Postconditioning protects endothelial cells from apoptosis during reperfusion injury- Role of inhibitor of apoptosis protein 2

Inaugural Dissertation

submitted to the

Faculty of Medicine

in partial fulfillment of the requirements

for the PhD-Degree

of the Faculties of Veterinary Medicine and Medicine

of the Justus Liebig University Giessen

by

Krishnaveni Gadiraju

of

Hyderabad, India

Giessen (2010)

From the Institute of Physiology
Director/Chairman: Prof. Dr. K. D. Schlüter
of the Faculty of Medicine of the Justus Liebig University Giessen

First Supervisor and Committee Member: Priv. Doz. Dr. Thomas Noll
Second Supervisor and Committee Member: Prof. Dr. Henning Morawietz
Committee Members: Prof. Dr. Ulrich Müller, Prof. Dr. Dr. Stefan Arnhold

Date of Doctoral Defense: 08.09.2010

Table of contents

Abbreviations

Apaf-1	Apoptosis protease activating factor-1
Apo-1	Apoptosis-inducing protein-1
APS	Ammonium per sulfate
Asp	Aspartic acid
ATP	Adenosine-5-triphosphate
Bak	Bcl-2 homologue antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma-2
bFGF	Basic fibroblast growth factor
BH3	Bcl-2 homology domain
BID	Bcl-2 interacting domain
BIR	Baculoviral IAP repeat
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CARD	Caspase recruitment domain
cIAPs	Cellular inhibitor of apoptosis proteins
CPC	Chromosomal passenger complex
Cyt C	Cytochrome C
CWFSG	Cold-water fish skin gelatin
DED	Death effector domain
DISC	Death-inducing signaling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DR3-6	Death receptors 3-6
DTT	Dithiothreitol
dUTP	Deoxy uridine triphosphate
EC	Endothelial cells
ECGS	Endothelial cell growth supplement
ECL	Enhanced chemiluminescence
ECO	Escherichia Coli Oxyrase
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid

eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERK 1\2	Extracellular signal-regulated kinases 1\2
FACS	Fluorescence activated cell sorting
FADD	Fas-associated death domain
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GSK-3β	Glycogen synthase kinase 3 beta
HBSS	Hank's balanced salt solution
hEGF	Human epidermal growth factor
HEPES	4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
HUVEC	Human umbilical vein endothelial cells
IAPs	Inhibitor of apoptosis proteins
IU	International unit
JAK/STAT	Janus kinases/ Signal transducers and activators of transcription
JNK	c-Jun N-terminal kinase
LAD	Left anterior descending artery
L-NAME	L-nitro-arginine methyl ester
K_{ATP} channels	Potassium ATP channels
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
kDa	Kilo Dalton
MAPK	Mitogen activated protein kinase
$MgCl_2$	Magnesium chloride
min	Minutes
$MnCl_2$	Manganese chloride
MPO	Myeloperoxidase
mPTP	Mitochondrial permeability transition pore
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NaF	Sodium fluoride
Na_2HPO_4	Di-sodium hydrogen phosphate
NaH_2PO_4	Sodium dihydrogen phosphate
Na-orthovanadate	Sodium orthovanadate

NF-κB	Nuclear factor κ-light chain enhancer of activated B-cells
NIAP	Neuronal inhibitor of apoptosis protein
NO	Nitric oxide
NOS	Nitric oxide synthase
NP-40	Nonidet P-40
OMI/HTRA2	High temperature requirement protein A 2
PBS	Phosphate-buffered saline
pH	Negative log of H^+ concentration
PI	Propedium Iodide
PI 3K	Phosphoinositide 3-kinase
PMSF	Phenylmethylsulfonyl fluoride
RING	Really interesting new gene
ROS	Reactive oxygen species
RT	Room temperature
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA
Smac/DIABLO	Second mitochondria-derived activator of caspase
TAB1	TAK1 binding protein
TAK1	TGF-β activated kinase 1
tBID	Truncated Bcl-2 interacting domain
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TEMED	N, N, N', N',-tetramethylethylenediamine
TGF-β	Transforming growth factor-β
TNF-α	Tumour necrosis factor- α
TNFR	TNF- α receptor
TRAIL	TNF- α related apoptosis-inducing ligand
Tris	Tris (hydroxymethyl) aminomethane
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
XIAP	X-linked inhibitor of apoptosis protein
% vol/vol	Volume by volume percentage
% wt/vol	Weight by volume percentage
25g	25 gauge

1. Introduction

1.1 Endothelial apoptosis

The endothelium is a monolayer of cells forming the innermost lining of the entire circulatory system. It acts as a selectively-permeable membrane barrier between the blood and the interstitial spaces. Although historically viewed as a passive monolayer merely reducing the turbulence of blood flow, the endothelium infact, is a dynamic membrane making many active contributions to cardiovascular function. The major contributions of the endothelium include selective blood tissue exchange, regulation of vascular tone by vasoactive secretions like nitric oxide (NO), endothelium derived hyperpolarizing factor, prostacyclin and endothelin, flow induced vasodilatation and constriction and hence control of blood pressure, blood clotting, modification of circulating plasma components by angiotensin-converting enzyme, inflammatory defence against pathogens and initiation of angiogenesis.

The function and integrity of the endothelium, therefore, are absolute necessities for the function of the cardiovascular system. However, this integrity is at stake in several pathological conditions like ischemia-reperfusion, leading to damage or loss of endothelial cells. Under these conditions, apoptosis is the predominant form of cell death in the endothelium due to the robust energy metabolism of these cells. The ability of endothelial cells to maintain high levels of ATP, even in the adverse conditions of hypoxia or ischemia, prevents them from the necrotic fate (Lelli et al., 1998) (Fig 1.1). Increasing evidence suggests that apoptosis of endothelial cells can be responsible for acute and chronic coronary diseases, e.g. through atherogenesis (Chen et al., 2004), thrombosis (Bombeli et al., 1997) and endothelial dysfunction (Werner et al., 2006), hence jeopardizing the survival of the whole myocardial tissue. It is now known that endothelial apoptosis is a critical part of reperfusion injury and it is the endothelial cells rather than the cardiomyocytes that begin to undergo apoptosis early during reperfusion (Scarabelli et al., 2001).

Inspite of the high clinical relevance associated, little is known about the mechanisms preventing apoptosis in endothelial cells. The present study focuses on hypoxia-reoxygenation induced endothelial apoptosis and its response to postconditioning.

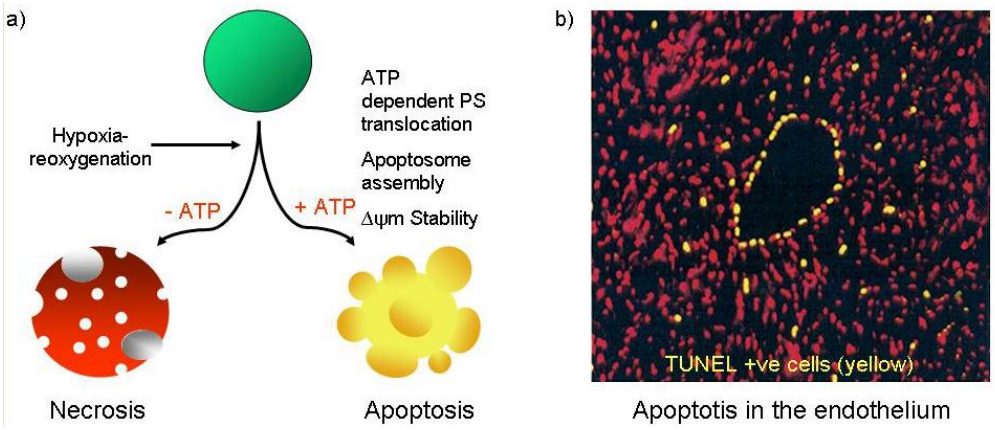


Fig 1.1 Endothelial apoptosis: a) Apoptosis – necrosis switch by ATP. b) Cross section of a rat heart subjected to 35 min ischemia followed by 60 min reperfusion, showing TUNEL positive (yellow) apoptotic endothelial cells around the vessel and TUNEL positive cardiomyocytes whose number decreases with increasing distance from the lumen (Scarabelli et al., 2001).

1.2 Reperfusion injury

'Ischemia', literally meaning restriction of blood flow, is one of the most frequent cardiovascular complications and the leading cause of death worldwide. Reperfusion or restoration of blood flow remains the definitive strategy for saving the myocardium. However, reperfusion has been referred as a 'double edged sword' (Braunwald and Kloner, 1985), because reperfusion itself is associated with a series of detrimental events that extend the damage beyond that observed during the ischemic period alone. These events are collectively called as reperfusion injury. Reperfusion injury is not a mere worsening of the ischemia-induced damage, but it constitutes processes that are specifically induced by reperfusion *per se*. It includes complex mechanisms involving mechanical, extracellular and intracellular processes. Some of the events that trigger reperfusion injury are:

- Rapid generation of reactive oxygen species (ROS) by activated vascular endothelial cells, neutrophils and stressed cardiomyocytes (Ambrosio et al., 1991).
- Activation of sodium hydrogen exchanger (Allen et al., 2003) and augmentation of ischemia induced cellular and mitochondrial Ca^{2+} overload (Piper et al., 1989).

- Increased osmotic gradient and cell swelling induced by the accumulation of products of anaerobic metabolism (Jennings et al., 1986).
- Opening of the mitochondrial permeability transition pore (mPTP), resulting in influx of otherwise impermeable proteins, mitochondrial swelling, uncoupling of oxidative phosphorylation and release of pro-apoptotic molecules like cytochrome C (Cyt C) and second mitochondria-derived activator of caspase (Smac/DIABLO) into the cytosol (Griffiths et al.,1995).
- Reduced NO availability (Lefer et al., 1993) leading to the augmented expression of cellular adhesion molecules, induction of local inflammation, leukocyte infiltration and no-reflow phenomenon.

The clinical consequences of these events, occurring within minutes of the onset of reperfusion are manifested by myocardial stunning or hypercontracture, infarction, reperfusion arrhythmias, endothelial dysfunction and cell death by necrosis and apoptosis.

1.3 Apoptosis in reperfusion injury

Ischemia/reperfusion induces cell death via apoptosis, oncosis and necrosis. However, endothelial cells due to their robust energy metabolism undergo apoptosis, rather than necrosis.

Apoptosis is a controlled process of programmed cell death. The morphology of this death process was originally recognized by nineteenth century microscopists. Thus, what we now call apoptosis was first described in the epithelial cells of atretic ovarian follicles by Flemming in 1885 (Eefting et al., 2004; Scarabelli et al., 2006).

Morphologically, apoptosis is characterized by cell shrinkage, chromatin condensation and migration, DNA fragmentation and blebbing of the plasma membrane. Nuclear and cytoplasmic material is surrounded by intact plasma membrane and these apoptotic bodies are engulfed by phagocytes. Due to this rapid vesiculation and phagocytosis, there is no spilling of intracellular material into the surrounding tissue. Therefore, there is less inflammation in the surrounding tissue compared to necrosis, where cell swelling and rupture of the plasma membrane lead to leakage of cellular content, consequently resulting in a strong inflammatory response. Due to its programmed nature, apoptosis is also more amenable to therapeutic interventions. Though seemingly opposing insults, both prolonged hypoxia/ischemia and reperfusion result in apoptosis, with the burst of reactive

oxygen species caused by reperfusion, enhancing the rate of apoptosis initiated by hypoxia or ischemia.

DNA fragmentation in ischemia and reperfusion:

DNA fragmentation is one of the defining characteristics of apoptosis. It is commonly identified by DNA laddering in gel electophoresis and by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay.
Gottlieb et al., (1994) have identified the absence of TUNEL-positive apoptotic cells or DNA laddering in the rabbit heart exposed to ischemia alone but did detect DNA fragmentation by both these assays during reperfusion following ischemia. In contrast, Kajstura et al., (1996) have observed DNA fragmentation by both these assays in rat hearts exposed to prolonged ischemia without reperfusion. In a more recent study (Zhao et al., 2002), very few TUNEL-positive cells and no DNA laddering were detected in the dog heart exposed to ischemia alone, but a very large number of TUNEL-positive cells and extensive DNA laddering were observed in the peri-necrotic area, after 6 hours of reperfusion. These studies suggest that although there may be differences between species and different experimental systems, it is likely that the vast majority of DNA fragmentation is confined to the post-ischemic period rather than to ischemia itself. This conclusion is reinforced by the work of Scarabelli et al., (2001) who observed no TUNEL positivity in both cardiomyocytes and endothelial cells of the rat heart exposed to ischemia alone. However, TUNEL positivity was detected in endothelial cells after as little as 5 minutes of reperfusion, peaked at 60 minutes of reperfusion and decreased at 2 hours of reperfusion. In contrast, the proportion of TUNEL-positive cardiac myocytes slowly increased over 2 hours of reperfusion. As expected, DNA laddering was detected in samples prepared after reperfusion but not in samples exposed to ischemia alone. These studies, therefore, indicate that apoptosis does occur in the heart, particularly during reperfusion, and has a different time-course in endothelial cells compared to cardiac myocytes.

The importance of apoptosis as a key step in reperfusion injury is supported by studies in which such DNA fragmentation was inhibited by treatment with aurintricarboxylic acid (ATA), an inhibitor of DNA endonucleases. In these experiments, addition of ATA at the onset of reperfusion resulted in reduced infarct size and enhanced regional contractile function (Zhao et al., 2003).

Translocation of phosphotidylserine in reperfusion injury:

Translocation of phosphotidylserine residues from the inner to the outer side of the plasma membrane occurs as an outcome of apoptosis. Hence apoptotic cells exhibit staining with Annexin V, which binds to phosphotidylserine.

It has been shown that surface staining with Annexin V appeared in the intact mouse heart only during reperfusion and not during the ischemic episode (Dumont et al., 2000). This reinforces the DNA fragmentation studies that apoptosis occurs predominantly during reperfusion. Increased uptake of labeled Annexin V in the infarct area was shown in patients with acute myocardial infarction when treated with primary coronary angioplasty, confirming the role of apoptosis in reperfusion injury (Hofstra et al., 2000).

Caspase activation in reperfusion injury:

Caspases are cysteine (Cys)-dependent aspartic acid (Asp)-specific proteases that are the key executers of apoptosis. They are constitutively expressed as inactive precursor zymogens that are activated in response to an apoptotic stimulus by proteolytic cleavage and dimerization, to generate active caspases. Upon activation, they execute the cell death process through cleavage of specific structural and regulatory proteins necessary for cell survival. Fourteen caspases have been identified so far and they are divided into two groups, namely, the initiator and the effector caspases.

The initiator caspases like -2, -8, -9, -10 and -12 are characterized by the presence of long N-terminal regions that contain one or more adaptor domains (death effector domain, DED or caspase recruitment domain, CARD), which are absent in the effector enzymes. Activation of initiator caspases takes place in a multiprotein complex, such as the apoptosome for caspase-9 and the death-inducing signaling complex (DISC) for caspase-8. Active initiator caspases consequently activate downstream effector caspases, such as caspase-3, -6, and -7 by cleavage at internal Asp residues. Effector caspases are expressed as homodimers and their activation involves intrachain cleavage that generates fragments of ~10 and ~20 kDa still in a dimeric form. Active effector caspases recognize a 4-amino-acid motif in their substrates, P4-P3-P2-P1, and cleave after the C-terminal (P1) Asp. Over 280 such caspase substrates have been identified and most of them are structural or regulatory proteins whose function is inactivated by caspase cleavage (Fischer et al.,

2003). In some cases the cleaved fragment also helps to augment the apoptotic process (Scarabelli et al., 2006).

The initiator caspases -8, -9 and -12 and the effector caspases -3, -6 and -7 have been shown to be involved in ischemia-reperfusion induced apoptosis. Activation of these caspases in response to ischemia-reperfusion involves both the intrinsic and the extrinsic pathways (Scarabelli et al., 2006).

1.4 The intrinsic pathway

The intrinsic pathway is also called the mitochondrial pathway and is employed by a wide range of apoptotic stimuli that converge on the mitochondria. These stimuli affect the mitochondria in different ways by either altering the permeability or by membrane swelling and disruption. Caspase-9 is the principle initiator caspase of the intrinsic pathway. Release of Cyt C from the inner mitochondrial membrane into the cytosol is the critical initiating step of mitochondria-mediated apoptosis. In the cytosol, Cyt C binds to apoptosis protease activating factor (Apaf-1), in the presence of ATP. Procaspase-9 is recruited to, and activated, in this complex, called the apoptosome (Adrain et al., 2001). Active caspase-9 subsequently cleaves the effector caspases -3, -6 and -7 thereby activating them.

Cyt C release has been observed in the intact heart exposed to ischemia/reperfusion, with its translocation from mitochondria to the cytosol becoming maximal during the reperfusion phase (Narula et al., 1999) and was associated with caspase-9 activation (Scheubel et al., 2002). The key role for Cyt C is further supported by studies in which inhibition of Cyt C release was found to block apoptosis (Borutaite et al., 2003), whereas its addition to heart cytosol was shown to be sufficient to induce apoptosis (Borutaite et al., 2001). Release of Cyt C is governed by the integration of the Bcl-2 family proteins, Bax and Bak that form channels in the mitochondrial membrane or by the opening of the mPTP.

mPTP is a protein pore spanning across the inner and outer mitochondrial membranes together with proteins of the intermembrane space. Its opening, apart from Cyt C release, results in release of other pro-apoptotic molecules like Smac/ DIABLO, production of ROS, release of mitochondrial NADH and influx of ions such as calcium, causing swelling of the mitochondria. Inhibition of the mPTP in rat hearts by cyclosporine A (Griffiths et al., 1995) and sanglifehrin A (Hausenloy et al., 2003)

during reoxygenation was observed to be protective against ischemia-reperfusion injury.

1.5 The extrinsic pathway

The extrinsic pathway or the death receptor pathway operates via ubiquitously expressed cell surface receptors characterized by the presence of a death domain. Six death receptors have been identified, including CD95 (also known as APO-1, Fas), TNFα receptor-1 (TNFR1), and death receptors 3-6 (DR 3-6), and all of these are expressed in the heart (Spierings et al., 2004). Their corresponding ligands, CD95 ligand (CD95L), tumor necrosis factor-α (TNFα), and TNFα-related apoptosis-inducing ligand (TRAIL), are also expressed in the heart. On ligation to the receptors, the death domains transduce the apoptotic signal by recruiting adaptor molecules (e.g. Fas-associated death domain (FADD) or TNF receptor-associated death domain (TRADD)), which in turn, recruit the enzymatically inactive procaspase-8. The resulting complex is known as the death inducing signaling complex (DISC). The recruitment of procaspase-8 to the DISC results in its oligomerization and activation through selfcleavage. The enzymatically active caspase-8 then cleaves downstream caspases, such as caspases-3, -6, and -7.

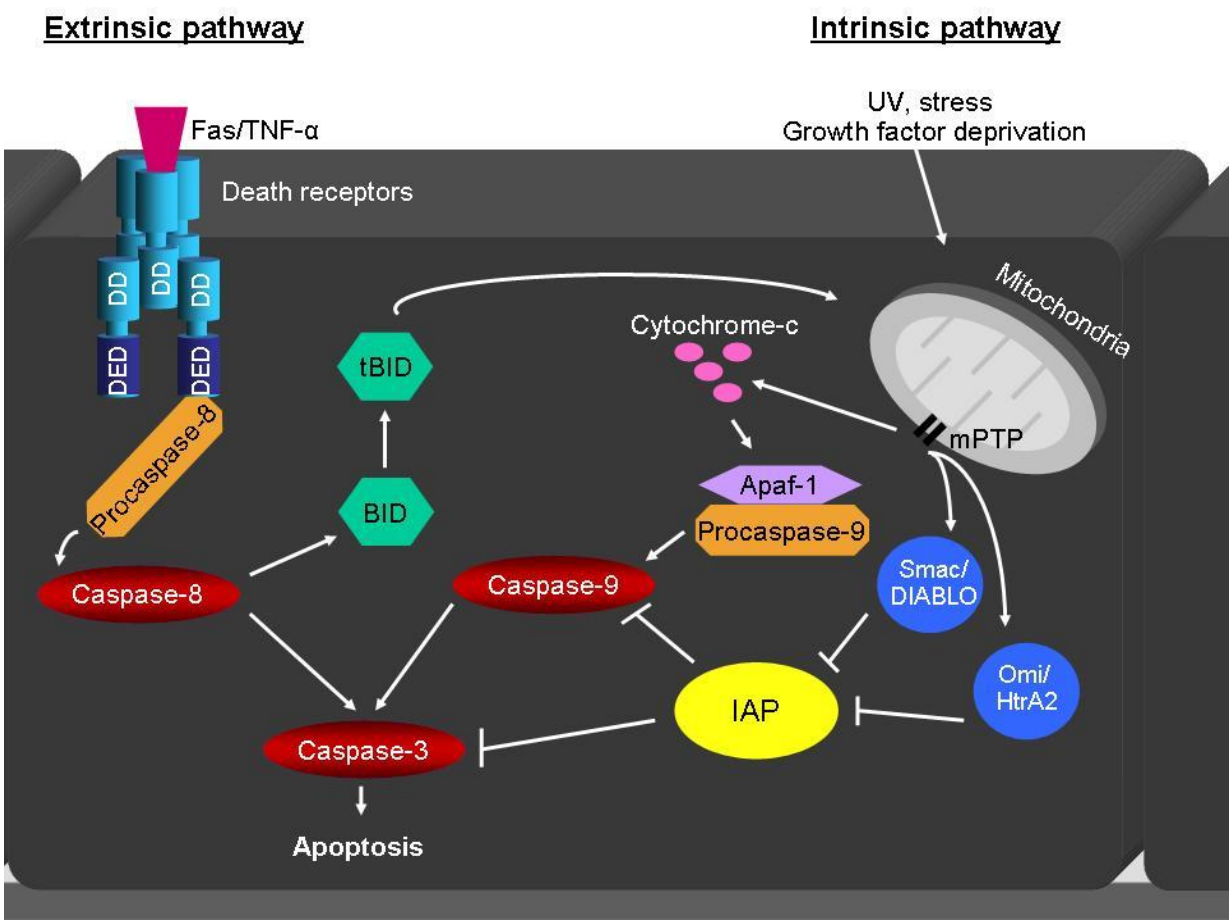


Fig 1.2 Cross talk between the intrinsic and extrinsic apoptotic pathways converging at effector caspases and their inhibitors, the IAPs.

