosphorylation and modulates subsequent NO production (Amour, Brzezinska et al. 2010). The resulting imbalance between NO bioavailability and the ROS production is diminishing positive EC-CM interaction and abolishing APC- induced cardioprotection.

Scope of the thesis

The main goal of the thesis was to evaluate the mechanisms responsible for cardioprotection during APC, and specifically to elucidate the role of endothelial cells and eNOS, and determine how eNOS-related signaling events are adversely modulated by hyperglycemia and diabetes. Furthermore, we investigated whether ApoA1 mimetics redress the hyperglycemia induced abrogation of APC evoked cardioprotection, thus representing a potential strategy for further clinical investigation.

In **chapter 2** (full-text of the published paper: Isoflurane Favorably Modulates Guanosine Triphosphate Cyclohydrolase-1 and Endothelial Nitric Oxide Synthase during Myocardial Ischemia and Reperfusion Injury in Rats (Baotic, Weihrauch et al. 2015)), employing *in vivo* coronary ligation to produce cardiac ischemia/reperfusion in adult male rats with and without APC, we investigated the hypothesis that isoflurane modulates NO synthesis and protection against myocardial infarction through time-dependent changes in the expression of key NO regulatory proteins, guanosine triphosphate cyclohydrolase (GTPCH)-1, the rate-limiting enzyme involved in the biosynthesis of tetrahydrobiopterin and eNOS (Baotic, Weihrauch et al. 2015).

In **chapter 3** (full-text of the published paper: Endothelial–cardiomyocyte crosstalk enhances pharmacological cardioprotection (Leucker, Bienengraeber et al. 2011)) we investigated endothelial cell–cardiomyocyte cross-talk using isoflurane as a pharmacological stimulus to enhance endothelial protection of cardiomyocytes against hypoxia and reoxygenation injury in a co-culture cell model (Leucker, Bienengraeber et al. 2011). Subsequently, we elucidated that triggering of intracellular signal transduction

pathways, culminating in the enhanced production of NO, appears to be a central component of pharmacologically induced cardioprotection (Leucker, Bienengraeber et al. 2011).

Diabetes has previously been shown to alter mitochondrial bioenergetics and consequently disrupts cardioprotective signaling, however, the contribution of mitochondrially encoded proteins to the disruptive signaling has never been investigated (Leucker, Bienengraeber et al. 2011). Therefore, in **chapter 4** (full-text of published paper: Cardioprotection during Diabetes, The Role of Mitochondrial DNA (Muravyeva, Baotic et al. 2014)) we investigated whether mitochondria harboring different mtDNA genomes modify APC and cardiac susceptibility to ischemia/reperfusion injury by using two strains of rats, both sharing nuclear genome of type 2 diabetes mellitus (T2DN) rats but with distinct mitochondrial genomes of Wistar and fawn-hooded hypertensive (FHH) rat strains ($T2DN^{mtWistar}$ and $T2DN^{mtFHH}$, respectively) (Muravyeva, Baotic et al. 2014). In **chapter 5** („work in progress" manuscript: Mitochondrial Bioenergetics in Diabetic Myocardium – Implications for Protective Conditioning Strategies), we review the specific adaptations in mitochondria that may contribute to the abrogation of APC induced cardioprotection in hyperglycemia and diabetes.

Apolipoprotein A-1 (ApoA-1) mimetics that scavenge oxidized lipids and modulate cholesterol transport to membrane microdomains, have been suggested to decrease cardiovascular risk during diabetes and AHG (Peterson, Husney et al. 2007; Bloedon, Dunbar et al. 2008; Baotic, Ge et al. 2013). As AHG decreases the availability of NO and impairs APC-elicited protection against myocardial infarction, we investigated whether D-4F, an ApoA-1 mimetic, rescues the myocardium by promoting APC-induced endothelial NO signaling during AHG (Baotic, Ge et al. 2013) **(chapter 6)** (full-text of the published paper: Apolipoprotein A-1 mimetic D-4F enhances isoflurane-induced eNOS signaling and cardioprotection during acute hyperglycemia (Baotic, Ge et al. 2013)). Lastly, in **chapter 7** we summarize and discuss the results of our investigations and suggest a framework for their interpretation and future studies.

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