Abrogation of Anthracycline and Ischemia-Reperfusion Induced Injury by Cell Signaling Modulators

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SCIENTIFIC ENVIRONMENT

The work presented in this thesis was performed at the Department of Biomedicine, part of the Faculty of Medicine and Dentistry at the University of Bergen. The experimental work has been conducted in the Heart and Circulation Group as well as the Translational Research Group, with Anne Kristine Jonassen and Stein Ove Døskeland as supervisors.

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Norwegian Women's Public Health Association

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With love, Anita

ABSTRACT

Chemotherapy has a long history in cancer treatment, and the anthracycline used in **Paper I and II** are among the most effective anti-cancer drugs developed. Unfortunately, the use of anthracyclines is dose-restricted due to the risk of cumulative toxicity in healthy tissue, most notably in the heart. Targeted therapy using *all-trans* retinoic acid (ATRA) is used to differentiate, and hence, eliminate acute promyelocytic leukemia (APL) cells, and is combined with low dose anthracyclines to remove live or ATRA resistant cells. Recently, cAMP was recommended as adjuvant to standard APL therapy since it enhances ATRA induced differentiation of APL cells. In **Paper I** we demonstrate that cAMP in fact *abrogate* the anti-cancer effect of the anthracycline Daunorubicine (DNR) in blasts from APL patients and also in ATRA-sensitive and insensitive APL cell lines. The protection was dependent on the cytoplasmic PKA-type I rather than perinuclear PKA type-II, and was associated with (inactivating) phosphorylation of pro-apoptotic Bad and (activating) phosphorylation of the acute myeloid leukemia (AML) oncogene cAMP-responsive element binding protein (CREB). Mice with orthotopic NB4 cell leukemia showed a more rapid disease progression when given cAMP-increasing agents (prostaglandin E2 analog and aminophylline), both with and without DNR chemotherapy. Together this suggests that the beneficial pro-differentiating and non-beneficial pro-survival APL cell effects of cAMP should be weighed against each other. Although the mechanism behind anthracycline mediated cardiotoxicity is highly contested, intramyocardial production of reactive oxygen species (ROS) is generally accepted as a strong candidate, and has increased the focus on antioxidants in cardioprotective strategies. In **Paper II,** we demonstrate that Red Palm Oil (RPO) supplemented diet during chemotherapy attenuate cardiotoxic side-effects of daunorubicin, by improving aortic output and coronary flow in the isolated working rat heart model. Improved hemodynamic was accompanied by stabilization of important antioxidant systems (SOD1 and NOS1) and reduction of stress-induced MAPK activation.

While cancer is a consequence of restricted cell death, the opposite scenario, with increased cell death is an important component of ischemia-reperfusion induced injury. Since prolonged ischemia may lead to cardiac cell death, rapid and adequate reperfusion is a necessity to salvage the compromised cardiac tissue. Paradoxically, reperfusion *per se* also induces cell death (lethal reperfusion injury) a process involving opening of the mitochondrial permeability transition pore (mPTP). Different ways to limit or delay cardiomyocyte cell death have emerged in the laboratory setting, and are evaluated as clinical candidates to further improve the outcome of patients with acute myocardial infarction (AMI). **In paper III and IV** we utilize the Langendorff perfusion setup for the *ex vivo* rat heart, to evaluate different therapeutic strategies to reduce ischemia-reperfusion induced injury. In **Paper III** we show that corticotropin releasing factor (CRF) significantly reduce infarct size when applied to the heart prior to a lethal ischemic insult, and was cytoprotective in neonatal mouse cardiomyocytes when added prior to a lethal simulated ischemic event (hypoxia). CRF was however not protective when administered at the point of ischemic reperfusion or hypoxic reoxygenation. The cardioprotective effects of CRF was mediated via activation of PKA and PKC dependent signaling pathways downstream of CRF receptor type 2 (CRFR2). In **Paper IV** we evaluated the possible additive effects of combining known cardioprotective treatments. We found that combining insulin reperfusion therapy with direct Glycogen synthase kinase 3 β (GSKβ) inhibition at reperfusion did not confer any additive effect, but showed similar cardioprotection as seen when the treatments were administered separately. Surprisingly, we found that combining either of the two pharmacologic interventions with ischemic postconditioning (IPost) abrogated all cardioprotective effect. This loss of cardioprotection was accompanied with blunted Akt phosphorylation. To our knowledge, we are the first to demonstrate the loss of protection when combining two otherwise cardioprotective regimes.