Though the molecular cascades employed are distinct to each pathway, the mitochondrial and the death receptor mediated apoptotic pathways are not mutually exclusive. Procaspase-8 activated by stimulation of the death receptors, can activate the BH3 only protein, BID, whose cleavage product, tBID, migrates into the mitochondria, disrupting the membrane. This disruption causes the translocation of Cyt C to the cytoplasm interlinking both the apoptotic pathways (Luo et al., 1998).

Evidence is now available that both caspase-8, the initiator caspase of the extrinsic pathway and caspase-9, the initiator caspase of the intrinsic pathway, play important but distinct roles in reperfusion injury. Specific inhibitors of either caspase-9 or caspase-8 given at reperfusion were able to reduce infarct size in the isolated rat heart (Scarabelli et al., 2002). More detailed studies in cultured cardiac cells (Stephanou et al., 2001) have indicated that both chemical and gene-based inhibitors of caspase-9 can reduce apoptotic cell death in cardiomyocytes exposed to

simulated ischemia alone, whereas inhibition of caspase-8 has no effect. In contrast, inhibition of either caspase-8 or caspase-9 was able to reduce apoptotic cell death in response to ischemia/reperfusion. These studies in cultured cardiac cells were supported by further studies in the intact isolated heart exposed to ischemia/reperfusion that demonstrated activation of caspase-9 during ischemia alone with further activation during reperfusion, whereas caspase-8 was only activated by reperfusion following ischemia (Scarabelli et al., 2002). In addition to this difference in the activation of the two caspases during ischemia and reperfusion, another interesting observation was that their activation differs in endothelial cells and cardiomyocytes. Activation of caspase-9 was observed primarily in endothelial cells and only to a much lesser extent in cardiac myocytes, whereas activation of caspase-8 was only observed in cardiac myocytes. In agreement with this, a specific caspase-9 inhibitor prevented endothelial apoptosis in this system, whereas a specific caspase-8 inhibitor affected only cardiac myocyte apoptosis (Scarabelli et al., 2002). In this study, it was also shown that endothelial apoptosis preceeds cardiomyocyte apoptosis, in the heart exposed to ischemia/reperfusion.

Taken together, these findings on the time-course of apoptosis in the different cell types suggest a model in which activation of caspase-9 during ischemia itself leads to initiation of apoptosis, primarily in the endothelium, which futher extends to cardiomyocytes and continues during reperfusion as well. On the other hand, caspase-8, which is the principal initiator caspase in cardiomyocytes, is activated only at reperfusion and not during ischemia. This clearly indicates that endothelial apoptosis is one of the initiating events of reperfusion injury and is central to the outcome of myocardial damage.

1.6 Inhibitors of apoptosis proteins

As their name implies, the IAPs (inhibitor of apoptosis proteins), are a family of proteins that confer protection to the cell by counteracting apoptotic execution. They are characterized by the presence of at least one baculovirus IAP repeat (BIR) domain, reflecting their original discovery in baculovirus. A genetic screen to identify regulators of host cell viability led to this discovery of IAPs (Crook et al., 1993), which was followed by identification of cellular orthologues in species as diverse as yeast (Uren et al., 1999), nematodes (Fraser et al., 1999), flies (Hay et al., 1995) and humans (Duckett et al., 1996; Liston et al., 1996). Eight human IAPs have been

identified so far and their regulation has been implicated in the maintenance of tissue homeostasis in several physiological and pathophysiological situations like acute myelogenous leukaemia (Tamm et al., 2000), MALT lymphoma (Dierlamm et al., 1999), melanomas (Vucic et al., 2000), oesophageal squamous-cell carcinoma (Imoto et al., 2001), neuro-degenerative disorders (Roy et al., 1995) and in ischemia (Dong et al., 2001).

1.6.1 Structure and function of mammalian IAPs

Three major domains, namely, the BIR domain, the RING finger and the CARD domain constitute mammalian IAPs, though not all of them are present in all the eight proteins of the family (Fig 1.3).

BIR Domain: The BIR domain is a ~ 70 to 80 amino acid zinc-binding domain, the existence of which in a protein constitutes membership of the IAP family (Miller et al., 1999; Hinds et al., 1999; Sun et al., 1999). It comprises three short β-strands and four α-helices that fold into a compact structure containing a zinc ion coordinated by conserved histidine and cysteine residues (Hinds et al., 1999). The BIR domain sequences are strongly conserved from viral to mammalian IAPs and have a remarkable structural similarity. However, specific sequence differences underlie the distinct binding properties of each of the BIR domain. One to three copies of this motif have been identified in numerous proteins, not all of which have clear links with apoptosis. Indeed, IAPs are sometimes referred by the alternative nomenclature of BIRPS (BIR-containing proteins), as some BIR-containing proteins do not seem to function as bona fide inhibitors of apoptosis, but all IAPs are BIR-containing proteins (Uren et al., 1998). The BIRs are essential for the anti-apoptotic properties of the IAPs (Duckett et al., 1996) and in several cases this has been directly attributed to the binding and inhibition of caspases (Devereaux et al., 1997).

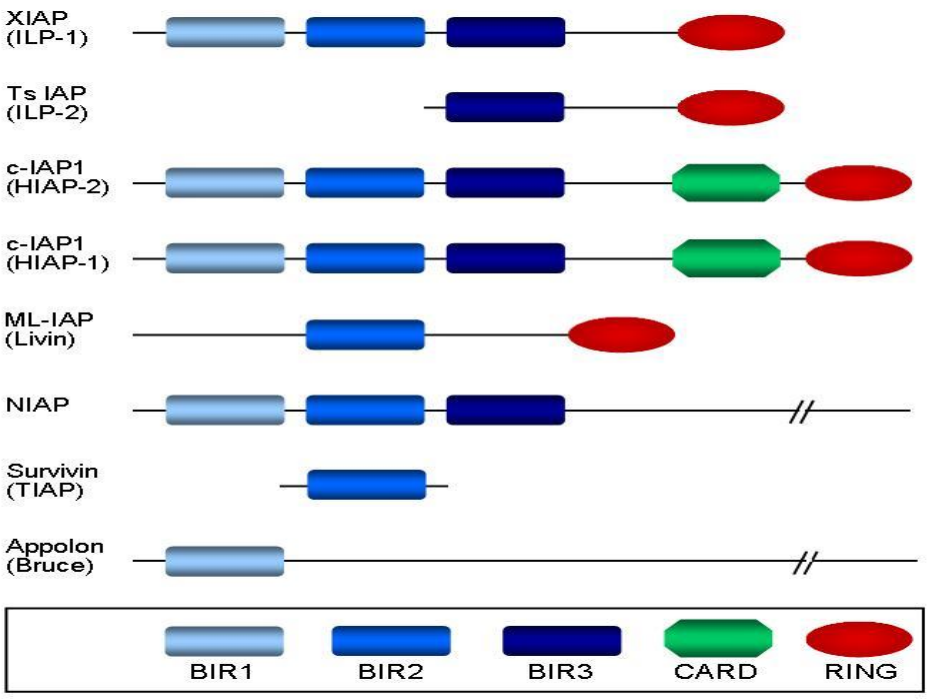


Fig 1.3 Domain repersentation of the mammalian IAPs: Each member of the family has atleast one BIR or baculoviral IAP repeat. CARD or caspase recruitment domain is found only in cellular IAP1 (cIAP1) and cellular IAP2 (cIAP2). RING finger is present at the carboxy terminal in all except neuronal IAP (NIAP), Survivin and Appolon.

RING Domain: The prototype baculoviral IAPs, and several cellular IAPs, contain a second type of zinc-binding motif known as the RING (really interesting new gene) domain (Joazeiro et al., 2000). In an IAP that contains a RING domain, this motif is invariantly found at the extreme carboxyl terminus of the protein. It is a small ~ 40 amino acid domain that is defined by eight cysteine and histidine residues that coordinate two zinc ions. Although RING domains have been identified in various proteins with different functions, the RINGs of IAPs are much more closely related to each other than they are to the RINGs of other proteins. This indicates that they might have evolved, and retained, a specialized function. The RING domain possesses dimerization and E3 ligase activity (Silke et al., 2005) that enables RING-containing proteins to catalyse the degradation of both themselves and selected target proteins through ubiquitination.

CARD Domain: The caspase recruitment domain (CARD) is another conserved domain that is found only in cIAP1 and cIAP2. The structure and function of CARD in these molecules has not been determined. However, given the conserved nature of this domain, it is highly likely that in IAPs the CARD forms a six-helix bundle, which serves as a protein-protein interaction motif (Park et al., 2007).

Non-apoptotic functions: Keeping up to their name as inhibitors of apoptosis, IAPs are the key regulators of programmed cell death occurring in various physiological and pathophysiological processes including cancers, neurological disorders and ischemia-reperfusion diseases. However, the function of IAPs is not restricted to anti-apoptosis. There is a rapidly growing body of evidence that an important, if not a predominant, role of IAPs is the regulation of a diverse set of non-apoptotic signaling pathways, including those involved in cell cycle regulation, morphogenesis, MAP Kinase activation, NF-kB activation, innate immunity and even in heavy metal metabolism (Srivasula et al., 2008).

1.6.2 Mechanism of caspase inhibition by IAPs

The BIR domains of IAPs allow them to bind to and inhibit caspases, the proteases that orchestrate apoptosis, providing a direct explanation for how IAPs prevent apoptosis. Importantly, X linked IAP (XIAP), cIAP1 and cIAP2 are shown to directly inhibit specific caspases (Devereaux et al., 1997; Roy et al., 1997). This differs from the effects of Bcl-2-related apoptotic suppressors, which function at points that are distal in the death pathway, upstream of the apoptosome, to prevent integration of the caspase-activating signal.

Structure–function analysis of XIAP showed two distinct domains in XIAP that can suppress caspase activity (Devereaux et al., 1999). The BIR3 domain of XIAP binds directly to the small (carboxy-terminal) subunit of caspase-9 (Srinivasula et al., 2001). The cleavage of caspase-9 is not required for its activation (Stennicke et al., 1999), yet, paradoxically, cleavage seems to be required for the inactivation of caspase-9 by XIAP. The small subunit of caspase-9 is generated by means of proteolytic cleavage at a conserved Asp residue at position 315 — an event that exposes a segment, starting at residue 316, that is recognized by the BIR3 domain of XIAP (Srinivasula et al., 2001).

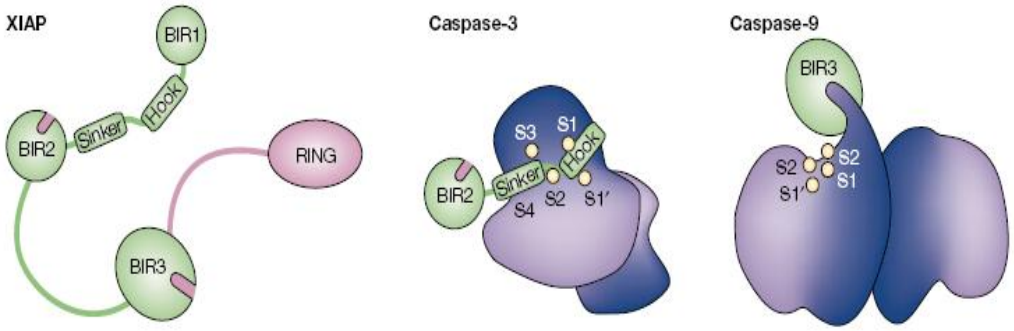


Fig 1.4 Interaction of IAP with Caspase-3 and -9: The order of XIAP domains starts with BIR1 at the amino terminus and terminates with the RING domain at the carboxyl terminus. The caspases are shown in their dimeric structure, with large subunits in blue-grey, small subunits in blue, and active-site substrate pockets (S-designation) as yellow dots. The catalytic machinery is between the S1 and S1′ pockets (Salvesen and Duckett, 2002).

The mechanism by which XIAP binds to caspase-3 and caspase-7 is entirely different to the manner in which it binds caspase-9. The crystallographic resolution of XIAP with caspase-3 and caspase-7 shows that the domain in XIAP that is essential for interaction lies in a small segment, which is immediately amino-terminal to BIR2 (Huang et al., 2001; Riedl et al., 2001). This domain functions by reversible, high-affinity binding to caspase-3 and caspase-7, and results in the steric occlusion of normal substrates of these caspases. The crucial sequence in XIAP binds the caspase in the opposite orientation to a caspase substrate, and has little requirement for the substrate-binding residues in the caspase. So, this important inhibitory domain in XIAP binds with high affinity to a surface that is conserved between caspase-3 and caspase-7, but XIAP is not a pseudosubstrate and functions solely to mask the active site in the caspase (Chai et al., 2001).

1.6.3 Regulation of IAPs

Regulation of IAPs is necessary for the occurrence of controlled apoptosis which is crucial for modulation of cell number and elimination of damaged cells. IAPs are unstable proteins with very short half lives (Hu and Yang, 2003), explaining the existence of efficient regulatory mechanisms that control their abundance. They are known to be regulated at the transcriptional, post-transcriptional and post-translational levels by auto-ubiquitination and control of IAP activity by regulatory proteins.

Transcriptional and post-transcriptional control: Expression levels of certain IAPs are subject to tight transcriptional control. For example, expression of survivin is regulated in a cell-cycle-dependent manner, and seems to be induced in normal cells at the G2–M boundary (Li et al., 1998). In many situations, the activation of NF-κB has been shown to exert a pro-survival effect, and this has been proposed to be mediated by the transcriptional activation of one or more genes that encode anti-apoptotic proteins including cIAP2 (Chu et al., 1997) and XIAP (Stehlik et al., 1998). Also, transcriptional upregulation of cIAP2, but not cIAP1 or XIAP was shown under severe hypoxia (Dong et al., 2001).

Post-translational control: The highly conserved RING domain at the carboxy termini of several mammalian IAPs has a key role in the targeted degradation of proteins by the ubiquitin–proteasome system. This process involves the sequential covalent addition of ubiquitin, a 76-residue protein; onto specific lysine residues on the target protein (Weissman et al., 2001). The RING domains of XIAP, cIAP1 and cIAP2 show ubiquitin ligase enzyme (or E3) activity. Targets of IAP-mediated ubiquitination include caspases (Huang et al., 2000; Suzuki et al., 2001), Smac/DIABLO (McFarlane et al., 2002; Hu et al., 2003) and TRAFs (Li et al., 2002). Importantly, IAPs themselves are controlled by auto-ubiquitination leading to degradation (Yang et al., 2000). Mammalian IAPs have been reported to form homodimers and heterodimers through their RING domains (Silke et al., 2005), which might lead to their auto-ubiquitination in trans. The RING domain of IAPs is also found to be required for IAP antagonists to induce their degradation (Zachariou et al., 2003).

IAP regulatory proteins: A well studied IAP-interacting molecule is the mitochondrial protein Smac/DIABLO (Du et al., 2002; Verhagen et al., 2000). Smac/DIABLO is a 239-residue protein that is translocated from the inter-membrane space of mitochondria to the cytosol, apparently along with Cyt C, in response to an apoptotic stimulus. On its release from mitochondria, mature Smac/DIABLO binds XIAP, and probably several other IAPs, in a manner that displaces caspases from XIAP. So, Smac/DIABLO is a negative regulator of IAP, and is therefore an apoptosis-enhancing molecule.

Smac/DIABLO and several other IAP regulatory proteins like OMI/HTRA2 (Martins et al., 2001; Loo et al., 2002) have been found to bind to IAPs through a conserved sequence, known as an IAP-binding motif or IBM (Srinivasula et al., 2002) (Fig 1.5).

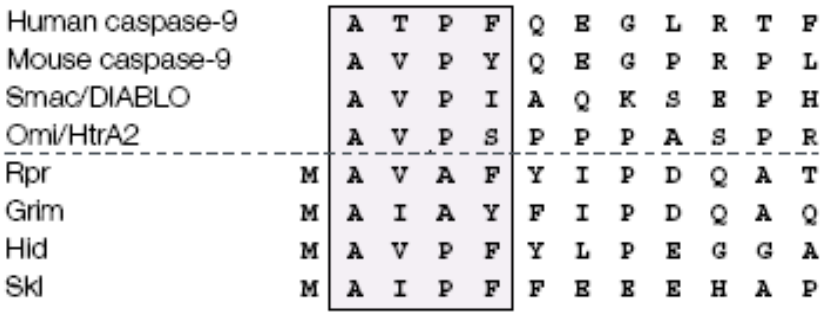


Fig 1.5 IAP binding motifs (IBMs): Caspases and many mammallian & insect IAP-regulatory proteins contain a conserved tetrapeptide sequence that interacts with the BIR motifs of IAPs. Also known as RHG (Reaper-Hid-Grim) motif.

1.7 Postconditioning

Deleterious manifestations initiated by reperfusion *per se* are proven to be efficiently abrogated by hypoxic pre and postconditioning (Zhao ZQ et al., 2002). Events like rapid generation of ROS, activation of sodium hydrogen exchanger, Ca^{2+} overload and mPTP opening, which are initiated in the first minutes of reperfusion, can also trigger later events, such as increased capillary permeability, no-reflow, necrosis and apoptosis. The more proximal the position of the above events in the time course of reperfusion injury, the greater likelihood that they are effective targets for reperfusion therapy, since they tend to attenuate downstream responses as well. As stated by Prof. Dr. H. M. Piper “What comes first must be treated first, as otherwise the opportunity for specific treatment is lost” (Piper et al., 2004).

Postconditioning is controlled reperfusion (Buckberg 1986), defined as a series of brief interruptions of ischemia/hypoxia applied at the very onset of reperfusion. The concept of postconditioning was first revealed in 2002 by Vinten-Johansen and colleagues. The first studies published by Zhao et al., (2002) and Halkos et al., (2004) from this group compared postconditioning to the gold standard cardioprotective strategy of ischemic preconditioning. Using a canine model of one hour of left anterior descending (LAD) coronary artery occlusion and three hours of reperfusion, one group was assigned to abrupt coronary artery reflow while the other

was assigned to postconditioning using an algorithm of 30 seconds LAD reperfusion and 30 seconds re-occlusion, repeated for three cycles. Full reflow was continued for the remainder of the three hours. A third group of canines was preconditioned by a single 5 minute LAD occlusion followed by 10 minutes of reperfusion prior to the prolonged ischemic period. As expected, ischemic preconditioning reduced infarct size by approximately 40% compared to controls and surprisingly, the infarct size observed with the postconditioning algorithm was comparable to that observed with ischemic preconditioning. The infarct size reduction was confirmed by plasma creatine kinase activity at the end of reperfusion. Neutrophil accumulation (tissue myeloperoxidase (MPO) activity) in the area at risk in both the ischemic preconditioning and the postconditioning groups was significantly reduced compared to untreated controls. Postconditioning also preserved post-ischemic coronary artery endothelial function (vasorelaxation to endothelium-dependent stimulators of nitric oxide synthase) comparable to that observed with preconditioning. The surface expression of P-selectin on coronary artery vascular endothelium was comparably attenuated with both preconditioning and postconditioning, suggesting a reduction in the pro-inflammatory state of the coronary artery endothelium. Furthermore, superoxide radical generation by the endothelium of the postischemic LAD was less in postconditioned groups than in controls subjected to abrupt coronary artery reperfusion. The comparable reduction in tissue edema observed in both preconditioning and postconditioning groups is commensurate with an attenuation of vascular endothelial activation and dysfunction. It was also observed that oxidant generation by the postischemic myocardium at the end of reperfusion, measured by dihydroethidium fluorescence, was reduced with postconditioning in both parenchyma and vascular/perivascular tissue, suggesting a reduction in the oxidant burden in reperfused tissue (Zhao et al., 2002). The plasma lipid peroxidation product malondialdehyde was accordingly reduced comparably in preconditioned and postconditioned groups, consistent with reduced oxidant generation. Additionally, Halkos et al., (2004) also reported that postconditioning reduced the incidence of reperfusion arrhythmias, a finding that was confirmed by Galagudza et al., (2004) in isolated perfused rat hearts.

1.7.1 Triggers and mediators of postconditioning

Adenosine: Endogenously released adenosine is involved in the cardioprotection of postconditioning. It has been shown that the release of endogenous adenosine into the buffer perfusate of isolated mouse hearts was delayed during postconditioning (Kin et al., 2004). Additionally, in a rat model of coronary artery occlusion-reperfusion, blockade of adenosine receptors with 8-sulfophenyl theophylline administered intravenously five minutes before reperfusion reversed the infarct reduction observed with postconditioning. This was also observed in an *in situ* rabbit model of coronary artery occlusion-reperfusion (Philipp et al., 2004). The cardioprotective actions of adenosine during postconditioning were linked to activation of the adenosine A2A and A3 receptors, but not the A1 receptor subtype (Kin et al., 2005). Interestingly, the physiological effects of adenosine reperfusion therapy essentially recapitulate those observed for postconditioning (Zhao et al., 1993; Jordan et al., 1997). Therefore, higher concentrations of endogenous adenosine may act as a trigger of cardioprotection, potentially through its interactions with adenosinergic G-protein coupled receptors to attenuate the release of oxidants and cytokines by activated endothelium and myocytes, in addition to its well-known inhibitory effects on neutrophils.

K_{ATP} channels: The adenosine triphosphate-sensitive potassium (K_{ATP}) channels are activated during postconditioning (Yang et al., 2004). Blockade of K_{ATP} channels with the nonselective inhibitor, glibenclamide, abrogated the infarct sparing effect of postconditioning. Further, the selective inhibitor of mitochondrial K_{ATP} channel activation, 5-hydroxydecanoate, also reversed the infarct sparing effect of postconditioning, suggesting that protection involved specific activation of the mitochondrial K_{ATP} channels. However, it was not shown whether the sarcolemmal K_{ATP} channel had any role, parallel or synergistic.

Nitric oxide: Nitric oxide synthase (NOS) is reported to be involved in the protective effect of postconditioning (Yang et al., 2003). Blocking NOS with L-NAME administered just before reperfusion alone had no effect on infarct size, but in conjunction with postconditioning, L-NAME completely inhibited the infarct-sparing effects of postconditioning. The involvement of the endothelial isoform (e-NOS) is

supported by a reported increase in phospho-eNOS seven minutes after reperfusion with postconditioning compared to abrupt reperfusion in isolated perfused rat hearts (Tsang et al., 2004). In addition, postconditioning could be blocked by L-NAME and the guanylyl-cyclase inhibitor 1H-[1,2,4]oxadiazolo- [4,3-a]quinoxaline-1-one (ODQ), suggesting an NO – cGMP pathway (Pagliaro et al., 2004). NOS may be involved at several levels in ischemia/reperfusion. The release of NO by coronary vascular endothelium is impaired after ischemia/reperfusion (Guo et al., 1996; Ma et al., 1993). Although not shown directly, postconditioning may reverse the NO release, speculated by attenuated P-selectin expression, decreased neutrophil adherence, and improved vasodilator responses to acetylcholine observed after postconditioning in canine models (Halkos et al., 2004; Zhao et al., 2003), all of which are physiological responses associated with increased NO generation. eNOS is also a downstream target of PI3 kinase – Akt pathway and other reperfusion injury kinase pathways (Hausenloy et al., 2004), which were shown to be involved in postconditioning.

mPTP: The mitochondrial permeability transition pore is one of the most important mediator of cardioprotection elicited by postconditioning. This selectively permeable pore remains closed during ischemia, but opens during the first few minutes of reperfusion (Griffiths et al., 1995; Halestrap et al., 1998). Opening of the mPTP has been associated with onset of cell death by both necrosis and apoptosis. Accordingly, inhibition of mPTP opening has been shown to be cardioprotective (Hausenloy et al., 2003). Postconditioning does inhibit opening of the mPTP (Argaud et al., 2005). This would be consistent with the involvement of NO, which inhibits mPTP opening (Piantadosi et al., 2002), reduced oxidant burden and reduced intracellular and mitochondrial Ca^{2+} (Sun et al., 2005). Taken together, inhibition of mPTP opening, generation of NO, reduction of ROS and Ca^{2+} and activation of several possible signaling molecules are major contributors of the cardioprotection mediated by postconditioning.

1.7.2. Signaling pathways in postconditioning

A number of signal transduction pathways have been shown to be recruited by postconditioning, which mostly converge at the mitochondria and are pivotal to the cardioprotection elicited.

Akt (Tsang et al., 2004) and ERK 1/2 (Yang et al., 2003) pathways are the first among these, providing the first line of evidence that postconditioning is capable of recruiting pro-survival signal transduction cascades. In these studies, PD98059 and LY294002 / Wortmannin, inhibitors of ERK1/2 and Akt pathway respectively, were shown to reduce the infarct sparing effect of postconditioning. Akt and ERK1/2 pathways together were subsequently named as RISK or reperfusion-induced survival kinase pathway (Hausenloy et al., 2004). Although the actual mechanism through which the RISK pathway is recruited by postconditioning is unresolved, experimental data suggest the activation of cell-surface receptors, including the adenosine A2 receptor (Morrison et al., 2007). The down stream targets of RISK-mediated protection are clearer and converge on the mitochondria. The phosphorylation and thus inhibition of GSK-3β, known to elicit cardioprotective effects via inhibition of mPTP opening (Juhaszova et al., 2004), is reported to occur in postconditioning (Gomez et al., 2008).

The roles of p38 MAPK and the JNK pathway in postconditioning have been much debated (Hausenloy et al., 2006; Bassi et al., 2008). One study shows the inhibition of JNK and p38 MAPK in postconditioning (Sun et al., 2006), indicating that activation of these pathways is detrimental to cardioprotection. Other signal transduction pathways involved in postconditioning include the JAK-STAT pathway (Boengler et al., 2008; Goodman et al., 2008), sphingosine kinase 1 (Jin et al., 2008) and protein kinase C (Penna et al., 2006). Sphingosine kinase 1 was infact demonstrated to have an obligatory role in postconditioning, which is potentially upstream to the RISK pathway (Jin et al., 2008)

Taken together, postconditioning is not only as powerful as preconditioning, but infact seems to be a comparatively better strategy, since it has the potential of being clinically applicable in the most common situation of unexpected coronary occlusion and acute myocardial infarction. The clinical usage of the maneuver gained further acclaim since the application of postconditioning to human heart following acute myocardial infarction (Laskey et al., 2005).

1.8 Aims and objectives of the project

The present study aims to examine the effect of postconditioning on hypoxia-reoxygenation induced endothelial apoptosis. Coronary endothelial apoptosis, preceding myocyte apoptosis, is a critical event in reperfusion injury.

Postconditioning, a strategy known to effectively reduce reperfusion injury, is well proven in cardiomyocytes, However, little is known about postconditioning in endothelial cells and whether it plays a role in anti-apoptosis. The project aims to study the anti-apoptotic effect of postconditioning in endothelial cells and the molecular mechanisms involved, focusing on the inhibitors of apoptosis proteins (IAPs) as potential antiapoptotic candidates induced by hypoxia. More specifically, the following questions were addressed;

- What is the effect of postconditioning on hypoxia/reoxygenation-induced endothelial apoptosis?
- What are the anti-apoptotic proteins involved?
- What is their mechanism of action?
- Which signal transduction pathways might be recruited?

The study was performed using cultured human umbilical vein endothelial cells (HUVEC), that were subjected to hypoxia-reoxygenation and/or postconditioning. Apoptosis was measured by Annexin V staining in flow cytometry and caspase-3 cleavage in Western blotting. Downregulation with siRNA and pharmacalogical inhibition were employed to determine the molecular and signaling mediators involved. Co-immunoprecipitation and co-localization studies were performed to analyze the interaction of proteins. An intact vessel model of rat aorta was established to demonstrate the physiological relevance of the molecules identified to be involved in the anti-apoptotic effect of endothelial postconditioning.

2. Materials

2.1 Chemicals and reagents

All chemicals used were of the highest analytical purity and best quality available.

Acrylamide	Roth, Karlsruhe
Agarose	Invitrogen, Paisley, United Kingdom
6-Aminohexanoic acid	Merck-Schuchardt, Hohenbrunn
APS	Serva, Heidelberg
Benzonase	Merck, Darmstadt
Bisacrylamide	Roth, Karlsruhe
Bromphenol blue	Sigma, Deisenhofen
BSA	Sigma, Deisenhofen
Calcium chloride	Merck, Darmstadt
Collagenase	PAA Laboratories, Pasching, Austria
CWFSG	Sigma, Deisenhofen
Di-Sodium hydrogen phosphate	Roth, Karlsruhe
DMSO	Stigma, Deisenhofen
DTT	Stigma, Deisenhofen
Dynabeads protein G	Invitrogen, Karlsruhe
EC Oxyrase	Oxyrase, Mansfield, USA
EDTA-Sodium chloride	Sigma, Deisenhofen
Endothelial growth medium kit	PromoCell, Heidelberg
Ethanol	Riedel de Haën, Seelze
FCS	PAA Laboratories, Pasching, Austria
Filter paper	Biotec-Fischer, Reiskirchen
Glucose	Merck, Darmstadt
Glycerine	Roth, Karlsruhe
HBSS	PAA Laboratories, Pasching, Austria
HEPES	Roth, Karlsruhe
High molecular weight standard	Sigma, Deisenhofen
Isoflurane	Baxter, Unterschleißheim
Low molecular weight standard	Sigma, Deisenhofen
Magnesium chloride	Fluka, Neu-Ulm

Magnesium sulfate	Merck, Darmstadt
Methanol	Riedel de Haën, Seelze
Mercaptoethanol	Merck-Suchard, Hohenbrunn
NCS	PAA Laboratories, Pasching, Austria
Nitrocellulose membrane	Schleicher und Schuell, Dassel
Page Ruler	Fermentas, St.Leon-Rot
Paraformaldehyde	Merck, Darmstadt
Penicillin-streptomycine	Gibco BRL, Eggenstein
Ponceau S solution	Serva, Heidelberg
Potassium chloride	Merck, Darmstadt
Potassium dihydrogen phosphate	Merck, Darmstadt
Protease inhibitor cocktail Complete	Roche Diagnostics, Mannheim
Protein blockt serum-free	Dako, Hamburg
Skimmed milk powder	Applichem, Darmstadt
Sodium bicarbonate	Merck, Darmstadt
Sodium chloride	Roth, Karlsruhe
Sodium hydroxide	Roth, Karlsruhe
Sodium dodecyl sulphate	Merck, Darmstadt
Sodium flouride	Merck, Darmstadt
Super Signal-West	Pierce, Niedderau
TEMED	Sigma, Deisenhofen
Tissue Tek	Sakura Finetek, Staufen
Tris	Roth, Darmstadt
Triton X 100	Serva, Heidelberg
Trypsin-EDTA solution	Biochrom AG, Berlin
Tween 20	Amersham, Braunschweig

2.2 Pharmacalogical inhibitors

LY294002	Calbiochem, Bad Soden
PD98059	Calbiochem, Bad Soden
SB203580	Calbiochem, Bad Soden
SP600125	Sigma, Steinheim
UO126	Calbiochem, Bad Soden

2.3 Antibodies

Primary antibodies:

Anti-Actin (mouse IgG)	Sigma, Deisenhofen
Anti-Akt~p (rabbit IgG)	Cell Signaling Technology, USA
Anti-cIAP1 (goat IgG)	Santa Cruz biotechnology, Heidelberg
Anti-cIAP2 (rabbit IgG)	Santa Cruz biotechnology, Heidelberg
Anti-c-jun~p (rabbit IgG)	Cell Signaling Technology, USA
Anti-cleaved Caspase-3 (rabbit IgG)	Cell Signaling Technology, USA
Anti-ERK1/2~p (mouse IgG)	Cell Signaling Technology, USA
Anti-p38 MAPK~p (mouse IgG)	Cell Signaling Technology, USA
Anti-procaspase-3 (mouse IgG)	Imgenex, Darmstadt
Anti-vinculin (mouse IgG)	Sigma, Steinheim
Anti-von Willebrand Factor (rabbit IgG)	Dako, Hamburg
Anti-XIAP (rabbit IgG)	Cell Signaling Technology, USA

Secondary antibodies:

Anti-goat IgG HRP-conjugated	Dianova, Hamburg
Anti-mouse IgG HRP-conjugated	Amersham, Freiburg
Anti-rabbit IgG HRP-conjugated	Amersham, Freiburg
Anti-mouse IgG Alexa 633-conjugated	Invitrogen, Karlsruhe
Anti-rabbit IgG Alexa 488-conjugated	Invitrogen, Karlsruhe
Anti-rabbit IgG Alexa 546-conjugated	Invitrogen, Karlsruhe

2.4 SiRNA transfection

cIAP2 siRNA	Santa Cruz biotechnology, Heidelberg
Control siRNA	Eurogentec, Cologne
JetSI Endo	Eurogentec, Cologne
Opti-MEM	Invitrogen, Karlsruhe

2.5 Flow cytometry

AnnexinV/PI-FITC kit	BD Pharmingen, Heidelberg
FACS Clean	BD Pharmingen, Heidelberg
FACS Flow	BD Pharmingen, Heidelberg
FACS Rinse	BD Pharmingen, Heidelberg
FACS tubes	BD Biosciences, Heidelberg

2.5 Laboratory instruments

CCD camera	Bio Rad, Hercules, USA
Culture dishes	Becton-Dickinson, Heidelberg
Demineralisation unit	Millipore, Eschborn
Electroblot chamber	Biotec-Fischer, Reiskirchen
Electrophoresis chamber	Biotec-Fischer, Reiskirchen
Electrophoresis apparatus	Biometra, Göttingen
FACS Calibur flowcytometer®	BD Biosciences, Heidelberg
Glass articles	Schott, Mainz
Glass coverslips	Menzel, Braunschweig
Hamilton syringe	Hamilton, Bonaduz, Switzerland
Incubator	Heraeus,Hanau
LSM-510 confocal microscope	Carl-Zeiss, Heidelberg
Magnetic rack	Dynalbiotech ASA Oslo, Norway
Magnet stirrer	Jahnke und Kunkel, Staufen
Microscope	Olympus, Japan
Neubauer-chamber	Superior, Marienfeld
pH-Meter	WTW-Weilheim
Pipettes	Eppendorf Netheler-Hinz, Hamburg
Pipette tips	Eppendorf Netheler-Hinz, Hamburg
Power supply	Biometra, Göttingen
Shaker	Biometra, Göttingen
Sterile bench	Heraeus, Hanau
Sterile filter (0.2 µm)	Sartorius, Göttingen
Sterile pipettes	Becton-Dickinson, Heidelberg

Table centrifuge	Hereaus, Hanau
Tubes	Eppendorf-Netheler-Hinz, Hamburg
Vortex	Heidolph, Kehlheim
Water bath	Julabo, Seelbach

2.6 Software

Cell-Quest-Pro	BD Biosciences, Heidelberg
LSM 510	Carl-Zeiss, Heidelberg
Quantity One analysis software	Bio Rad, Hercules, USA

3. Methods

3.1 Preparation of human umbilical vein endothelial cells

Collagenase solution:	
HBSS	x ml
Collagenase II (293 Units/mg) (wt/vol)	0.025%
$MgCl_2$. 6 H_2O	0.5 mM
$CaCl_2$	1.5 mM

Cell culture medium:	
Endothelial cell basal medium	x ml
FCS (vol/vol)	10%
penicillin/streptomycin (vol/vol)	2%
ECGS/Heparin (wt/vol)	0.4%
Hydrocortisone (wt/vol)	0.1%
Basic fibroblast factor	1 ng/ml
Epidermal growth factor	0.1 ng/ml

Protocol: The procedure conforms to the principles outlined in the ‘’Declaration of Helsinki’’ (Cardiovascular Research 1997; 35:2–3). Human umbilical vein endothelial cells (HUVEC) were isolated from freshly collected umbilical cords according to Jaffe et al., (1973) with minor modifications. After cleaning, the untraumatized umbilical vein was canulated and perfused with HBSS to remove traces of blood. The lumen of the vein was then filled with collagenase solution and incubated for 20 min at 37° C. After incubation, the collagenase solution containing endothelial cells was removed by perfusing the vein with 30 ml of HBSS containing 3% (vol/vol) FCS, added to inactivate collagenase. The effluent was collected in a 50 ml falcon tube and centrifuged for 5 min at 250 x g at RT. The supernatant was discarded and the cell pellet was resuspended in culture medium containing 0.1% (vol/vol) penicillin/streptomycin. Thereafter, cells were seeded onto 3-4 primary culture dishes. After incubation for 2 h at 37° C and 5% CO_2 cells were extensively washed with HBSS to remove the unattached non-endothelial cells and cell debris. Adherent cells were incubated in 15-20 ml of cell culture medium containing 2% (vol/vol)

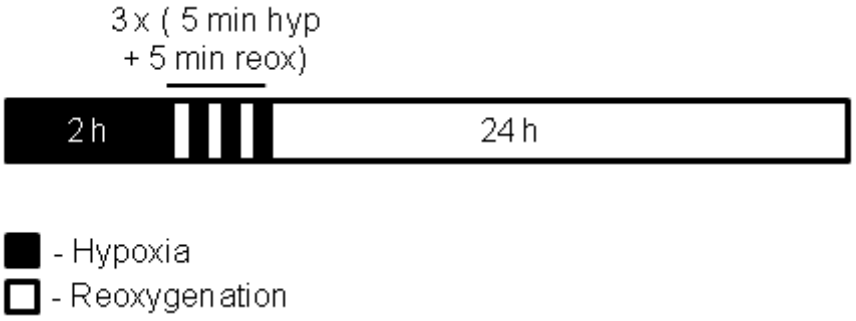
penicillin/streptomycin at 37° C and 5% CO_2. After 24 h the medium was replaced with fresh cell culture medium.

3.2 Subcultivation of endothelial cells

Confluent monolayers of primary endothelial cell cultures were trypsinized 5 to 7 days after isolation. Cells were washed with HBSS and subsequently incubated with 3 ml of trypsin/EDTA solution (composition in mM: 137 NaCl, 2.7 KCl, 1.5 KH_2PO_4, 8.0 Na_2HPO_4, pH 7.4, 0.05% (wt/vol) trypsin and 0.02% (wt/vol) EDTA). Trypsinized cells were collected into cell culture medium and seeded at a density of 5.5 $x10^4$ cells/cm^2 on 35 mm^2 or 60 mm^2 dishes, according to the experiment being performed. For immunostaining and confocal microscopy cells were seeded on 25 mm^2 glass cover slips. Experiments were performed with confluent endothelial monolayers of either primary or passage 1, 3-4 days after seeding.

3.3 Experimental protocol for hypoxia/reoxygenation and postconditioning

Subconfluent monolayers of HUVEC were subjected to severe hypoxia (Po_2 < 1 mmHg) for 2 h followed by 24 h of reoxygenation. Hypoxia was applied with 1U/ml EC Oxyrase, a biocatalyst capable of consuming molecular oxygen in the presence of an available hydrogen donor (Jacobson et al., 1987). Following the hypoxic phase, postconditioning was applied at the onset of reoxygenation by the intermittent application of three short periods of severe hypoxia, 5 min each, separated by a 5 min reoxygenation period (see below). For this, endothelial monolayers were incubated with regular growth medium containing 1U/ml EC Oxyrase, thrice for 5 min, interrupted by 3 periods of incubation with Oxyrase-free medium, 5 min each. Oxyrase-free medium was used during all the 6 incubation periods for control samples, to nullify the effects of medium change.



3.4 siRNA transfection of endothelial cells

Downregulation of cIAP2 was achieved by transfecting endothelial cells with cIAP2 specific siRNA duplexes ordered from Santa Cruz Biotechnolgy. The calculations given below are for subconfluent HUVEC monolayers seeded on 30 mm^2 dishes.

Solution A: X (X = siRNA in ng x 0.003) µl of JetSI-ENDO in 100µl Opti-MEM
Solution B: 2.5µl (25 nM) of siRNA in 100µl Opti-MEM

Protocol: 24 h prior to the application of hypoxia/reoxygenation or postconditioning, 70 - 80 % confluent cell monolayers were transfected with cIAP2 specific siRNA for 5.5 h according to the manufacturer's instructions. For this, cells were washed and incubated in a low-serum transfection medium, Opti-MEM, since serum hinders the uptake of siRNA by the cells. Solution A containing the transfection reagent, Jet SI-ENDO is then added to solution B containing siRNA by gentle vortexing. The mixture was incubated for 30 min to allow formation of complexes and is then gently added to the cells drop-wise. After 5.5 h of incubation, the low-serum transfection medium is replaced with regular medium allowing normal growth for 16-18 h. Control samples were treated with the same concentration of non-specific control siRNA following the same protocol.

3.5 Application of pharmacological inhibitors

PI3 kinase inhibitor	LY 294002	10 µM
ERK 1/2 inhibitor	PD 98059	20 µM
ERK 1/2 inhibitor	UO 0126	10 µM
p38 MAPK inhibitor	SB 203580	10 µM
JNK inhibitor	SP600125	10 µM

Stock solutions were prepared immediately before use in basal medium with DMSO. Appropriate volumes of these solutions were added to the cells yielding a final solvent concentration of ≤ 0.1% (vol/vol), 30 min prior to the experiment. The same final concentrations of DMSO were included in all respective control experiments.

3.6 Fluorescence-activated cell sorting (FACS) analysis

Effect of hypoxia/reoxygenation and postconditioning on endothelial cell apoptosis was determined by FACS analysis of annexin V-FITC and propidium iodide staining according to the manufacturer's instructions. Annexin V and PI were added to trypsinized and suspended endothelial cells which were analyzed by flow cytometry on a FACS Calibur using Cell Quest Pro software. Cells that were annexin-FITC positive were identified as apoptotic, while cells that were PI positive and Annexin-FITC negative were categorized as necrotic. Non-stained cells were identified as vital.

3.7 Protein analysis

3.7.1 Preparation of Samples

Lysis buffer:

Tris/HCl pH 6.8	250 mM
Glycerol	20 % (vol/vol)
SDS	4 % (wt/vol)
β-mercaptoethanol	1 % (vol/vol)
Bromphenol blue	0.001 % (wt/vol)
DTT	10 mM (added freshly before use)
Benzonase®	50 IU/ml (added freshly before use)
$MgCl_2$	2 mM (added freshly before use)

Protocol: Endothelial cells were washed with HBSS and subsequently lysed in 150 µl of preheated 2X SDS lysis buffer. Subsequently, 50 IU/ml Benzonase® and 2 mM $MgCl_2$ were added and lysate was collected in a 1.5 ml Eppendorf tube. Samples were denatured for 3 minutes at 95 °C and used immediately or stored at –20 °C.