LIST OF PUBLICATIONS

Paper I **Gausdal G, Wergeland A, Skavland J, Nguyen E, Pendino F, Rouhee N, McCormack E, Herfindal L, Kleppe R, Havemann U, Schwede F, Bruserud O, Gjertsen BT, Lanotte M, Ségal-Bendirdjian E, Døskeland SO.** Cyclic AMP can promote APL progression and protect myeloid leukemia cells against anthracycline-induced apoptosis. *Cell Death Dis.* 2013 Feb 28;4:e516. doi: 10.1038/cddis.2013.39.

Paper II **Wergeland A, Bester DJ, Sishi BJN, Engelbrecht AM, Jonassen AK, Van Rooyen J.** Dietary red palm oil protects the heart against the cytotoxic effects of anthracycline. *Cell Biochem Funct. 2011 Jul 29(5):356-64*

Paper III **Jonassen AK, Wergeland A, Helgeland E, Mjøs OD, Brar BK.** Activation of corticotropin releasing factor receptor type 2 in the heart by corticotropin releasing factor offers cytoprotection against ischemic injury via PKA and PKC dependent signaling. *Regul Pept. 2012 Feb 10; 174(1-3):90-7*

Paper IV **Helgeland E[1], Wergeland A[1], Breivik L, Askeland M, Jonassen AK.** Abrogated Cardioprotection and Blunted Akt Phosphorylation when Combining Ischemic Postconditioning with Pharmacological Reperfusion Therapy

[1]These authors contributed equally to the paper

Manuscript

CONTENT

ABBREVIATIONS

ACTH	adrenocorticotropic hormone
ACD	autophagic cell death
AKAP	A-kinase anchoring protein
Akt/PKB	Protein Kinase B
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
ATO	arsenic trioxide
ATRA	all-trans retinoic acid
Bcl-2/X_L	B-cell lymphoma 2/extra large
BH domain	Bcl-2 homology domain
cAMP	3'-5'-cyclic adenosine monophosphate
cNMP	cyclic nucleotide monophosphate
CHF	congestive heart failure
CREB	cAMP-responsive element binding protein
CRF	corticotropin releasing factor
CRFR	corticotropin receptor
CVD	cardiovascular disease
CR	complete remission
DFS	disease-free survival
DISC	death inducing signaling complex
DNA	deoxyribonucleic acid
DNR	daunorubicin
DOX	doxorubicine
eNOS	endothelial nitrogenoxide synthase

Epac	exchange proteins directly activated by cAMP
ERK	extracellular signal-regulated kinase
GEF	guanine nucleotide exchange factor
GIK	glucose-insulin-kalium(potassium)
GSK3	glycogen synthase kinase 3
GTP	guanosine-5'-triphosphate
IDA	idarubicin
IL-6	interleukin-6
INSR	insulin receptor
IC	ischemic conditioning
IPC	ischemic preconditioning
IPost	ischemic postconditioning
JAK	janus kinase
KHB	krebs-henseleit buffer
MAPK	mitogen-activated protein kinases
Mcl-1	induced myeloid leukemia cell differentiation protein
MEK	mitogen-activated protein kinase kinase
mPTP	mitochondrial permeability transition pore
NADH	nicotinamide adenine dinucleotide
NB4-RAr	NB4-retinoic acid resistant
NO	nitrogen oxide
PCD	programmed cell death
PCI	percutaneous coronary intervention
PDE	phosphodiesterase
PI3K	phosphatidylinositide 3-kinases
PKA	protein kinase A