3.7.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Resolving gel buffer:	Tris/HCl; pH 8.8 120 mM
Stacking gel buffer:	Tris/HCl; pH 6.8 120 mM

10X Gel running buffer:

Tris	250 mM
Glycine	2.0 M
SDS	10 % (wt/vol)

SDS gels:

	Resolving gels		Stacking gel
Percentage	12.5 %	15 %	6 %
Acrylamide	12.7 ml	15.3 ml	3.8 ml
Bisacrylamide	7.0 ml	8.4 ml	2 ml
Millipore water	9.8 ml	5.8 ml	17.5 ml
Resloving gel buffer	9.5 ml	9.5 ml	--------
Stacking gel buffer	--------	--------	6.0 ml
SDS 10% (wt/vol)	0.4 ml	0.4 ml	0.25 ml
TEMED	30 µl	30 µl	20 µl
APS 10% (wt/vol)	0.4 ml	0.4 ml	0.25 ml

Protocol: After cleaning the glass plates and spacers with water and ethanol, the gel apparatus was assembled and the resolving gel solution was poured (~ 10 cm height), and layered with water. The gel was let to polymerize for 3-4 h or overnight at room temperature. The layer of water was removed and the stacking gel solution was poured on top of the resolving gel, followed by the insertion of a comb. The stacking gel was let to polymerize for 1 h at room temperature. After removing the comb, 1X running gel buffer was added to the chamber and the wells were washed with a syringe. Protein samples were loaded into the wells and the gel was run overnight at 45 volts. The run was stopped when bromophenol blue had passed through the gel.

3.7.3 Western blotting

Proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane by semi-dry blotting. Afterwards proteins of interest were immunodetected using specific antibodies.

Solutions and materials:

- Nitrocellulose transfer membrane, cut to the dimensions of the gel
- Six pieces of Whatman® 3 MM filter paper, cut to the dimensions of the gel
- Blotting chamber
- Anode buffer 1: 0.3 M Tris/HCl; pH 10.4, 20% (vol/vol) methanol
- Anode buffer 2: 30 mM Tris/HCl; pH 10.4, 20% (vol/vol) methanol
- Cathode buffer: 25 mM Tris/HCl; 40 mM 6-amino-n-hexanoic acid; pH 9.4, 20% (vol/vol) methanol

Protocol: The blotting chamber was assembled as follows: Two sheets of filter paper (Whatman® 3MM) soaked in anode buffer 1, were placed onto the centre of the graphite anode of the blotting chamber. On top of these sheets, two sheets of filter paper, soaked in anode buffer 2, were placed followed by nitrocellulose membrane equilibrated in anode buffer 2 for 10-15 min. After briefly equilibrating with cathode buffer, the SDS-gel (devoid of stacking gel) was layered on top of the nitrocellulose membrane, avoiding air bubbles. Two sheets of filter paper, pre-soaked in cathode buffer, were then placed on top of the gel followed by the graphite cathode of the blotting chamber. Transfer was achieved by application of 0.8-0.9 mA /cm^2 current for approximately 2- 2.5 h.

3.7.4 Staining of transferred proteins

To estimate the efficiency of protein transfer after blotting, the membrane was stained with ponceau S, a reversible stain that produces pink bands on a light background. The nitrocellulose membrane was washed with Millipore water for 1 min, incubated in Ponceau-S solution for 2-3 min with constant shaking at room temperature. Subsequently the membrane was destained by washing in Millipore water to the desired contrast and photographed. To remove the stain completely, the membrane was washed with TBST (1X TBS plus 0.1% Tween 20) under constant shaking.

3.7.5 Immunodetection of proteins

Solutions:
10X Tris-buffered saline (TBS): 100 mM Tris/HCl (pH 7.4), 1.6 M NaCl
TBS Tween (TBST): 1X TBS, 0.1% (vol/vol) Tween 20
Blocking-buffer and antibody-dilution buffer:
3% (wt/vol) BSA in 1X TBST or 5% (wt/vol) non-fat dried milk powder in 1X TBST

Primary Antibodies:

Antibody	Dilution	Dilution buffer
Anti-Actin (mouse IgG)	1:1000	BSA
Anti-cIAP1 (goat IgG)	1:500	BSA
Anti-cIAP2 (rabbit IgG)	1:2000	BSA
Anti-cleaved caspase-3 (rabbit IgG)	1:1000	BSA
Anti-phospho Akt (rabbit IgG)	1:1000	Milk
Anti-phospho c-jun (rabbit IgG)	1:1000	BSA
Anti-phospho ERK1/2 (mouse IgG)	1:2000	BSA
Anti-procaspase-3 (mouse IgG)	1:1000	BSA
Anti-vinculin (mouse IgG)	1:1000	BSA
Anti-XIAP (rabbit IgG)	1:1000	Milk

Secondary antibodies, horseradish peroxidase (HRP)-labeled:

Antibody	Dilution	Dilution buffer
Anti-rabbit IgG	1:2000	BSA or Milk
Anti-mouse IgG	1:2000	BSA or Milk
Anti-goat IgG	1:1000	BSA or Milk

Protocol: After a brief washing with Millipore water and TBST, the membranes were blocked with either 5% (wt/vol) non-fat milk powder or 3% (wt/vol) BSA in TBST for 2 h at room temperature. After blocking, the membranes were incubated with primary antibody overnight at 4 °C, followed by washing with TBST 3-4 times for 5-10 minutes each time at room temperature and incubated with secondary antibody for 1

h at room temperature. The membranes were then washed with TBST 3-4 times for 10-15 min each and incubated with enhanced chemiluminescence (ECL) solution (30 sec to 1 min) and the luminescence was detected and recorded with Bio-Rad Quantity One gel documentation system.

3.7.6 Stripping and reprobing

Stripping solution:	(50 ml)
Millipore H_2O	44 ml
1M Tris HCl (pH 6.8)	3.1 ml
10% SDS (wt/vol)	2.5 ml

To reprobe the membranes with antibodies against other proteins of the same or equal size, bound antibodies were removed by incubating the membranes with prewarmed (60°C) stripping buffer 2-5 min at RT under constant shaking. Subsequently membranes were washed extensively with TBST buffer, blocked and reprobed with appropriate antibodies.

3.8 Co-immunoprecipitation

To determine the binding of cIAP2 with procaspase-3 under conditions of hypoxia/reoxygenation and postconditioning, co-immunoprecipitation was performed with procaspase-3 coated G protein dynabeads.

Solutions and materials:

G protein coated magnetic beads:	6 µl beads suspention / ~ 1mg total cell lysate
Anti-procaspase-3 antibody:	3 µg / ~ 1 mg total cell lysate

Sodium phosphate buffer (0.1M) pH 7.4:

Na_2HPO_4	80 mM
NaH_2PO_4	20 mM

Phospahate Buffer Saline (PBS) pH 7.4:

NaCl	137 mM

KCl	2.7 mM
KH_2PO_4	1.5 mM
Na_2HPO_4	8.0 mM

Lysis Buffer (ice cold):

Tris/HCl (pH 7.5)	50 mM
NaCl	150 mM
Triton X-100	50 mM
NaF	1 mM
EDTA	1 mM
EGTA	150 mM
Na_3VO_4	0.5 mM
PMSF (vol/vol)	0.5%
NP-40 (vol/vol)	0.5%

1 tablet proteinase inhibitor Complete^{TM} per 10 ml buffer

Preparation of beads: Protein G-coated magnetic beads were washed 3-4 times with 0.1 M sodium phosphate buffer using a magnetic rack and incubated with anti-procaspase-3 antibody overnight at 4 °C with end-over-end rotation. The antibody coated beads were blocked with 6% (wt/vol) skimmed milk powder in 0.1 M sodium phosphate buffer and 0.1% (vol/vol) tween 20 for 1 h at room temperature. Afterwards the beads were washed 3-4 times with 0.1 M sodium phosphate buffer containing 0.1 % (vol/vol) Tween 20 and stored in 50 µl of 1X PBS.

Protocol: Confluent endothelial monolayers cultured on a 10 cm cell culture dish that were subjected to hypoxia/reoxygenation or postconditioning were incubated with 600 µl lysis buffer for 10 minutes on ice and subsequently harvested by scraping with a rubber policeman. Cells were further lysed in a douncer or using a 25 g needle and syringe. The lysate was centrifuged at 1000 X g for 2 min at 4 °C. The cleared supernatant was added to the antibody coated beads and incubated for 1.5 h at 4 °C with end-over-end rotation. Supernatant containing unbound protein was discarded and the beads were washed three times with PBS containing 0.1 % (vol/vol) Tween 20, followed by heating with 2X SDS lysis buffer for 5 min at 90 °C to detach the bound protein. The dissolved protein in buffer was analyzed by SDS-PAGE.

3.9 Immunofluorescence:

Solutions and materials:

Primary antibodies:

Antibody	Cell culture		Aorta sections	
	Dilution	Dilution buffer	Dilution	Dilution buffer
Anti-cIAP2 (rabbit IgG)	1:500	BSA+CWFSG	1:50	Protein block
Anti-procaspase-3 (mouse IgG)	1:500	BSA+CWFSG	1:50	Protein block
Anti-von Willebrand factor (rabbit IgG)	------	----------	1:50	Protein block

Secondary antibodies:

Antibody	Cell culture		Aorta sections	
	Dilution	Dilution buffer	Dilution	Dilution buffer
Anti-mouse Alexa fluor 488	1:500	BSA+CWFSG	-----	---------
Anti-rabbit Alexa fluor 633	1:500	BSA+CWFSG	-----	---------
Anti-rabbit Alexa fluor 546	------	----------	1:250	Protein block

Buffered glycerol:

Na_2CO_3 1.5 M

$NaHCO_3$ 1.5 M

Glycerol (water-free)

1.5 M $NaHCO_3$ solution is slightly heated and set to a pH of 8.6 using 1.5 M Na_2CO_3 solution. 1 portion of the above buffer is then mixed with 1 portion of glycerol.

Protocol: Endothelial monolayers on glass cover slips were washed with 1X PBS and fixed with 4 % (wt/vol) paraformaldehyde at 37 °C for 20 min, followed by permeabilization with 0.1 % (wt/vol) Triton X-100 at 37 °C for 20 min. Cells were then washed 3-4 times with 1X PBS and blocked with 5 % (wt/vol) bovine serum albumin + 5 % (wt/vol) cold water fish skin gelatin for 1 h to prevent non-specific binding. Tissue sections were blocked similarly with Protein block serum-free. Samples were then probed with primary antibody (1:50 dilution for tissue sections and 1:500 dilution for cells) overnight at 4 °C, followed by thorough washing in 1X PBS. Secondary antibodies tagged with Alexa fluor 488/ Alexa fluor 546/ Alexa fluor 633 were used in a dilution of 1: 250 – 1:500 for 1 h at room temperature. The samples were then washed 3-4 times with 1X PBS and mounted with buffered glycerol. Immunoreactivity was visualized and analyzed by confocal microscopy with Carl Zeiss LSM 510.

3.10 Intact vessel model

Zamboni fixative (pH 7.3):

Paraformaldehyde:	20 mg
Picric acid (saturated solution):	150 ml

The solution is heated to 60°C and alkalized with 2.5% (vol/vol) NaOH until the solution is clear and made upto 1 litre with phosphate buffer (80 mM NaH_2PO_4; 20 mM Na_2HPO_4).

Protocol: Freshly excised aortas were cleaned and rinsed with 1X PBS and immersed in a 2 ml Eppendorf tube containing PromoCell growth medium. Hypoxia was applied following the same protocol used for cell cultures, by flushing the medium with 1U/ml of EC oxyrase. Postconditioning was applied at the onset of reoxygenation after 2 h of hypoxia, while control vessels were normally reoxygenated with EC-oxyrase free medium. After the experiment, aortas were fixed with 1X Zamboni for 24 h and washed with 1X PBS until the colour of Zamboni is cleared, followed by over night washing. The aortas were thereafter incubated in 18% (wt/vol)

saccharose over night, embedded in Tissue Tec and were frozen in liquid nitrogen. 6 µm tissue sections were obtained at -20 °C on a Leica CM 3050S crystat and mounted with buffered glycerol onto frost free glass slides.

3.11 Statistical analysis

Data are given as means ± S.D. of 3-5 experiments using independent cell preparations. The comparison of means between groups was performed by oneway analysis of variance (ANOVA) followed by a Student-Newman-Keuls post-hoc test. Changes in parameters within the same group were assessed by multiple ANOVA analysis. Probability (P) values of less than 0.05 were considered significant ($P< 0.05$).

4. Results

4.1 Effect of postconditioning on hypoxia/reoxygenation-induced apoptosis in endothelial cells

To test whether postconditioning protects endothelial cells from hypoxia/reoxygenation-induced apoptosis, HUVEC were first induced to apoptosis by subjecting them to severe hypoxia (Po_2 < 1 mmHg) for 2 h followed by 24 h reoxygenation. Postconditioning was applied at the onset of reoxygenation and cell death was analysed by annexin V/ PI staining in FACS, 24 h after reoxygenation, in comparision to cells that were reoxygenated after hypoxia, without the application of postconditioning.

Hypoxia/reoxygenation led to an increase in apoptosis from 8.1± 0.9% to 19.2± 1.4% in cultured endothelial cells. Application of hypoxic postconditioning at the onset of reoxygenation reduced the percentage of annexin V positive cells (Fig. 4.1) to basal values. Correspondingly, the loss in percentage of vital cell population due to hypoxia/reoxygenation was revived by postconditioning (Fig. 4.1). No significant changes were observed in percentage of necrotic cells.

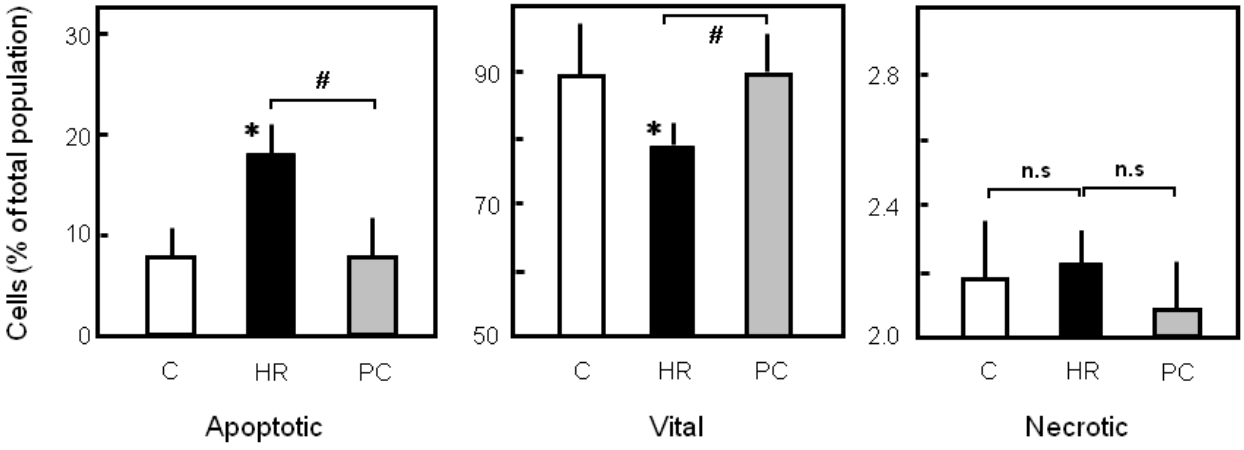


Fig. 4.1 Effect of hypoxia/reoxygenation and postconditioning on percentage of apoptotic cells: HUVEC were subjected to 2 h of severe hypoxia followed by 24 h of reoxygenation without (HR) and with (PC) postconditioning. Non-treated cells were taken as control (C). Distribution of vital, apoptotic and necrotic cells determined by FACS analysis. Data are means ± SD of 3 separate experiments with independent cell preparations. $^{*}P < 0.05$ vs C; $^{\#}P < 0.05$; n.s: not significantly different.

4.2 Effect of postconditioning on hypoxia/reoxygenation-induced cleavage of caspase-3

Cleavage of caspase-3, a central effector caspase of the apoptotic cascade, was measured to analyze the effect of postconditioning on hypoxia/reoxygenation-induced apoptosis in endothelial cells. For this, HUVEC were induced to apoptosis by subjecting them to 2 h severe hypoxia, followed by 24 h reoxygenation. Postconditioning was applied at the onset of reoxygenation and caspase-3 cleavage was measured at 6 and 24 h after reoxygenation in comparision to cells that were reoxygenated after hypoxia without the application of postconditioning.

As shown in fig. 4.2, severe hypoxia followed by 6 h reoxygenation caused a distinct 2.1± 0.3 fold increase in cleavage of caspase-3. This hypoxia/reoxygenation-induced caspase-3 cleavage was abolished by the application of hypoxic postconditioning. The changes in cleavage of caspase-3 in response to hypoxia/reoxygenation and postconditioning were less distinct when measured after 24 h of reoxygenation.

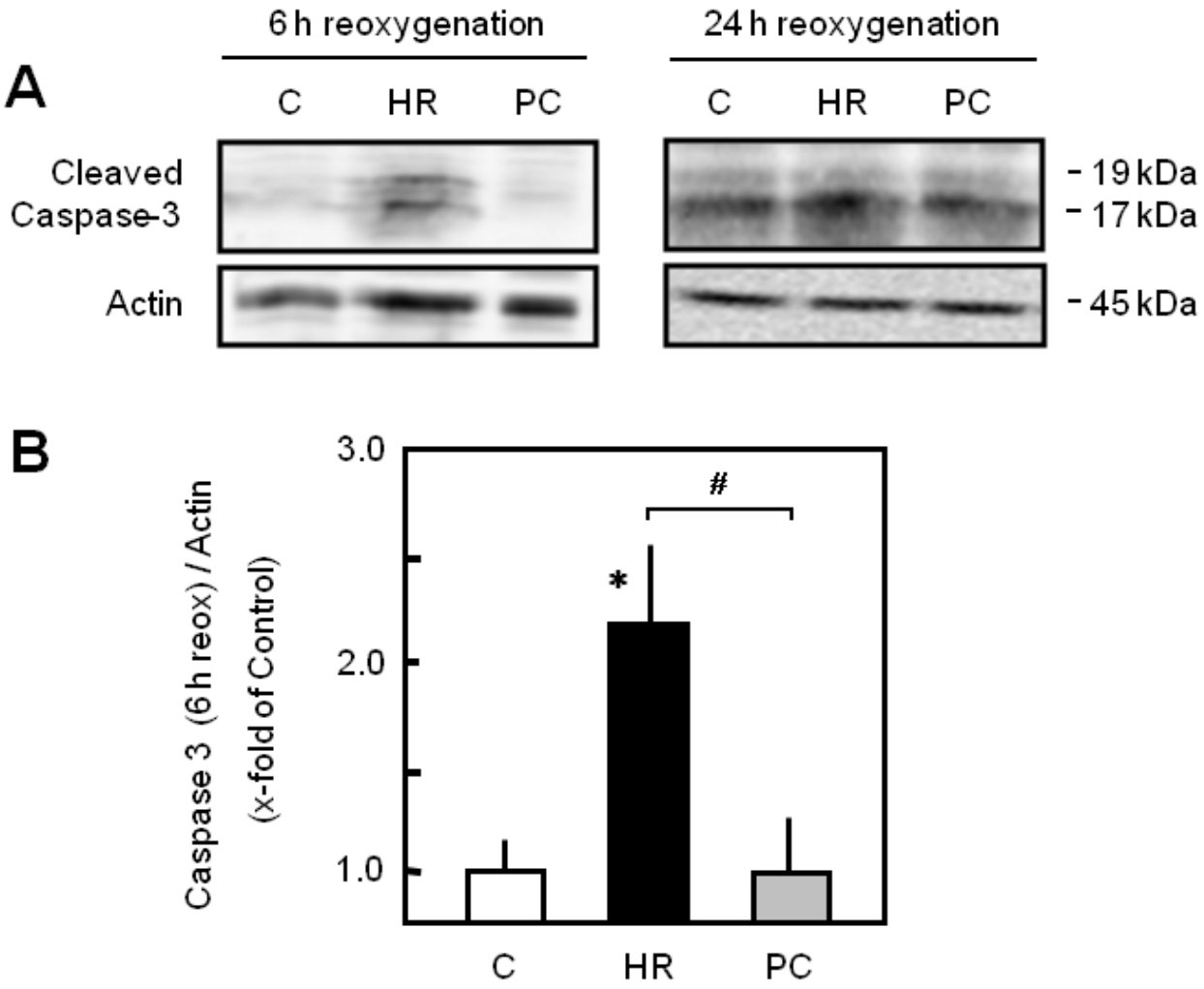


Fig. 4.2 Effect of hypoxia/reoxygenation and postconditioning on caspase-3 cleavage: HUVEC were subjected to 2 h of severe hypoxia followed by 6 h and 24 h of reoxygenation without (HR) and with (PC) postconditioning. Non-treated cells were taken as control (C). **(A)** Representative Western blot showing cleavage of caspase-3. Actin was taken as loading control. **(B)** Densitometric analysis of caspase-3 cleavage at 6 h reoxygenation. Cleaved caspase-3 relative to actin is given as x-fold of control. The ratio of control was set to 1. Data are means ± SD of 3 separate experiments with independent cell preparations. $^{*}P < 0.05$ vs. C; $^{\#}P < 0.05$.

4.3 Effect of postconditioning on inhibitor of apoptosis proteins, cIAP1, cIAP2 and XIAP

Inhibitors of apoptosis proteins, known to be potential caspase-3 inhibiting proteins, were screened in this study for their expression in postconditioning. For this, HUVEC were subjected to postconditioning, following 2 h of severe hypoxia. Cells were harvested for protein lysates, at the end of postconditioning or at the end of the corresponding time period (30 min reoxygenation) for cells that were reoxygenated without postconditioning.

Western blot analysis showed that, cIAP2 but not its close homologues, cIAP1 or XIAP, is upregulated by severe hypoxia and declines to basal levels with reoxygenation. As shown in fig. 4.3, a striking increase of 6.4 ± 0.3 fold in the protein level of cIAP2 was observed with 2 h of severe hypoxia compared to normoxia. The protein level reduced to basal values in cells that were reoxygenated for 30 minutes. On the other hand, cells that were subjected to postconditioning showed a 7.6 ± 0.7 fold increase in cIAP2 levels compared to normal hypoxia/reoxygenated cells that were taken as control. In contrast, the levels of cIAP1 and XIAP remained unchanged under corresponding conditions (Fig. 4.3).

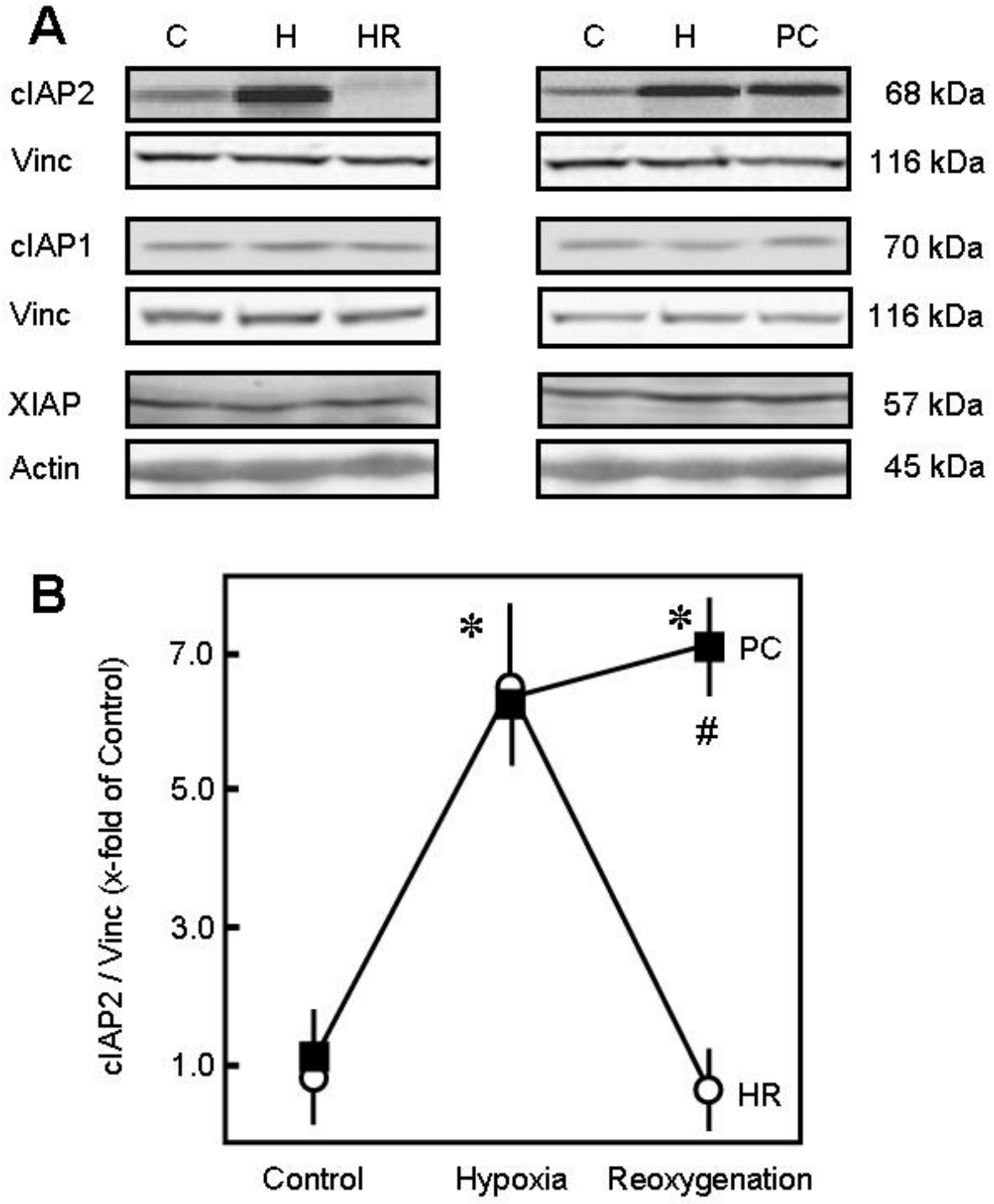


Fig. 4.3 Effect of hypoxia/reoxygenation and postconditioning on protein levels of cIAP1, cIAP2 and XIAP: **(A)** Representative Western blots showing cIAP2, cIAP1 and XIAP under 2 h severe hypoxia (H) followed by 30 min reoxygenation without (HR) and with postconditioning (PC). Actin or vinculin (Vinc) was used for loading control. **(B)** Densitometric analysis of cIAP2 expression. cIAP2 relative to vinculin is given as x-fold of control. The ratio of control was set to 1. Data are means ± SD of 4 separate experiments with independent cell preparations. *$P < 0.05$ vs. C; $^{\#}P < 0.05$ vs HR.

4.4 Effect of cIAP2 silencing on hypoxia/reoxygenation-induced apoptosis and postconditioning

To confirm the role of cIAP2 in endothelial postconditioning, the protein was downregulated by transfecting with specific siRNA 24 h prior to the experiment. The efficiency of downregulation was checked by Western blot analysis by measuring cIAP2 protein content in transfected cells. Cells transfected with non-specific siRNA were taken as control, to nullify the effects of transfection procedure. A reduction of 80 ± 12% in the protein levels of cIAP2 was achieved with cIAP2 siRNA in comparison to non-specific siRNA transfected cells (Fig. 4.4 A, B).

The effect of postconditioning on hypoxia/reoxygenation-induced apoptosis was measured in these cIAP2 downregulated cells. For this, HUVEC were transfected with non-specific or cIAP2 siRNA, 24 h prior to the application of hypoxia/reoxygenation or postconditioning. Cell death was analysed by annexin V/ PI staining in FACS, 24 h after reoxygenation. As shown in fig. 4.4 C, cIAP2 downregulation caused a 1.7 ± 0.4 fold increase in the basal level of apoptosis in non-treated cells. As expected, hypoxia/reoxygenation lead to a significant increase in percentage of apoptosis compared to normoxic controls, in cells transfected with non-specific siRNA and cIAP2 siRNA as well. As observed previously in non-transfected cells (Fig. 4.1), postconditioning abolished hypoxia/reoxygenation-induced apoptosis in cells transfected with non-specific siRNA. However, this reduction of apoptosis by postconditioning was significantly less effective in cIAP2 downregulated cells, confirming the protein's role in postconditioning. Correspondingly, postconditioning could revive the loss in percentage of healthy cells due to hypoxia/reoxygenation (HR), in non-specific siRNA transfected cells, while the revival effect was lost in cIAP2 downregulated cells (Fig. 4.4 C).

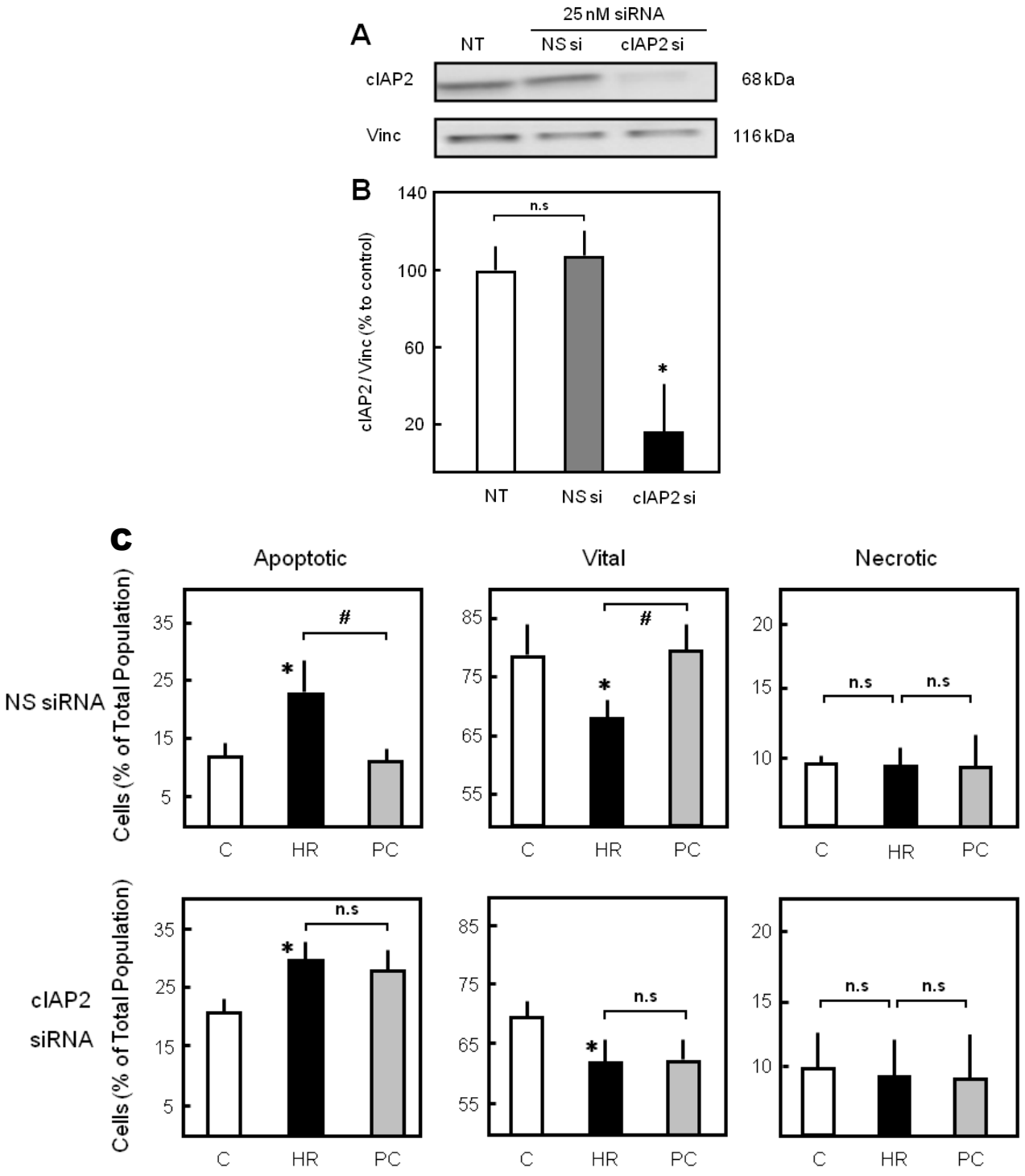


Fig. 4.4 Effect of cIAP2 downregulation on hypoxia/reoxygenation-induced apoptosis and postconditioning. HUVEC were transfected with cIAP2 siRNA 24 h prior to the application of hypoxia/reoxygenation (HR) or postconditioning (PC). Non-treated cells were taken as control (C). **(A)** Representative Western blot showing downregulation of cIAP2 with cIAP2 specific siRNA (cIAP2 si) in comparison to non-specific control siRNA (NS si). Non-transfected cells were taken as control (NT). Vinculin was taken as internal loading control. **(B)** Densitometric analysis of cIAP2 relative to Vinculin, given as percentage of control. Data are means ± SD of 5 separate experiments with independent cell preparations. $^{*}P < 0.05$ vs. NS si; n.s: not significantly different. **(C)** Distribution of vital, apoptotic and necrotic cells determined by FACS analysis of cells treated with cIAP2 siRNA compared to those treated with non-specific siRNA (NS si). Data are means ± SD of 3 separate experiments with independent cell preparations. $^{*}P < 0.05$ vs. C; $^{\#}P < 0.05$; n.s: not significantly different.

4.5 Effect of cIAP2 silencing on hypoxia/reoxygenation-induced caspase-3 cleavage and postconditioning

In addition to FACS analysis, the effect of postconditioning on hypoxia/ reoxygenation-induced caspase-3 cleavage was measured in cIAP2 downregulated cells, to confirm the role of cIAP2 in endothelial postconditioning.

Following 24 h of transfection with non-specific siRNA or cIAP2 specific siRNA, HUVEC were subjected to severe hypoxia. Caspase-3 cleavage was measured 6 h after reoxygenation or postconditioning. Consistent with the previous observation (Fig. 4.2), hypoxia/reoxygenation caused an increase in cleavage of caspase-3 which was effectively abolished by postconditioning (Fig 4.5). Treatment with non-specific siRNA showed no influence on the protective effect of postconditioning in reducing hypoxia/reoxygenation-induced caspase-3 cleavage. However, postconditioning failed to abolish hypoxia/reoxygenation-induced caspase-3 cleavage in cells treated with cIAP2 siRNA (Fig. 4.5)

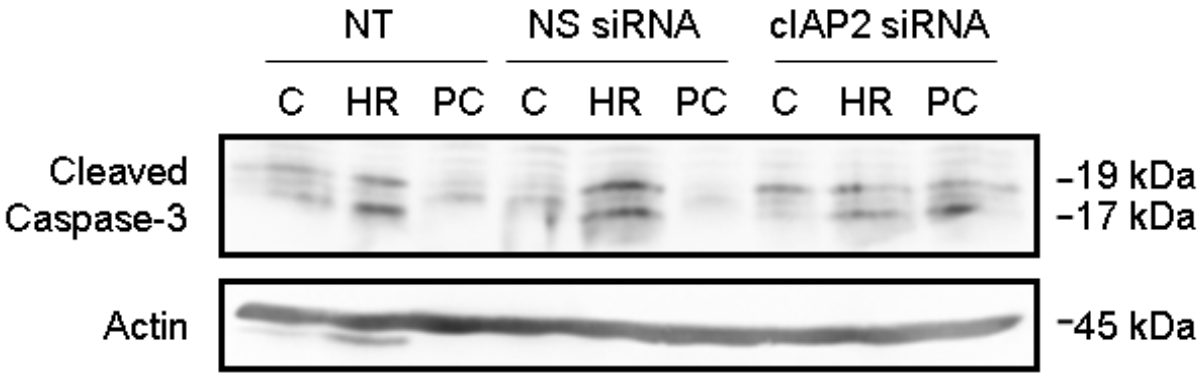


Fig. 4.5 Effect of cIAP2 silencing on caspase-3 cleavage under hypoxia/reoxygenation and postconditioning. Cells were transfected with cIAP2 siRNA or non-specific siRNA (NS si), 24 h prior to the application of hypoxia/reoxygenation (HR) or postconditioning (PC). Non-treated cells were taken as control (C). Representative Western blot showing cleavage of caspase-3. Actin was taken for internal loading control.

4.6 Effect of hypoxia/reoxygenation and postconditioning on cIAP2 - procaspase-3 interaction

cIAPs are known to execute their anti-apoptotic function by directly binding to specific procaspases and inhibiting their proteolytic activation (Roy et al., 1997). To test whether cIAP2 directly binds to procaspase-3 in endothelial cells under hypoxia/reoxygenation and postconditioning, co-immunoprecipitation of both the proteins and their co-localization were studied. HUVEC were subjected to 2 h of severe hypoxia followed by reoxygenation or postconditioning.

Anti-procaspase-3 antibody coated beads were used to coimmunoprecipitate procaspase-3 with its interacting partners from protein lysates of cells subjected to hypoxia/reoxygenation or postconditioning. As shown in fig 4.6 A, cIAP2 was co-immunoprecipitated with the caspase-3 zymogen under conditions of hypoxia and postconditioning, while no binding was observed under control or reoxygenation conditions.

In accordance with the coimmunoprecipitation studies, co-staining of cIAP2 and procaspase-3 in immunocytochemistry showed increased co-localization of both proteins in the peri-nuclear regions, during hypoxia and postconditioning compared to control or reoxygenated cells (Fig. 4.6B).

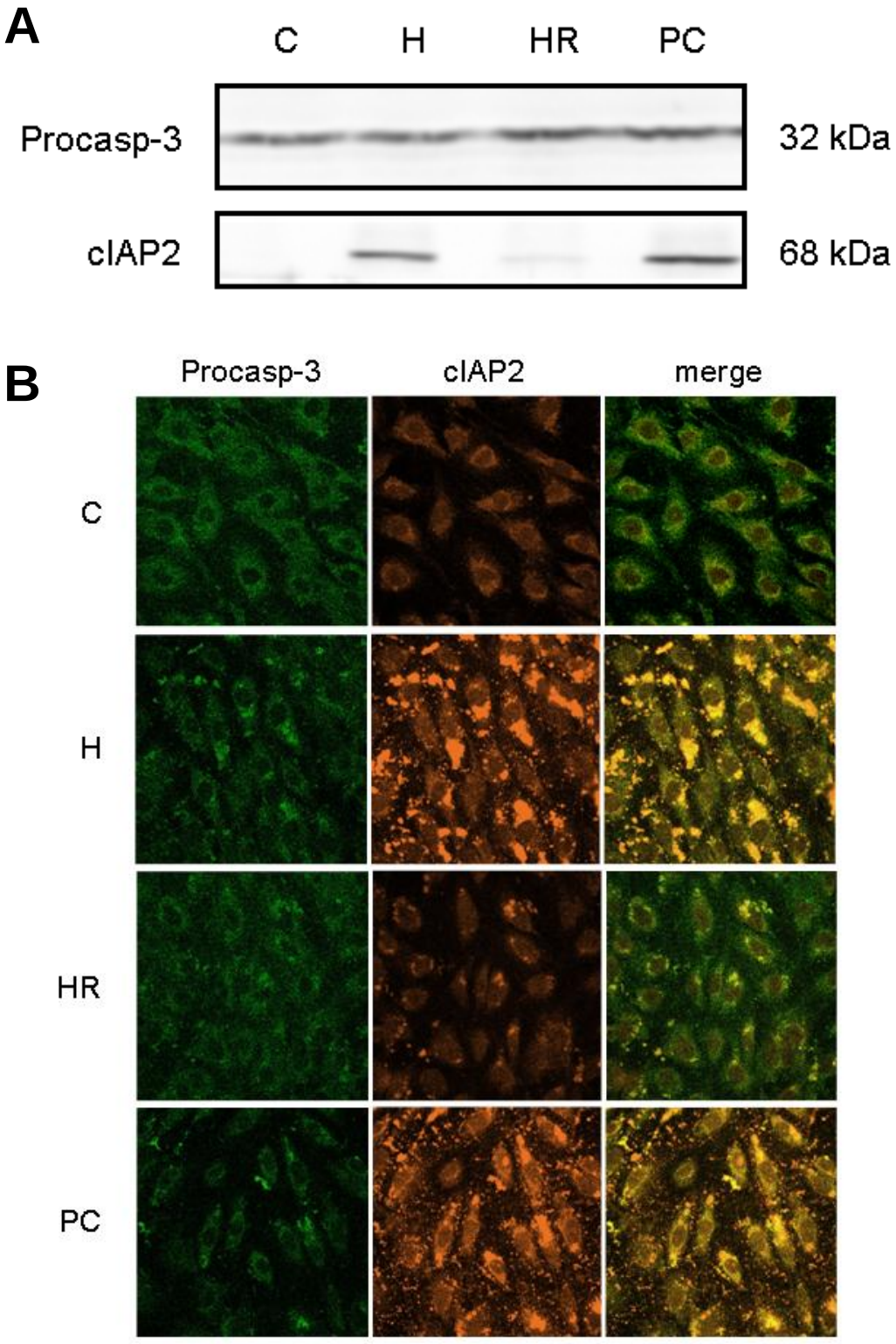


Fig. 4.6 Effect of hypoxia/reoxygenation and postconditioning on cIAP2 - procaspase-3 interaction. HUVEC were subjected to 2 h hypoxia (H) followed by reoxygenation without (HR) and with postconditioning (PC). Non-treated cells were taken as control (C). **(A)** Representative Western blot showing co-immunoprecipitation of cIAP2 with procaspase-3 under hypoxia and postconditioning, while no binding was observed under control and reoxygenation conditions **(B)** Immunostaining of procaspase-3 (green) and cIAP2 (red), showing increased co-locolization of both the proteins (yellow), under hypoxia and postconditioning compared to reoxygenation and control conditions.

4.7 Effect of postconditioning on PI3-kinase and MAPKs in endothelial cells

Mitogen activated protein kinases and the PI3-Kinase pathway play a major role in various anti-apoptotic/pro-survival mechanisms. The activation of Akt, ERK1/2, p38 MAPK and JNK pathways in endothelial postconditioning was examined as an initial step to identify the mechanism by which cIAP2 is maintained in postconditioning.

For this, HUVEC were subjected to postconditioning, following 2 h of severe hypoxia. Cells were harvested for protein lysates at the end of postconditioning or at the end of the corresponding time period (30 min reoxygenation) in cells that were reoxygenated without postconditioning. Phosphorylation of the kinases was detected in Western blotting as a measure of their activation. As shown in fig 4.7, a significant increase in the phosphorylation of Akt, ERK1/2 and p38 MAPK was observed during postconditioning in comparison to cells that were normally reoxygenated after hypoxia. Phosphorylation of c-jun remained unchanged under all corresponding conditions, negating the role of the stress activated protein kinase in endothelial postconditioning.