PKC	protein kinase C
PKG	protein kinase G
PML	promyelocytic leukemia protein
RA	retinoic acid
RAR	retinoic acid receptor
RAS	retinoic acid syndrome
RISK	reperfusion injury salvage kinase
ROS	reactive oxygen species
RPO	red palm oil
RTK	receptor tyrosine kinase
SAFE	survivor activating factor enhancement
SOD	superoxide dismutase
STAT	signal transducer and activator of transcription
TNF-α	tumor necrosis factor- α
WHO	world health organization

1. INTRODUCTION

1.1 Preface

This thesis represents basic research devoted to reduce the harmful consequence of myocardial ischemia, and also modulate classical anti-cancer therapy to alleviate drug associated toxicity. I have focused on modulation of cell death in the two different pathologic situations represented by ischemia-reperfusion induced injury and anthracycline induced apoptosis/cardiotoxicity.

Cardiovascular diseases are the leading cause of death globally, estimated to 17.3 mill deaths in 2008, from where 42% were due to myocardial ischemia [1]. Myocardial ischemia is defined as "a condition in which the coronary blood flow is inadequate to permit the maintenance of a steady state metabolism" [2]. Therefore, rapid and adequate reperfusion of the compromised cardiac tissue is necessary, as prolonged ischemia will lead to cardiac cell death. However, reperfusion itself induces cardiac injury, e.g. lethal reperfusion injury, contributing to the total infarct size and presents as an important target for additional cardioprotection. Although the precise mechanism needs to be fully elucidated, it is clear that cell death is the most important consequence of both ischemic as well as reperfusion induced injury. Contrary, attenuation of cell death is the key feature of cancer. Cancer is a heterogeneous group of diseases which are increasing both in incidence and mortality. Chemotherapy includes more than hundred different drugs used for cancer treatment, and the anthracyclines described and used in this thesis (daunorubicine-DNR) has been among the most effective antitumor drugs ever developed. Unfortunately, due to the risk of cumulative cardiotoxicity the use of high dose anthracycline is hampered and may reduce its therapeutic potential. This thesis explores the possibility of, indirectly or directly, reducing the toxic side-effects of anthracycline treatment, by combining classical treatment with novel agents (**Paper I and II**) and also reducing ischemia-reperfusion induced cardiac injury using novel therapeutic strategies (**Paper III and IV**).

1.2 Cell death

Precise description of naturally occurring cell death was reported in more than 100 papers as early as the 19th century (reviewed in [3]). However, the introduction of the term Programmed Cell Death (PCD) came in 1964 when Locksin and Williams realized that cell death occur at predicted time and places during development, and are programmed into the developmental plan of an organism [4]. Eight years later, Kerr, Wyllie and Currie further specialized the term by introducing apoptosis as a special variant of PCD. They described precise morphological features of apoptosis such as cell shrinkage and fragmentation, followed by phagocytosis of the apoptotic bodies. This process is highly distinguished from the pathological variant of cell death called necrosis (also called oncosis), where the cells tend to swell and rupture with subsequent inflammation [5] (Fig. 1.1). Necrosis has traditionally been considered as an uncontrolled process, but accumulating evidence is now suggesting that necrosis can be finely regulated [6]. This process has been termed necroptosis and involves signaling via receptor-associated adaptor kinase RIP1 [7]. Finally, autophagy is also linked to PCD. Autophagy was described already in the 1960s, and is a catabolic mechanism that involves degradation of damaged and dysfunctional cellular components through the lysosomal machinery [8]. The idea of autophagic cell death (ACD) gained its momentum in the 1990s with the discovery of the autophagy-related genes (ATG) [9] and the observation of caspase-independent cell death with non-apoptotic morphology [10]. However, ACD is only a morphological definition based on accumulation of autophagosomes in dying cells, and there is still an on-going debate whether ACD is actually cell death *with* autophagy rather than cell death *by* autophagy [11].