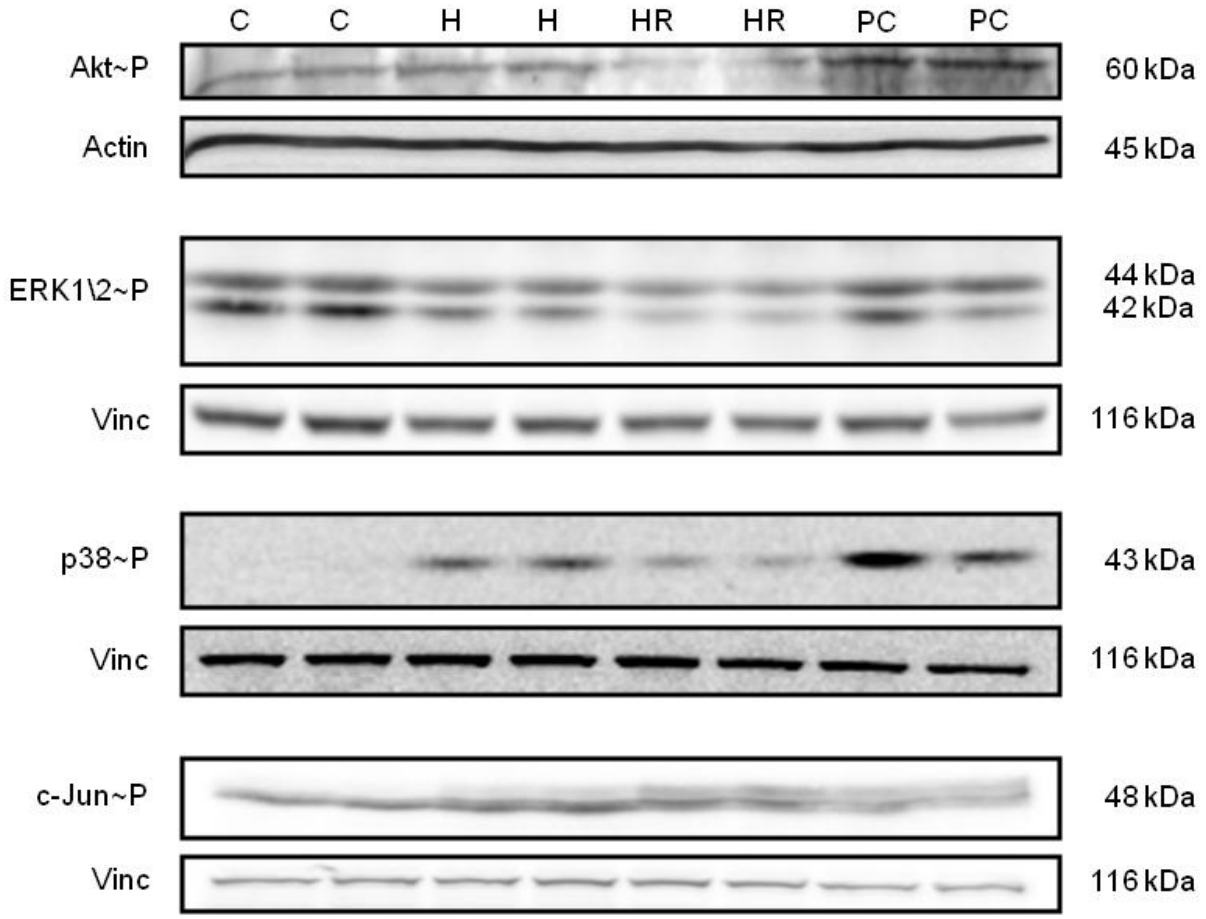


Fig. 4.7 Effect of postconditioning on phosphorylation of PI3 kinase and MAPKs. HUVEC were subjected to 2 h severe hypoxia (H) followed by 30 min reoxygenation, without (HR) or with postconditioning (PC). Representative Western blots showing phosphorylation of Akt, ERK1/2, p38MAPK and c-Jun. Duplicate samples were taken for each condition. Actin or vinculin was taken for internal loading control.

4.8 Role of PI3 kinase and MAPKs in the maintenance of cIAP2 by postconditioning

Pharmacological inhibition of the kinases that were found to be activated during postconditioning in endothelial cells was used as a strategy to test the involvement of those kinases in the maintenance of cIAP2 by postconditioning. Accordingly, HUVEC were subjected to postconditioning in the presence of the Akt inhibitor LY294002, MEK-1/2 inhibitors PD98059 or UO126, the p38 MAPK inhibitor SB203580 or the c-jun inhibitor SP600125. DMSO treated cells were taken as vehicle control.

As observed previously (Fig. 4.3), postconditioning prevented the loss of cIAP2 during reoxygenation (Fig. 4.8). However, inhibitors of both Akt and ERK 1/2 phosphorylation attenuated the high levels of cIAP2 in postconditioning, indicating their role as upstream targets of cIAP2 (Fig. 4.8). Despite the increase in p38 MAPK phosphorylation during postconditioning (Fig. 4.7), its inhibition had no significant effect on the maintenance of cIAP2 in postconditioning (Fig. 4.8), suggesting a non-causal role of the kinase activation in cIAP2 mediated protection in postconditioning. Inhibition of c-jun phosphorylation had no effect on cIAP2 in postconditioning (Fig. 4.8). Treatment with DMSO has no effect on the maintenance of cIAP2 by postconditioning.

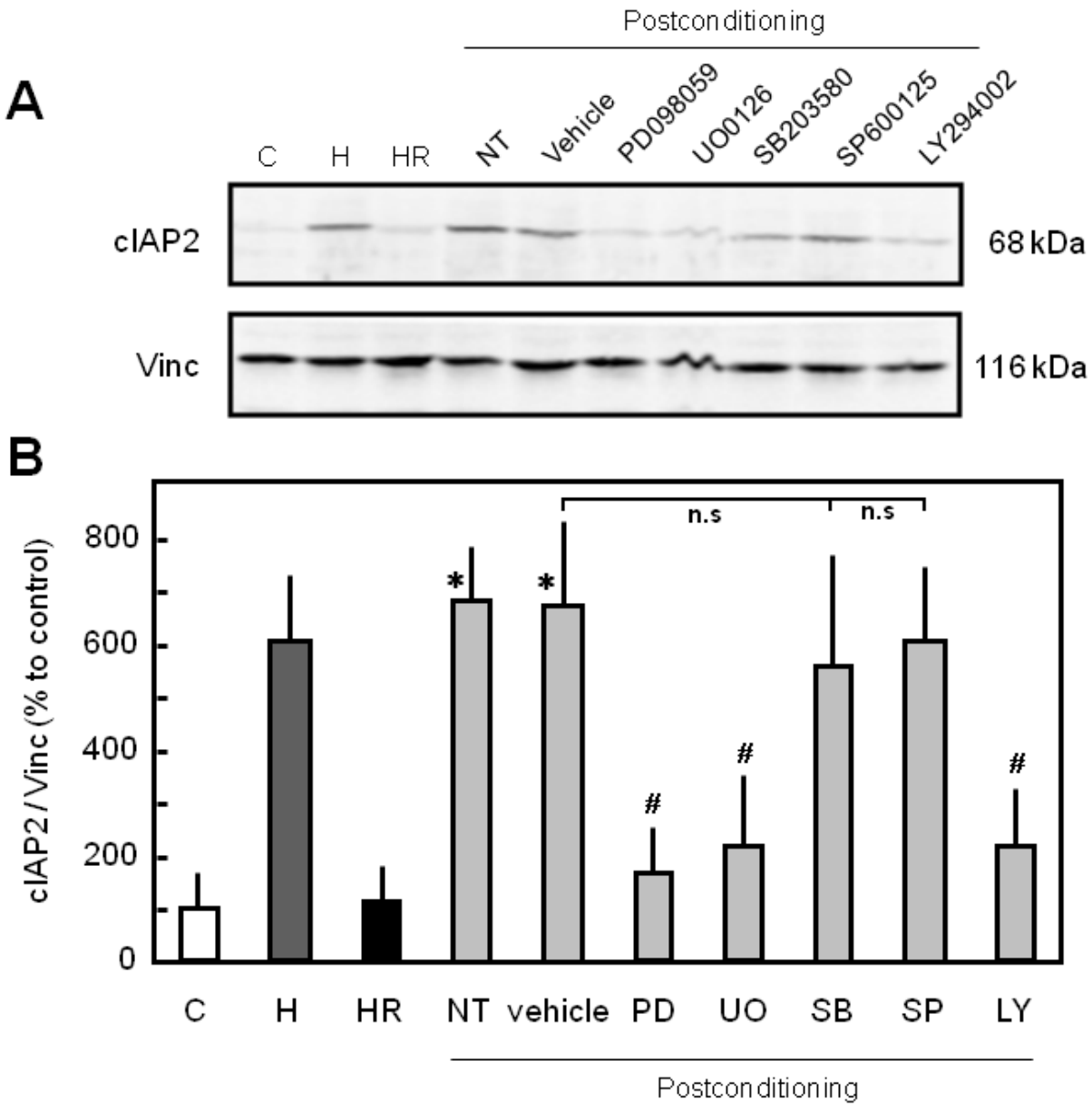


Fig. 4.8 Effect of PI3 kinase and MAPK inhibitors on cIAP2 under postconditioning. HUVEC were subjected to postconditioning in the presence of PD98059 (20 µM), UO126 (10 µM), SB203580 (10 µM), SP600125 (10 µM), LY294002 (10 µM) or DMSO (10 µM). Cells subjected to postconditioning in the presence DMSO were taken as vehicle control. **(A)** Representative Western blot showing cIAP2 in non-treated control (C), hypoxia (H), hypoxia/reoxygenation (HR) and during postconditioning in the absence (NT) and presence of specific kinase inhibitors. Vinculin (Vinc) was taken for internal loading control. **(B)** Densitometric analysis of cIAP2 relative to Vinculin, given as percentage of control. The ratio of control was set to 100. Data are means ± SD of 3 separate experiments with independent cell preparations. $^{*}P < 0.05$ vs. HR; $^{\#}P < 0.05$ vs. vehicle n.s: not significantly different vs. vehicle.

4.9 Effect of hypoxia/reoxygenation and postconditioning on cIAP2 expression in the rat aorta

To confirm the relevance of cIAP2 expression in reponse to hypoxia, reoxygenation and postconditioning, in endothelial monolayers, an intact vessel model was established using rat aortas. Freshly excised rat aortas were subjected to 2 h of hypoxia, followed by reoxygenation or postconditioning, using the same protocol applied for cultured endothelial cells. Immunofluorescence labeling of the sectioned aortas showed an increase in staining of cIAP2 in the endothelium (identified by von Willebrand factor) with 2 h hypoxia. In accordance with the effect observed in cultured endothelial cells, reoxygenation caused a decrease in cIAP2, while postconditioning prevented the loss of cIAP2 with reoxygenation in the intact vessel as well (Fig. 4.9).

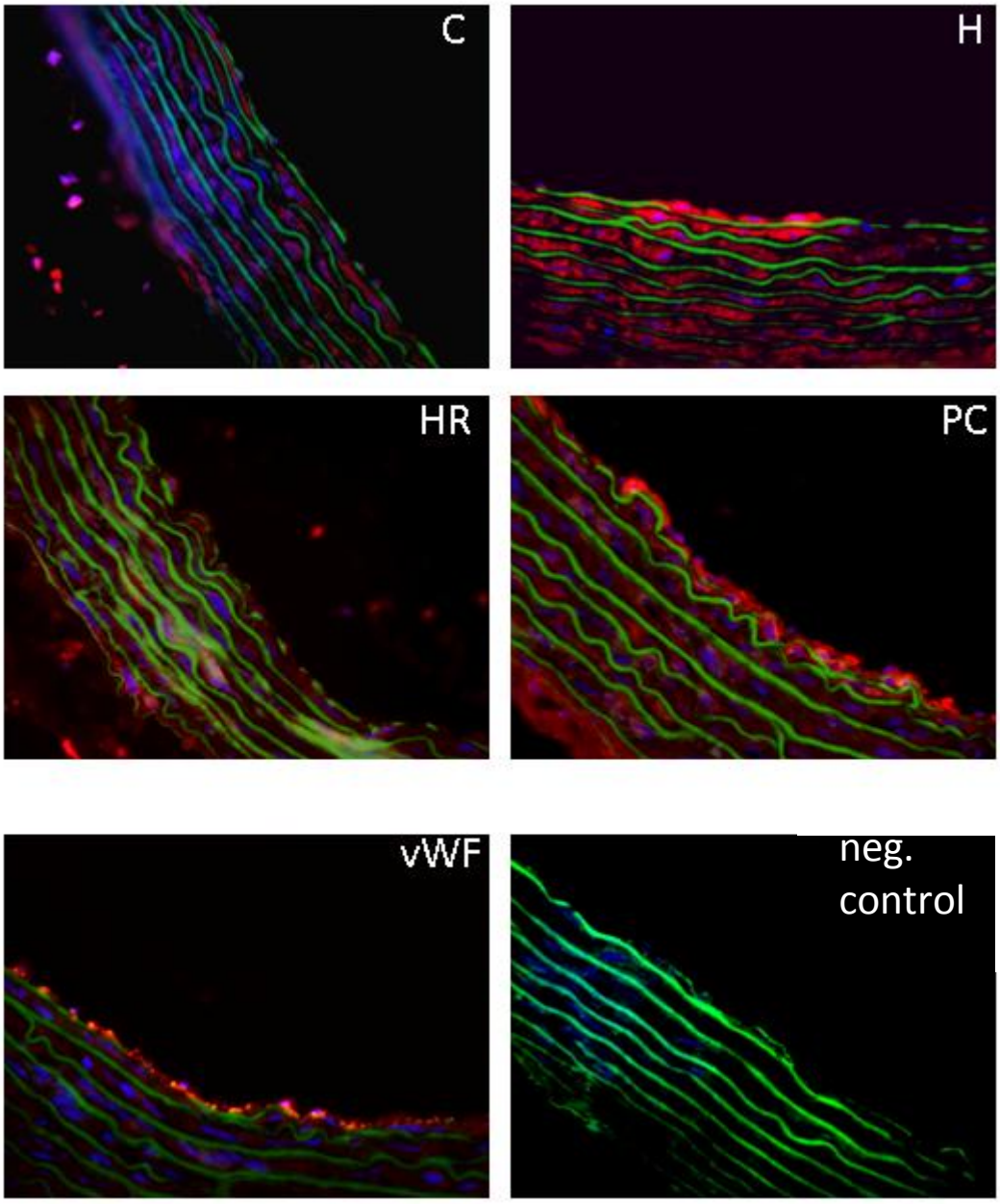


Fig. 4.9 Effect of hypoxia/reoxygenation and postconditioning on immunostaining of cIAP2 in the rat aorta. (Upper panel) Immunostaining of rat aorta cryosections showing differences in cIAP2 staining (red) in the endothelium of vessels subjected to hypoxia (H), hypoxia/reoxygenation (HR) or postconditioning (PC). Non-treated aortas were taken as control (C). DAPI (blue) was used as a nuclear stain. (Lower panel left) Immunostaining of von Willebrand factor (red) indicates endothelium. (Lower panel right) Non-stained section of the aorta was taken for negative control (neg. control).

5. Discussion

In the present study, the effect of postconditioning on endothelial cell survival and the molecular mechanisms involved were investigated. Postconditioning has emerged as a clinically feasible intervention, well-proven to effectively reduce reperfusion injury in cardiomyocytes (Zhao et al., 2003). However, little is known about the effects of this maneuver in endothelial cells and whether it has an influence on apoptosis, which is the predominant form of endothelial cell death in the reperfused myocardium. In an attempt to study this, endothelial cells were exposed to hypoxia/reoxygenation and the effects of postconditioning on cell survival as well as the underlying molecular mediators were examined.

The major findings of the present study are: (a) Postconditioning protects cultured endothelial cells from hypoxia/reoxygenation-induced apoptosis. (b) Cellular inhibitor of apoptosis protein 2, cIAP2, but not cIAP1 or XIAP is upregulated during hypoxia and its loss during reoxygenation is prevented by postconditioning. This effect of postconditioning on cIAP2 stabilization was observed in cultured endothelial cells as well as in intact vessels. (c) Downregulation of cIAP2 with siRNA augments hypoxia/reoxygenation-induced apoptosis and diminishes the protective effect of postconditioning. (d) cIAP2 directly interacts with pro-caspase3 in hypoxia and postconditioning. (e) ERK1/2 and PI3 Kinase pathways, but not p38 MAPK or the JNK pathway, are involved in the maintenance of cIAP2 in endothelial postconditioning.

5.1 Postconditioning protects endothelial cells from hypoxia/reoxygenation-induced apoptosis

This study shows for the first time that postconditioning, known to rescue cardiomyocytes from reperfusion injury, also has an effect on endothelial cells. According to the principle that the first few minutes of reperfusion provide the ‘window of opportunity’ (Piper et al., 2004), intermittent hypoxia applied at the very onset of reoxygenation reduced the caspase-3 cleavage and apoptosis induced by hypoxia/reoxygenation. Correspondingly, postconditioning could revive the loss in percentage of healthy cells induced by hypoxia/reoxygenation, while there were no significant changes in the percentage of necrotic cells.

The increase in percentage of apoptosis in response to hypoxia/reoxygenation can be explained by the robust energy metabolism of endothelial cells, which allows

them to maintain high phosphorylation potential, even in adverse conditions like ischemia or hypoxia. This ability to maintain their ATP levels prevents endothelial cells from the necrotic fate (Lelli et al., 1998), resulting in no significant changes in percentage of necrosis, in response to hypoxia/ reoxygenation or postconditioning.

Increasing evidence suggests that loss of endothelial integrity leads to leakage of cytokines, interleukins and other pro-inflammatory mediators, jeopardizing the survival of the entire myocardial tissue (Bombeli et al., 1997, Werner et al., 2006). Hence it is likely that prevention of apoptosis, which is the predominant form of cell death in the endothelium, comprises a significant fraction of the protection exerted by postconditioning. This is further supported by studies reporting endothelial apoptosis as a critical event preceding myocardial apoptosis during ischemia reperfusion injury (Scarabelli et al., 2001). It was also reported that during ischemia, inhibition of caspase-9, the principal initiator caspase of the endothelium, and not inhibition of caspase-8, which is found in the cardiomyocytes, leads to a reduction in infarct size (Stephanou et al., 2001), suggesting that prevention of endothelial apoptosis, could be critical in determining the outcome of reperfusion injury in the myocardium. The systematic nature of this cell death process, unlike necrosis, allows greater chances for therapeutic interventions and revival.

The algorithm of postconditioning applied in this study effectively abolished reoxygenation-induced caspase-3 cleavage and apoptosis, reviving the loss in percentage of vital cell population. Though postconditioning was originally performed on the canine heart, by the application of 3 cycles of ischemia and reperfusion, each lasting for 30 sec (Zhao et al., 2003), the optimal algorithm for postconditioning can vary widely depending on the species and the experimental model under study (Skyschally et al., 2009). 3 cycles of severe hypoxia and reoxygenation, 5 min each, were applied in this study, which is established to be optimal for cell culture models (Sun et al., 2005).

5.2 Inhibitors of apoptosis proteins in postconditioning

The Inhibitors of apoptosis family of proteins are known to play pivotal roles in a wide variety of pro-survival and anti-apoptotic pathways by inhibiting caspases, the key mediators of apoptosis. Here, the involvement of IAPs in the protective effect of postconditioning on endothelial cells was examined. Among the IAPs screened in the study, only cIAP2 was found to be upregulated by hypoxia. Importantly,

reoxygenation leads to a loss in cIAP2, which is prevented by the application of postconditioning. The protein levels of cIAP1 and XIAP remained unchanged in response to hypoxia/reoxygenation or postconditioning.

Focus was laid on the IAP family of proteins among all other known hypoxia-sensitive anti-apoptotic molecules, because of the converging downstream positioning of IAPs in both the intrinsic and extrinsic apoptotic pathways. IAPs act to directly suppress the activity of caspases, which are the key apoptotic executors. Further, the loss of cIAP2 at reoxygenation supports the hypothesis that a maneuver to prevent the loss could protect the cells from apoptosis. This makes cIAP2 an interesting candidate for postconditioning. Bcl2, for instance is one of the other hypoxia sensitive anti-apoptotic proteins, but the continued endogenous expression of this protein in reoxygenation has no protective effect, still allowing apoptosis to occur in reperfusion injury (Mishra et al., 2006). Similar is the case with Bcl-xL, making these proteins less plausible candidates to be involved in postconditioning. cIAP2, on the other hand, is upregulated as an adaptive response to hypoxia but reduces to basal levels with the onset of reoxygenation. As demonstrated by the present study, postconditioning indeed strengthens this adaptive response induced by hypoxia and prevents the loss of cIAP2 with the onset of reoxygenation.

Although other mammalian IAPs and particularly cIAP1, share a highly conserved structural and functional homology with cIAP2 including an NFκB response element, it is not yet clear what distinguishes cIAP2 from the others in terms of its hypoxia sensitivity. A previous study by Dong et al., (2001) supports our observation that only cIAP2, but not cIAP1 is upregulated by hypoxia. The upregulation was reported to be HIF-independent, but the specific mechanism remains to be elucidated.

5.3 Effect of cIAP2 silencing on hypoxia/reoxygenation-induced apoptosis and postconditioning

The functional significance of cIAP2 expression in endothelial postconditioning was tested by silencing the protein with specific siRNA before the application of hypoxia/reoxygenation or postconditioning. About 80% downregulation in the protein levels of cIAP2 was achieved in comparison to non-specific siRNA treated cells taken as control. As expected, cIAP2 silencing slightly increased the basal level of apoptosis in normal growing cells. Hypoxia/reoxygenation led to an increase in cleavage of caspase-3 and percentage of apoptosis, in both cIAP2 siRNA and non-

specific siRNA treated cells. This was accompanied by a corresponding loss in percentage of vital cell population, in both cases. As observed in non-treated cells, postconditioning effectively abolished hypoxia/reoxygenation-induced apoptosis in cells transfected with non-specific siRNA. However, the protective effect of postconditioning was diminished in cIAP2 downregulated cells, as seen by the failure of the maneuver to reduce hypoxia/reoxygenation-induced caspase-3 cleavage and apoptosis in these cells. Correspondingly, postconditioning was effective in reviving hypoxia/reoxygenation-induced loss in percentage of vital cell population in non-specific siRNA treated cells, but not in cIAP2 downregulated cells.

cIAP2 appears to be one of the anti-apoptotic molecules central in balancing the apoptotic machinery of endothelial cells. This is evident from the fact that, cIAP2 silencing even causes an increase in the basal level of apoptosis in normal growing cells that are unexposed to hypoxia/reoxygenation. The drastically high levels of the protein in postconditioning and the significant antagonizing effect of its silencing on the protective effect of postconditioning strongly emphasize the crucial role of cIAP2 in endothelial postconditioning.

5.4 Interaction of cIAP2 and procaspase-3

Based on the previously reported observation that cIAP1 and cIAP2 inhibit apoptosis by directly binding to procaspase-3 (Roy et al., 1997), it was hypothesized that the elevated levels of cIAP2 in postconditioning might prevent reoxygenation induced apoptotic execution by directly binding to procaspase-3 and preventing its cleavage to active subunits. The hypothesis was tested by studying the interaction of both the proteins in endothelial cells subjected to hypoxia/reoxygenation or postconditioning. Co-immunoprecipitation of cIAP2 with procaspase-3 showed direct interaction of the two proteins under conditions of hypoxia and postconditioning. This was further supported by immunocytochemical images showing increased co-localization of both the proteins in the peri-nuclear regions of the cell, under hypoxia and postconditioning.

In several cases, the BIR domain of IAPs enables them to bind caspases and suppress their activity (Devereaux et al., 1997). However cIAP1 and cIAP2 also possess an exclusive caspase recruitment domain (CARD), the function of which is not yet completely understood. It is not yet known whether the BIR or the CARD domain of cIAP2 binds to procapase-3 in endothelial cells, but it may be speculated

that either of the two domains or both of them could be potential motifs responsible for the protein's binding to procaspase-3 thereby leading to its inactivation during postconditioning in endothelial cells.

5.5 Role of PI3-kinase and MAPKs in the maintenance of cIAP2 by postconditioning

As an initial step to identify the signaling mechanism involved in the maintenance of cIAP2 by postconditioning, the activation of Akt and MAPK pathways in endothelial postconditioning was examined. The expression of cIAP2 in postconditioning was then studied by performing postconditioning in the presence of pharmacological inhibitors of those kinases that are found to be activated during postconditioning.

Increase in phosphorylation of Akt, ERK1/2 and p38 MAPK was observed in postconditioning compared to hypoxia/reoxygenation in endothelial cells. There were no significant changes in the phosphorylation state of c-jun. In accordance to this, pharmacological inhibition of PI3-kinase and ERK1/2 prevented the maintenance of cIAP2 by postconditioning. On the contrary, inhibition of p38 MAPK had no effect on the protein levels of cIAP2 during postconditioning, in spite of the fact that phosphorylation of p38 MAPK was observed in endothelial postconditioning, excluding p38 MAPK as an upstream signaling element in cIAP2 maintenance. The levels of phospho c-jun remained unchanged, suggesting that the stress-activated JNK pathway is not involved in endothelial postconditioning.

Activation of PI3-kinase and ERK1/2 pathways during postconditioning in endothelial cells is similar to cardiomyocytes (Hausenloy et al., 2004), where both the pathways were found to be activated during postconditioning. ERK1/2 and PI3-kinase are collectively called as reperfusion injury salvage kinases (RISK) and their activation is reported to protect against reperfusion injury (Hausenloy et al., 2004). The mechanism by which these survival kinases are recruited by postconditioning is still unresolved, although it is suggested that it may be due to activation of cell-surface receptors including the adenosine A2A receptor (Morrison et al., 2007). Other studies have placed its activation downstream of other signaling elements such as sphingosine kinase (Jin et al., 2008) and the recovery of neutral pH in the first few minutes of myocardial reperfusion (Fujita et al., 2007). The downstream targets of RISK are better resolved and converge on inhibition of mPTP opening, principally via

GSK-3ß (Halestrap et al., 1998; Gomez et al., 2008). Further, the mechanism by which activation of PI3-kinase and ERK1/2 lead to stabilization of cIAP2 in endothelial postconditioning is yet to be determined. However, pharmacological inhibition clearly indicates their upstream role in the maintenance of cIAP2 during postconditioning.

In addition, p38 MAPK was also found to be activated in endothelial postconditioning. The role of p38 MAPK in cardiac conditioning has frequently countered controversy, with studies reporting both cardio-protective (da Silva et al., 2004) and pro-injurious (Sun et al., 2006) roles of the kinase at the time of reperfusion (Ping et al., 2000; Hausenloy et al., 2007). In our model, p38 MAPK was observed to be activated during postconditioning, however, blocking the kinase by pharmacological inhibition had no effect on cIAP2, indicating that the activation plays no role in the maintenance of cIAP2 during postconditioning.

5.6 cIAP2 expression in the intact vessel

To test the expression of cIAP2 during hypoxia/reoxygenation and postconditioning in the intact vessel, a vessel model using rat aorta was established. The endothelium in the intact vessel showed an increased expression of cIAP2 in response to hypoxia. Reoxygenation led to a loss in signal intensity, which was prevented in the postconditioned vessel.

The expression pattern of cIAP2 in response to hypoxia/reoxygenation and postconditioning was very similar to the cell culture model, negating the occurrence of any cell culture artifacts. The increased expression of cIAP2 under postconditioning, seen in the intact vessel model, confirms the functional significance of the anti-apoptotic protein in endothelial postconditioning. It was also observed that the endothelium, identified by von Willebrand factor, showed more cIAP2 than the surrounding tissue, suggesting the importance of endothelial postconditioning.

5.7 Future perspective

Postconditioning is gradually being adapted into clinical practice in cases of coronary intervention, cardiac surgery, organ transplantation and vascular-based procedures. However, it has not yet assumed the position of ‘standard care’ in any of the clinical settings. Further insight into the molecular mechanisms of postconditioning, the signaling pathways recruited and the cell types involved is

necessary to establish postconditioning as a standard clinical practice to counteract reperfusion injury. In this context, understanding endothelial cell responses is essential since they are in direct contact with the blood carrying triggers and mediators of hypoxia/reperfusion and postconditioning, including oxidants, cytokines, interleukins, chemokines and ionic dyshomeostasis. The present study shows that endothelial cells do respond to postconditioning and that cIAP2 is crucial for the anti-apoptotic effect exerted. It was also observed that ERK1/2 and PI3-kinase are involved in the maintenance of cIAP2 by postconditioning, however, the mechanism by which postconditioning leads to activation of the kinases and how this activation controls cIAP2 content in the cells, remain to be elucidated.

Prevention of the mPTP opening, which is a crucial step in postconditioning of cardiomyocytes, would also be a question of interest in endothelial postconditioning. Phosphorylation of GSK-3ß, which leads to its inactivation, is known to prevent the opening of mPTP, and thus exert cardioprotection (Gomez et al., 2008). Whether such inactivation of GSK-3ß occurs in endothelial postconditioning or not, is yet to be determined.

Finally, in vivo experiments designed to study the role of endothelial postconditioning on the outcome of myocardial protection, would help to further validate the patho-physiological relevance of the current study and to better adapt postconditioning into clinical practice.

6. References

Adrain C, Martin CJ (2001) The mitochondrial apoptosome: a killer unleashed by the cytochrome. Trends Biochem Sci; 26:390-7.

Allen DG, Xiao X-H (2003) Role of the cardiac Na+/H+ exchanger during ischemia and reperfusion. Cardiovasc Res; 57:934–941

Ambrosio G, Zweier JL, Flaherty JT (1991) The relationship between oxygen radical generation and impairment of myocardial energy metabolism following post-ischemic reperfusion. J Mol Cell Cardiol; 23: 1359–1374

Argaud L, Gateau-Roesch O, Raisky O, Loufouat J, Robert D, Ovize M (2005) Post-conditioning inhibits mitochondrial permeability transition. Circulation; 111:194–197

Bassi R, Heads R, Marber MS, Clark JE (2008) Targetting p38 MAPK in the ischemic heart: kill or cure? J Curr Opin Pharmacol 8: 141-6

Baxter GF, Yellon DM (2003) Current trends and controversies in ischemiareperfusion research. Meeting report of the Hatter Institute 3rd International Workshop on Cardioprotection. Basic Res Cardiol; 98:133–136

Braunwald E, Kloner RA (1985) Myocardial reperfusion: a double-edged sword? J Clin Invest; 76:1713–1719

Boengler K, Buechert A, Heinen Y, Roeskes C, Hilfiker-Kleiner D, Heusch G (2008) Cardioprotection by ischemic postconditioning is lost in aged and STAT 3-deficient mice. Circ Res; 102:3911-3918

Bombeli T, Karsan A, Tait JF, Harlan JM (1997) Apoptotic vascular endothelial cells become procoagulant. Blood; 89:2429–42

Borutaite V, Budriunaite A, Morkuniene R, Brown GC (2001) Release of mitochondrial cytochrome-C and activation of cytosolic caspases induced by myocardial ischemia. Biochim Biophys Acta; 1537:101-9.

Borutaite V, Jekabsone A, Morkuniene R, Brown GC (2003) Inhibition of mitochondrial permeability transition prevents mitochondrial dysfunction, cytochrome-C release and apoptosis induced by heart ischemia. J Mol Cell Cardiol; 35:357-66.

Buckberg GD (1986) Studies of controlled reperfusion after ischemia: When is cardiac muscle damaged irreversibly? J Thorac Cardiovasc Surg; 92:483–487

Chai J, Shiozaki E, Srinivasula SM, Wu Q, Datta P, Alnemri ES, Shi Y (2001) Structural basis of caspase-7 inhibition by XIAP. Cell; 104: 769–780

Chen J, Mehta JL, Haider N, Zhang X, Narula J, Li D (2004) Role of caspases in Ox-LDL-induced apoptotic cascade in human coronary artery endothelial cells. Circ Res; 94:370–6

Chu ZL, McKinsey TA, Liu L, Gentry JJ, Malim MH, Ballard DW (1997) Suppression of tumor necrosis factor induced cell death by inhibitor of apoptosis c-IAP2 is under NF-κB control. Proc Natl Acad Sci USA; 94: 10057– 0062

Cooley DA, Ruel GJ, Wukasch DC (1972) Ischemic contracture of the heart: “Stone Heart”. Am J Cardiol; 29:575–577

Crook NE, Clem RJ, Miller LK (1993) An apoptosis inhibiting baculovirus gene with a zinc finger-like motif. J Virol; 67: 2168–2174

da Silva R, Grampp T, Pasch T, Schaub MC, Zaugg M (2004) Differential activation of mitogen-activated protein kinases in ischemic and anesthetic preconditioning. Anesthesiology; 100:59–69

Devereaux QL, Takahashi R, Salvesen GS, Reed JC (1997) X-linked IAP is a direct inhibitor of cell-death proteases. Nature; 388: 300–304

Deveraux QL, Leo E, Stennicke HR, Welsh K, Salvesen GS, Reed JC (1999) Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. EMBO J; 18: 5242–5251

Dierlamm J, Baens M, Wlodarska I, Stefanova-Ouzounova M, Hernandez JM, Hossfeld DK, De Wolf-Peeters C, Hagemeijer A, Van den Berghe H, Marynen P (1999) The apoptosis inhibitor gene API2 and a novel 18q gene, MLT, are recurrently rearranged in the t(11;18)(q21;q21) associated with mucosa-associated lymphoid tissue lymphomas. Blood; 93: 3601–3609

Dong Z, Venkatachalam MA, Wang J, Patel Y, Saikumar P, Semenza GL, Force T, Nishiyama J (2001) Up-regulation of apoptosis inhibitory protein IAP-2 by hypoxia: Hif-1-independent mechanisms. J Biol Chem; 276:18702–18709

Duckett CS, Nava VE, Gedrich RW, Clem RJ, Van Dongen JL, Gilfillan MC, Shiels H, Hardwick JM, Thompson CB (1996) A conserved family of cellular genes related to the baculovirus iap gene and encoding apoptosis inhibitors. EMBO J; 15: 2685–2694

Dumont EA, Hofstra L, van Heerde WL, van den Eijnde S, Doevendans PA, DeMuinck E, Daemen MA, Smits JF, Frederik P, Wellens HJ, Daemen MJ, Reutelingsperger CP (2000) Cardiomyocyte death induced by myocardial ischemia and reperfusion: measurement with recombinant human annexin-V in a mouse model. Circulation; 102:1564-8.

Eefting F, Rensing B, Wigman J, Pannekoek WJ, Liu WM, Cramer MJ, Lips DJ, Doevendans PA (2004) Role of apoptosis in reperfusion injury. Cardiovasc Res; 61:414-26

Fischer U, Janicke RU, Schulze-Osthoff K (2003) Many cuts to ruin: a comprehensive update of caspase substrates. Cell Death Differ; 10:76-100

Follette DM, Fey K, Buckberg GD, Helly JrJJ, Steed DL, Foglia RP, Maloney JV Jr (1981) Reducing postischemic damage by temporary modification of reperfusate calcium, potassium, pH, and osmolarity. J Thorac Cardiovasc Surg; 82:221–238

Fraser AG, James C, Evan GI, Hengartner MO (1999) Caenorhabditis elegans inhibitor of apoptosis protein (IAP) homologue BIR-1 plays a conserved role in cytokinesis. Curr Biol; 9:292–301

Fujita M, Asanuma H, Hirata A, Wakeno M, Takahama H, Sasaki H (2007) Prolonged Transient Acidosis During Early Reperfusion Contributes to the Cardioprotective Effects of Postconditioning. Am J Physiol Heart Circ Physiol; 4: H2004-2008

Galagudza M, Kurapeev D, Minasian S, Valen G, Vaage J (2004) Ischemic postconditioning: brief ischemia during reperfusion converts persistent ventricular fibrillation into regular rhythm. Eur J Cardio-thorac Surg; 25:1006–1010

Garcia-Dorado D, Gonzalez MA, Barrabes JA, Ruiz-Meana M, Solares J, Lidon R-M, Blanco J, Puigfel Y, Piper HM, Soler-Soler J (1997) Prevention of ischemic rigor contracture during coronary occlusion by inhibition of Na+-H+ exchange. Cardiovasc Res; 35:80–89

Goodman MD, Koch SE, Fuller-Bicer GA, Butler KL (2008) Regulating RISK: a role for JAK-STAT signaling in postconditioning? Am J Physiol Heart Circ Physiol; 295:H1649-1656

Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL (1994) Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J Clin Invest; 94:1621-8.

Gomez L, Paillard M, Thibault H, Derumeaux G, Ovize M (2008) Inhibition of GSK3ß by Postconditioning Is Required to Prevent Opening of the Mitochondrial Permeability Transition Pore During Reperfusion. Circulation; 117:2761-2768

Griffiths EJ, Halestrap AP (1995) Mitochondrial non-specific pores remain closed during cardiac ischaemia, but open upon reperfusion. Biochem J; 307:93–98

Gross GJ, Gumina RJ (2001) Cardioprotective effects of Na+/H+ exchange inhibitors. Drugs of the Future; 26:253–260

Guo J-P, Murohara T, Buerke M, Scalial R, Lefer AM (1996) Direct measurement of nitric oxide release from vascular endothelial cells. J Appl Physiol; 81:774–779

Halestrap AP, Kerr PM, Javadov S, Woodfield KY (1998) Elucidating the molecular mechanism of the permeability transition pore and its role in reperfusion injury of the heart. Biochim Biophys Acta; 1366:79–94

Halkos ME, Kerendi F, Corvera JS, Wang N-P, Kin H, Payne CS, Sun H-Y, Guyton RA, Vinten-Johansen J, Zhao Z-Q (2004) Myocardial protection with postconditioning is not enhanced by ischemic preconditioning. Ann Thorac Surg; 78:961–969

Hausenloy DJ, Duchen MR, Yellon DM (2003) Inhibiting mitochondrial permeability transition pore opening at reperfusion protects against ischaemia-reperfusion injury. Cardiovasc Res; 60:617–625

Hausenloy DJ, Yellon DM (2004) New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. Cardiovasc Res; 61:448–460

Hausenloy DJ, Yellon DM (2006) Survival kinases in ischemic preconditioning and postconditioning. Cardiovasc Res; 70:240–253

Hausenloy DJ, Yellon DM (2007) Reperfusion injury salvage kinase signaling: taking a RISK for cardioprotection. Heart Fail Rev; 12:217–234

Hay BA, Wassarman DA, Rubin GM (1995) Drosophila homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. Cell; 83: 1253–1262

Hinds MG, Norton RS, Vaux DL, Day CL (1999) Solution structure of a baculoviral inhibitor of apoptosis (IAP) repeat. Nature Struct Biol; 6: 648–651

Hofstra L, Liem IH, Dumont EA, Boersma HH, van Heerde WL, Doevendans PA, De Muinck E, Wellens HJ, Kemerink GJ, Reutelingsperger CP, Heidendal GA (2000) Visualisation of cell death in vivo in patients with acute myocardial infarction. Lancet; 356:209-12.

Huang H, Joazeiro CA, Bonfoco E, Kamada S, Leverson JD, Hunter T (2000) The inhibitor of apoptosis, cIAP2, functions as a ubiquitin-protein ligase and promotes in vitro monoubiquitination of caspases 3 and 7. J Biol Chem; 275: 26661–26664

Huang Y, Park YC, Rich RL, Segal D, Myszka DG, Wu H (2001) Structural basis of caspase inhibition by XIAP. Differential roles of the linker versus the BIR domain. Cell; 104: 781–790

Hu S & Yang Y (2003) Inhibitors of apoptosis 1 and 2 ubiquitin ligases for apoptosis inducer Smac/DIABLO. J Biol Chem; 278:10055-10060

Imoto I, Yang ZQ, Pimkhaokham A, Tsuda H, Shimada Y, Imamura M, Ohki M, Inazawa J (2001) Identification of cIAP1 as a candidate target gene within an amplicon at 11q22 in esophageal squamous cell carcinomas. Cancer Res; 61: 6629–6634

Jacobson KB, Manos RE, Wadzinski FA (1987) Partial purification of an oxygen savaging cell membrane fraction for use in anaerobic biochemical reactions. Biotechnology and Applied Biochemistry; 9:368-379

Jennings RB, Reimer KA, Steenbergen C (1986) Myocardial ischemia revisited: the osmolar load, membrane damage and reperfusion. J Mol Cell Cardiol; 18:769–780

Jin ZQ, Karliner JS, Vessey DA (2008) Ischemic postconditioning protects isolated mouse hearts against ischemia-reperfusion injury via sphingosine kinase isoform-1 activation. Cardiovasc Res; 79:134-140

Joazeiro CA, Weissman AM (2000) RING finger proteins: mediators of ubiquitin ligase activity. Cell; 102: 549–552

Jordan JE, Zhao Z-Q, Sato H, Taft S, Vinten-Johansen J (1997) Adenosine A2 receptor activation attenuates reperfusion injury by inhibiting neutrophil accumulation, superoxide generation and coronary endothelial adherence. J Pharmacol Exp Ther; 280:301–309

Juhaszova M, Zorov DB, Kim SH, Pepe S, Fu Q, Fishbein KW (2004) Glycogen synthase kinase-3beta mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. J.Clin.Invest; 113:1525-1549

Kajstura J, Cheng W, Reiss K, Clark WA, Sonnenblick EH, Krajewski S, Reed JC, Olivetti G, Anversa P (1996) Apoptotic and necrotic myocyte cell death are independent contributing variables of infarct size in rats. Lab Invest; 74:86-107.