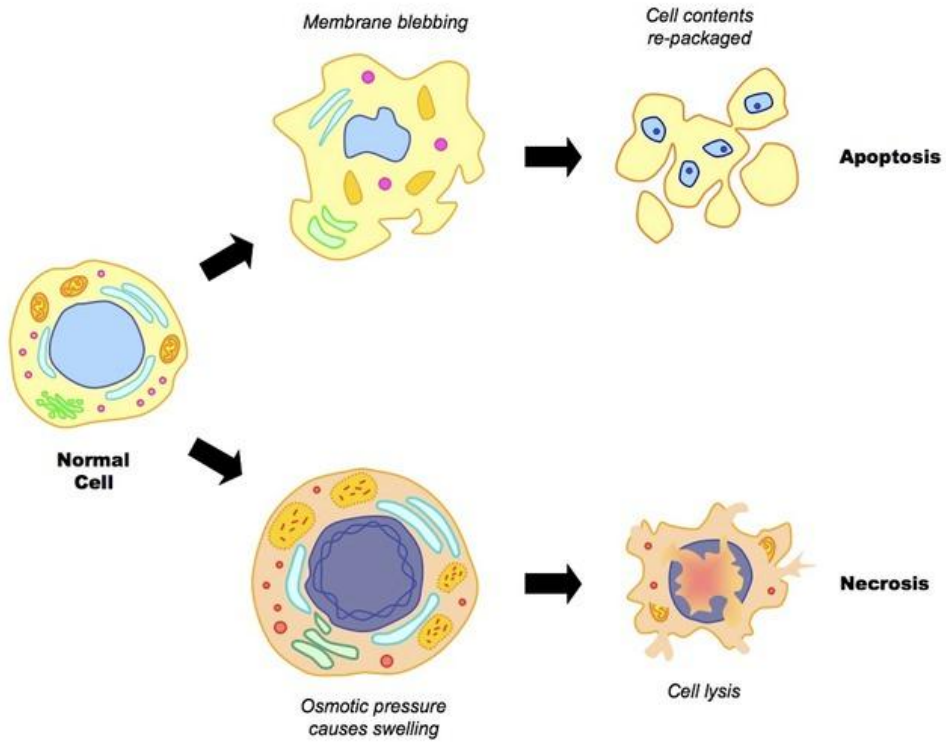


Figure 1.1 Two important types of cell death are depicted. Necrosis involves cell swelling and rupture of the cell membrane, with release of intracellular components leading to inflammatory reactions. Apoptosis is an organized and controlled process with cell shrinkage and chromatin condensation, followed by formation of apoptotic bodies which are phagocytized by surrounding cells without induction of inflammation (figure from [12]).

1.2.1 **Cell Death in "health and disease"**

In the adult organism, apoptosis is important for the clearance of damaged cells and is necessary for normal cell maintenance by acting as a counterbalance to cell proliferation [13]. In average, 50-70 billion cells undergo apoptosis every day in an adult organism [14]. Malfunction of the apoptotic signaling/machinery may have severe consequences as both too little and too much cell death result in pathological conditions. Neurodegenerative disorders such as Alzheimer's and Parkinson's, immunodeficiency syndrome (AIDS) and ischemia-reperfusion injury (myocardial infarction) are all associated with increased apoptosis. Ischemia-reperfusion induced injury is a result of both necrosis and apoptosis [15]. Whether apoptosis/necrosis

represents discriminate or more overlapping events leading to cell death is unclear, but it seems like necrosis is mainly found in the central part of the infarcted area, while apoptosis is more apparent at the border zone of the infarct [16]. In addition, recent reports indicate that apoptosis is accelerated by reperfusion [17-20]. Regarding autophagy, compelling evidence indicates up regulated levels of autophagy during ischemia-reperfusion [21, 22], however, the role of autophagy, whether being detrimental or beneficial to the ischemic myocardium, is a topic of growing interest and debate [23]. Contrary to ischemia-reperfusion injury, autoimmune disorders like Lupus, a variety of viral infections and last but not least, cancer, is associated with attenuation of apoptosis (for review see [24]). Evasion of apoptosis is actually one of the key components of malignant transformation [25], and also an important mediator in the development of therapy resistance [26]. Therefore, detailed knowledge about the molecular mechanisms of both successful and failed treatments will facilitate an improved approach for anti-cancer treatment.