Kin H, Lofye MT, Amerson BS, Zatta AJ, Kerendi F, Halkos ME, Zhao Z-Q, Headrick JP, Guyton RA, Vinten-Johansen J (2004) Cardioprotection by “postconditioning” is mediated by increased retention of endogenous intravascular adenosine and activation of A2a receptors during reperfusion. Circulation; 110:III-168

Kin H, Zatta AJ, Lofye MT, Amerson BS, Halkos ME, Kerendi F, Zhao Z-Q, Guyton RA, Headrick JP, Vinten-Johansen J (2005) Postconditioning reduces infarct size via adenosine receptor activation by endogenous adenosine. Cardiovasc Res; 67:124-133

Laskey WK (2005) Brief repetitive balloon occlusions enhance reperfusion during percutaneous coronary intervention for acute myocardial infarction: a pilot study. Catheter Cardiovasc Interv; 65:361– 7

Lefer AM, Tsao PS, Lefer DJ, Ma X-L (1991) Role of endothelial dysfunction in the pathogenesis of reperfusion injury after myocardial ischemia. FASEB J; 5:2029–2034

Lefer AM, Ma X-L, Weyrich A, Lefer DJ (1993) Endothelial dysfunction and neutrophil adherence as critical events in the development of reperfusion injury. Agents Actions (Suppl); 41:127–135

Lelli JL Jr, Becks LL, Dabrowska MI, Hinshaw DB (1998) ATP converts necrosis to apoptosis in oxidant-injured endothelial cells. Free Radic Biol Med; 25(6):694-702

Li F, Ambrosini G, Chu EY, Plescia J, Tognin S, Marchisio PC, Altieri DC (1998) Control of apoptosis and mitotic spindle checkpoint by survivin. Nature; 396:580–584

Li X, Yang Y, Ashwell JD (2002) TNF-RII and c-IAP1 mediate ubiquitination and degradation of TRAF2.Nature; 416:345–347

Liston P, Roy N, Tamai K, Lefebvre C, Baird S, Cherton-Horvat G, Farahani R, McLean M, Ikeda JE, MacKenzie A, Korneluk RG (1996) Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. Nature; 379:349–353

Loo VG, Gurp VM, Depuydt B, Srinivasula SM, Rodriguez I, Alnemri ES, Gevaert K, Vandekerckhove J, Declercq W, Vandenabeele P (2002) The serine protease Omi/HtrA2 is released from mitochondria during apoptosis. Omi interacts with caspase-inhibitor XIAP and induces enhanced caspase activity. Cell Death Differ; 9:20–26

Luo X, Budihardjo I, Zou H, Slaughter C, Wang X (1998) Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell; 94(4):481-90

Ma X-L, Weyrich AS, Lefer DJ, Lefer AM (1993) Diminished basal nitric oxide release after myocardial ischemia and reperfusion promotes neutrophil adherence to coronary endothelium. Circ Res; 72:403–412

Martins LM, Iaccarino I, Tenev T, Gschmeissner S, Totty NF, Lemoine NR, Savopoulos J, Gray CW, Creasy CL, Dingwall C, Downward J (2001) The serine protease Omi/HtrA2 regulates apoptosis by binding XIAP through a Reaper-like motif. J Biol Chem; 277:439–444

MacFarlane M, Merrison W, Bratton SB, Cohen GM (2002) Proteasome-mediated degradation of Smac during apoptosis; XIAP promotes Smac ubiquitination in vitro. J Biol Chem; 277:36611-36616

Miller LK (1999) An exegesis of IAPs: salvation and surprises from BIR motifs. Trends Cell Biol; 9: 323–328

Mishra OP, Randis T, Ashraf QM, Delivoria-Papadopoulos M (2006) Hypoxia-induced Bax and Bcl-2 protein expression, caspase-9 activation, DNA fragmentation and lipid peroxidation in mitochondria of the cerebral cortex of newborn piglets: the role of nitric oxide. Neuroscience; 141(3):1339-49

Morrison RR, Tan XL, Ledent C, Mustafa SJ, Hofmann PA (2007) Targeted deletion of A2A adenosine receptor attenuates the protective effects of myocardial postconditioning. Am J Physiol Heart Circ Physiol; 293: H2523-H2529

Narula J, Pandey P, Arbustini E, Haider N, Narula N, Kolodgie FD, Dal Bello B, Semigran MJ, Bielsa-Masdeu A, Dec GW, Israels S, Ballester M, Virmani R, Saxena S, Kharbanda S (1999) Apoptosis in heart failure: Release of cytochrome-C from mitochondria and activation of caspase-3 in human cardiomyopathy. Proc Natl Acad Sci USA; 96:8144-9.

Pagliaro PR, Rastaldo R, Penna C, Mancardi D, Cappello S, Losano GA (2004) Nitric oxide (NO)-cylic guanosine monophosphate (cGMP) pathway is involved in ischemic postconditioning in the isolated rat heart. Circulation; 110:III-136

Park HH, Lo YC, Lin SC, Wang L, Yang JK, Wu H (2007) The death domain superfamily in intracellular signaling of apoptosis and inflammation. Annu Rev Immunol; 25: 561-586

Penna C, Rastaldo R, Mancardi D, Raimondo S, Cappello S, Gattullo D (2006) Postconditioning induced cardioprotection requires signaling through a redox-sensitive mechanism, mitochondrial ATP-sensitive K+ channels and protein kinase C activation. Basic Res Cardiol; 101:180-189

Philipp SD, Downey JM, Cohen MV (2004) Postconditioning must be initiated in less than 1 minute following reperfusion and is dependent on adenosine receptors and P13-kinase. Circulation; 110:III-168

Piantadosi CA, Tatro LG, Whorton AR (2002) Nitric oxide and differential effects of ATP on mitochondrial permeability transition. Nitric Oxide-Biology and Chemistry; 6:45–60

Ping P, Murphy E (2000) Role of p38 mitogen-activated protein kinases in preconditioning: a detrimental factor or a protective kinase? Circ Res; 86:921–922

Piper HM (1989) Energy deficiency, calcium overload or oxidative stress: possible causes of irreversible ischemic myocardial injury. Klin Wochenschr; 67:465–476

Piper HM, Garcia-Dorado D, Ovize M (1998) A fresh look at reperfusion injury. Cardiovasc Res; 38:291–300

Piper HM, Garcia-Dorado D (1999) Prime cause of rapid cardiomyocytes death during reperfusion. Ann Thorac Surg; 68:1913–1919

Piper HM, Schafer AC (2004) The first minutes of reperfusion: a window of opportunity for cardioprotection. Cardiovasc Res; 61:365–371

Riedl SJ (2001) Structural basis for the inhibition of caspase-3 by XIAP. Cell; 104: 791–800

Rothe M, Pan MG, Henzel WJ, Ayres TM, Goeddel DV (1995) The TNFR2–TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. Cell; 83: 1243–1252

Roy N, Mahadevan MS, McLean M, Shutler G, Yaraghi Z, Farahani R, Baird S, Besner-Johnston A, Lefebvre C, Kang X (1995) The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy. Cell; 80: 167–178

Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC (1997) The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO J; 16: 6914–6925

Salvesen GS, Duckett CS (2002) IAP proteins: blocking the road to death's door. Nat Rev Mol Cell Biol; 3:401–410

Scarabelli T, Stephanou A, Rayment N, Pasini E, Comini L, Curello S, Ferrari R, Knight R, Latchman D (2001) Apoptosis of endothelial cells precedes myocyte cell apoptosis in ischemia/reperfusion injury. Circulation; 104:253Y256

Scarabelli TM, Stephanou A, Pasini E, Comini L, Raddino R, Knight RA, Latchman DS (2002) Different signaling pathways induce apoptosis in endothelial cells and cardiac myocytes during ischaemia/reperfusion injury. Circ Res; 90:745-8.

Scarabelli TM, Knight R, Stephanou A, Townsend P, Chen-Scarabelli C, Lawrence K, Gottlieb R, Latchman D, Narula J (2006) Clinical implications of apoptosis in ischemic myocardium. Curr Probl Cardiol; 31(3):181-264.

Scheubel RJ, Bartling B, Simm A, Silber RE, Drogaris K, Darmer D, Holtz J (2002) Apoptotic pathway activation from mitochondria and death receptors without caspase-3 cleavage in failing human myocardium: fragile balance of myocyte survival? J Am Coll Cardiol; 39:481-8.

Silke J, Kratina T, Chu D, Ekert PG, Day CL, Pakusch M, Huang DCS & Vaux DL (2005) Determination of cell survival by RING-mediated regulation of inhibitor of apoptosis (IAP) protein abundance. Proc Natl Acad Sci USA; 102: 16182-16187

Skyschally A, van Caster P, Iliodromitis EK, Schulz R, Kremastinos DT, Heusch G (2009) Ischemic postconditioning: experimental models and protocol algorithms. Basic Res Cardiol. 104(5):469-83

Spierings DC, de Vries EG, Vellenga E, van den Heuvel FA, Koornstra JJ, Wesseling J, Hollema H, de Jong S (2004) Tissue distribution of the death ligand TRAIL and its receptors. J Histochem Cytochem; 52:821-31

Srinivasula SM, Hegde R, Saleh A, Datta P, Shiozaki E, Chai J, Lee RA, Robbins PD, Fernandes-Alnemri T, Shi Y, Alnemri ES (2001) A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. Nature; 410: 112–116

Srinivasula SM, Datta P, Kobayashi M, Wu JW, Fujioka M, Hegde R, Zhang Z, Mukattash R, Fernandes-Alnemri T, Shi Y, Jaynes JB, Alnemri ES (2002) Sickle, a novel Drosophila death gene in the reaper-hid-grim region, encodes an IAP-inhibitory protein. Curr Biol; 12:125–130

Srinivasula SM & Jonathan D.Ashwell (2008) IAPs : What's in a name? Mol Cell; 30(2): 123-135

Steenbergen C, Hill ML, Jennings RB (1985) Volume regulation and plasma membrane injury in aerobic, anaerobic, and ischemic myocardium in vitro. Effects of osmotic cell swelling on plasma membrane integrity. Circ Res; 57:864–875

Stehlik C, de Martin R, Kumabashiri I, Schmid JA, Binder BR, Lipp J (1998) Nuclear factor (NF)-κB-regulated X-chromosome-linked iap gene expression protects endothelial cells from tumor necrosis factor-α-induced apoptosis. J Exp Med; 188:211–216

Stennicke HR, Deveraux QL, Humke EW, Reed JC, Dixit VM, Salvesen GS (1999) Caspase-9 can be activated without proteolytic processing. J Biol Chem; 274: 8359–8362

Stephanou A, Brar B, Liao Z, Scarabelli T, Knight RA, Latchman DS (2001) Distinct initiator caspases are required for the induction of apoptosis in cardiac myocytes in ischaemia versus reperfusion injury. Cell Death Differ; 8:434-5.

Sun C, Cai M, Gunasekera AH, Meadows RP, Wang H, Chen J, Zhang H, Wu W, Xu N, Ng SC, Fesik SW (1999) NMR structure and mutagenesis of the inhibitor-of-apoptosis protein XIAP. Nature; 401: 818–822

Sun HY, Wang N-P, Kerendi F, Halkos ME, Kin H, Guyton RA, Vinten-Johansen J, Zhao Z-Q (2005) Hypoxic postconditioning reduces cardiomyocytes loss by inhibiting the generation of reactive oxygen species and intracellular calcium overload. Am J Physiol; 288: H1900-H1908

Sun HY, Wang NP, Halkos M, Kerendi F, Kin H, Guyton RA (2006) Postconditioning attenuates cardiomyocytes apoptosis via inhibition of JNK and p38 MAPK pathways. Apoptosis; 11:1583-93

Suzuki Y, Nakabayashi Y, Takahashi R (2001) Ubiquitinprotein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in Fas-induced cell death. Proc Natl Acad Sci USA; 98:8662–8667

Tamm I, Kornblau SM, Segall H, Krajewski S, Welsh K, Kitada S, Scudiero DA, Tudor G, Qui YH, Monks A, Andreeff M, Reed JC (2000) Expression and prognostic significance of IAP-family genes in human cancers and myeloid leukemias. Clin Cancer Res; 6: 1796–1803

Tsang A, Hausenloy DJ, Mocanu MM, Yellon DM (2004) Postconditioning: a form of “modified reperfusion” protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. Circ Res; 95:230–232

Uren AG, Coulson EJ, Vaux DL (1998) Conservation of baculovirus inhibitor of apoptosis repeat proteins (BIRPs) in viruses, nematodes, vertebrates and yeasts. Trends Biochem Sci; 23: 159–162

Uren AG, Beilharz T, O'Connell MJ, Bugg SJ, van Driel R, Vaux DL, Lithgow T (1999) Role for yeast inhibitor of apoptosis (IAP)-like proteins in cell division. Proc Natl Acad Sci USA; 96:10170–10175

Vucic D, Stennicke HR, Pisabarro MT, Salvesen GS, Dixit VM (2000) ML-IAP, a novel inhibitor of apoptosis that is preferentially expressed in human melanomas. Curr Biol; 10: 1359–1366

Werner N, Wassmann S, Ahlers P, Kosiol S, Nickenig G (2006) Circulating CD31+/annexin V+ apoptotic microparticles correlate with coronary endothelial function in patients with coronary artery disease. Arterioscler Thromb Vasc Biol; 26:112–6

Weissman AM (2001) Themes and variations on ubiquitination. Nature Rev. Mol Cell Biol; 2:169–178

Yang Y, Fang S, Jensen JP, Weissman AM, Ashwell JD (2000) Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. Science; 288:874–877

Yang XM, Downey JM, Cohen MV (2003) Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by activation of ERK and production of nitric oxide. Circulation; (Suppl) 108:158

Yang XM, Proctor JB, Cui L, Krieg T, Downey JM, Cohen MV (2004) Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by targeting cell signaling pathways. J Am Coll Cardiol; 44:1103–1110

Zachariou A, Tenev T, Goyal L, Agapite J, Steller H, Meier P (2003) IAP antagonists exhibit non-redundant modes of action through different DIAP1 binding. EMBO J; 22: 6642-6652

Zhao ZQ, McGee DS, Nakanishi K, Toombs CF, Johnston WE, Ashar MS, Vinten-Johansen J (1993) Receptor-mediated cardioprotective effects of endogenous adenosine are exerted primarily during reperfusion after coronary occlusion in the rabbit. Circulation; 88:709–719

Zhao ZQ, Vinten-Johansen J (2002) Myocardial apoptosis and ischemic preconditioning. Cardiovasc Res; 55: 438-55.

Zhao Z-Q, Corvera JS, Wang N-P,Guyton RA, Vinten-Johansen J (2002) Reduction in infarct size and preservation of endothelial function by ischemic postconditioning: Comparison with ischemic preconditioning. Circulation; 106:II314

Zhao Z-Q, Corvera JS, Halkos ME, Kerendi F, Wang N-P, Guyton RA, Vinten-johansen J (2003) Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol; 285:579–588

Zhao ZQ, Morris CD, Budde JM, Wang NP, Muraki S, Sun HY, Guyton RA (2003) Inhibition of myocardial apoptosis reduces infarct size and improves regional contractile dysfunction during reperfusion. Cardiovasc Res; 59:132-42

Zhao Y, Conze DB, Hanover JA, Ashwell JD (2007) Tumor necrosis factor receptor 2 signaling induces selective c-IAP1-dependent ASK1 ubiquitination and terminates mitogen-activated protein kinase signaling. J Biol Chem; 282:7777–7782

7. Summary

Postconditioning (intermittent hypoxia at the onset of reperfusion), a strategy known to effectively reduce reperfusion injury, is well proven in cardiomyocytes. However, little is known about postconditioning in endothelial cells and whether it plays a role in anti-apoptosis, which is the predominant form of cell death in endothelial cells. Here the protective effect of postconditioning in endothelial cells and the molecular mechanisms involved were studied, focusing on the inhibitors of apoptosis proteins (IAPs) as potential anti-apoptotic candidates induced by hypoxia. Exposure of human umbilical vein endothelial cells to severe hypoxia (Po_2 < 1 mmHg) for 2 h caused a 2.1 ± 0.3 fold increase in caspase-3 cleavage, 6 h after reoxygenation and a 2.3 ± 0.2 fold increase in apoptosis (annexin V staining) 24 h after reoxygenation. Postconditioning abolished hypoxia/reoxygenation-induced apoptosis in endothelial cells. Quest for possible anti-apoptotic molecules led to the observation that cIAP2 but not its close homologue cIAP1 or XIAP, is upregulated during hypoxia and reduces to basal level with the onset of reoxygenation. Importantly, cIAP2 could be maintained by postconditioning in an ERK1/2 and PI3-Kinase dependant manner. Hypoxia as well as postconditioning induced an interaction between cIAP2 and procaspase-3 (co-immunoprecipitation and co-localization in immunochemistry), suggesting a mechanism by which cIAP2 counteracts hypoxia/reoxygenation-induced apoptosis. Downregulation of cIAP2 with siRNA enhanced hypoxia/reoxygenation-induced apoptosis and abolished the protective effect of postconditioning. Maintenance of cIAP2 by postconditioning in the intact vessel confirms the patho-physiological significance of the finding. The present study shows for the first time that postconditioning can protect endothelial cells against hypoxia/reoxygenation-induced apoptosis. This protective effect is conferred by the cIAP2, which is expressed during hypoxia and could be maintained at an elevated level by postconditioning, interacting with procaspase-3.

8. Zusammenfassung

Postkonditionierung (Phasen intermittierender Hypoxie zu Beginn einer Reperfusion) ist eine wirksame Strategie, die das Ausmaß des kardiomyozytären Reperfusionsschadens reduzieren kann. Es ist jedoch unklar, welche Wirkung die Postkonditionierung auf die endotheliale Apoptose hat, welche die vorherrschende Form des Zelluntergangs darstellt. Ziel dieser Arbeit war es daher, die protektive Wirkung der Postkonditionierung und die zu Grunde liegenden molekularen Mechanismen in Endothelzellen zu untersuchen. Der Fokus lag dabei auf einer Familie Apoptose-inhibierender Proteine (*Inhibitors of Apoptosis Proteins,* IAPs) als potentielle anti-apoptotische Kandidaten, die durch Hypoxie induziert werden. Humane Endothelzellen aus Nabelschnurvenen, die einer zweistündigen Hypoxie ($Po_2 < 1$ mmHg) ausgesetzt wurden, zeigten nach sechstündiger Reoxygenierung einen 2,1 ± 0,3-fachen Anstieg der Caspase 3-Aktivität (Westernblot) und nach 24 Stunden Reoxygenierung eine 2,3 ± 0,2-fache Steigerung der Apoptoserate (Annexin V-Färbung). Postkonditionierung im Anschluss an die zweistündige Hypoxie reduzierte die Hypoxie/Reoxygenation (H/R)-induzierte Apoptose. Die Suche nach möglichen antiapoptotischen Molekülen ergab, dass der zelluläre Gehalt an cIAP2, nicht jedoch der von cIAP1 oder XIAP, während Hypoxie anstieg. Mit Einsetzen der Reoxygenation sank der cIAP2-Gehalt wieder auf das Ausgangsniveau. Durch Postkonditionierung konnte ERK1/2- und PI3-Kinase-abhängig der cIAP2-Gehalt auf dem post-hypoxischen Niveau gehalten werden. Hypoxie sowie Postkonditionierunng induzieren die Interaktion zwischen cIAP2 und Pro-Caspase 3 (Co-Immunoprezipitation bzw. Co-Lokalisation im immunzytologischen Nachweis). Die gezielte Herunterregulation von cIAP2 durch Einsatz von siRNA führte zu einer Verstärkung der H/R-induzierten Apoptose und hob die protektive Wirkung der Postkonditionierung. Die Aufrechterhaltung von cIAP konnte in Untersuchungen an intakten Gefäßen bestätigt werden. Die vorliegende Arbeit erstmalig, dass Postkonditionierung Endothelzellen vor einer H/R-induzierten Apoptose schützt. Dieser Effekt wird durch cIAP2 vermittelt, das während Hypoxie verstärkt exprimiert wird und dessen Konzentration durch Postkonditionierung auf dem posthypoxischen Niveau gehalten werden kann und mit Procaspase-3 interagiert.

9. Declaration

"I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation."

Krishnaveni Gadiraju
Giessen, March 2010

10. Acknowledgements

I would like to first thank my supervisor Dr. Thomas Noll, for his valuable guidance and for providing a remarkable insight into my project. Apart from excellent scientific advice, he greatly helped me improve my analytical thinking, reasoning and presentation skills. I thank him for being such a kind guide and a wonderful human being.

I sincerely thank the former director of the Institute of Physiology, Prof. Dr. Dr. H. M. Piper, for providing me a place in the Institute and for all the facilities required to carry on my project

Many thanks to Dr. Frauke V. Härtel, who constantly challenged me with new ideas through out the course of my PhD and corrected my thesis.

I extend my gratitude to Dr. Sedding for kindly allowing me to practice Immonohistochemistry in his lab.

Very Special thanks to Hermann Holzträger, Anna Reis and Annika Krautwurst for their technical assistance, which greatly speeded up the progress of my work.

I thank all my lab mates, especially, Daniel, Tatyana, Marion, Aslam, Arshad, Assad, Kiran and Sabiha for their company and cooperation.

Finally, I thank all my family, especially, my father, mother, chinnana, my grandparents, and my husband, for being such a source of joy, strength and inspiration, through out the course of my PhD and always.

Thanks!

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

12. Publications

Abstracts:

- **Krishnaveni Gadiraju**, Frauke V. Haertel, H. M. Piper, Thomas Noll (2007) Stabilization of cIAP2 during postconditioining protects endothelial cells from reperfusion injury. **Circulation***.* 2007; 116(II) 29 Abstract 248 (American Heart Association Scientific sessions 2007, Orlando)

- **Krishnaveni Gadiraju**, Frauke V. Haertel, H. M. Piper, Thomas Noll (2008) Postconditioning protects endothelial cells from hypoxia-reoxygenation induced apoptosis - Role of cIAP2. **Hämostaseologie**. 2008; 28:1-98 (52nd Annual meeting of GTH 2008, Wiesbaden)

- **Krishnaveni Gadiraju**, Frauke V. Haertel, H. M. Piper, Thomas Noll (2008) Inhibitor of apoptosis 2 (cIAP2) elevated during postconditioning protects endothelial cells from reperfusion induced apoptosis. **Eur J Cell Biology**. 2008; 87S1: S58 (31st Annual meeting of German Society for Cell Biology (DGZ) 2008, Marburg)

- **Krishnaveni Gadiraju**, Frauke V. Haertel, H. M. Piper, Thomas Noll (2008) Role of Inhibitor of apoptosis 2 in endothelial postconditioning. **Acta Physiologica.** 2008; 192:S663 (87th Annual meeting of Deutsche Physiologische Gesellschaft 2008, Cologne)

- Daniel Urban, Frauke V. Haertel, **Krishnaveni Gadiraju**, H. M. Piper, Thomas Noll (2008) ATP released during hypoxia/reoxygenation activates a P2Y receptor mediated survival mechanism in human endothelial cells. **Circulation** 2008; 118 S. 563 Abstract 5506 (American Heart Association Scientific sessions 2008, New Orleans)

- **Krishnaveni Gadiraju**, Frauke V. Haertel, H. M. Piper, Thomas Noll (2008) Rolle des Inhibitor of Apoptosis 2 beim endothelialen postconditioning. (74th Annual meeting of German Cardiac Society (DGK) 2008, Mannheim)

PhD Publication:

- **Krishnaveni Gadiraju**, Frauke V. Haertel, Daniel Urban, Tatyana S. Dimitrova, Daniel Sedding, H. M. Piper, Thomas Noll (2009) Postconditioning protects endothelial cells from hypoxia/reoxygenation-induced apoptosis - Role of cIAP2. (Manuscript in preparation)