1.2.2 **Regulation of apoptosis**

Apoptosis is usually transmitted via two major signaling pathways (Fig.1.2). A variety of cellular stresses such as hypoxia and exposure to chemotherapeutic agents initiate the intrinsic pathway with release of several apoptosis related proteins from the mitochondrial inter-membrane space [27]. Released Cytochrome C, Apaf-1 and procaspase-9 oligomerize to form the “apoptosome” which activates caspase-3 and induce proteolysis of hundreds of proteins leading to apoptosis [28]. In the e*xtrinsic* pathway, cell-surface receptors connect death-promoting extracellular signals to apoptosis execution inside the cell. Ligand binding initiates assembly of the Death Inducing Signaling Complex (DISC), auto-activation of caspase-8 followed by caspase-3 activation and finally initiation apoptosis [29]. Another caspase-8 substrate is the BH3-only protein Bid. When activated, Bid translocate to the mitochondria, connecting the extrinsic pathway to the mitochondrial intrinsic pathway [30], and thereby amplifying the initial death receptor signal. The integrity of the mitochondrial membrane is regulated by the Bcl-2 family of proteins, which is divided into three groups. The anti-apoptotic proteins containing all four BH domains; Bcl-2, Bcl-X_L,

Bcl-W and Mcl-1[31], the pro-apoptotic multi-BH domain proteins Bax, Bak and Bok [32] and the pro-apoptotic BH3-only proteins Bim, Bad, Bid, Puma and Noxa [33]. Members from the different groups may interact as homo- or heterodimers, and these interactions between pro and anti- apoptotic proteins act as checkpoints determining the cell fate.

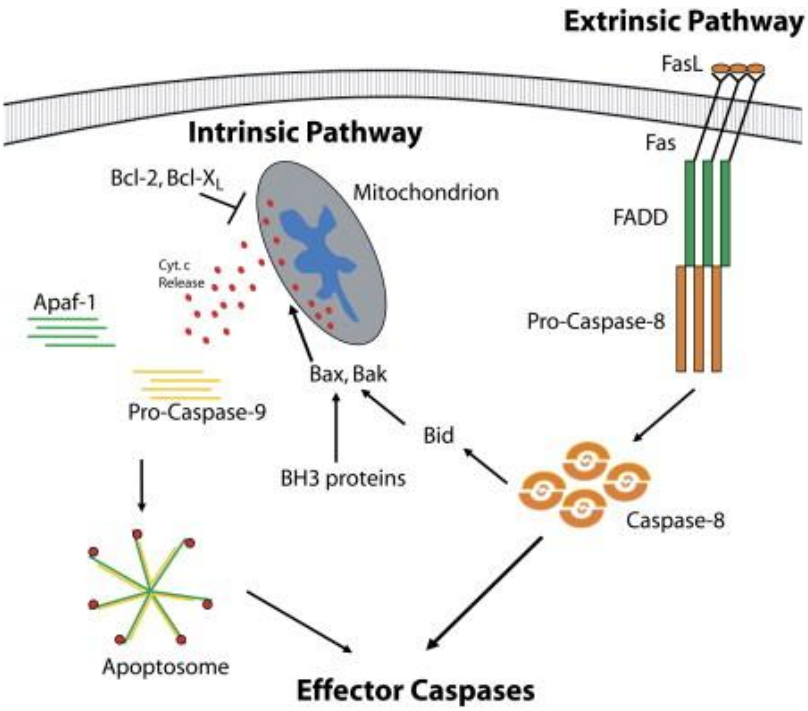


Figure 1.2 Apoptotic signaling induced via death receptor activation (extrinsic pathway) and stress-induced stimuli (intrinsic pathway). Activation of death receptors leads to recruitment of specific adaptor proteins (FADD) and consequently recruitment and activation of pro-caspase 8. In the intrinsic pathway the mitochondria is perturbed in response to stress, which leads to release of proteins such as cytochrome *c* from the inter-mitochondrial membrane space. The release of mitochondrial proteins is regulated by anti-apoptotic proteins such as Bcl-2, Bcl-X_L and Mcl-1 and pro-apoptotic proteins such as Bax, Bak and BH3-only proteins such as tBid and Bad. Once released, cytochrome *c* initiates the formation of the apoptosome complex and activation of the initiator caspase 9. Activated caspase 8 and 9 further activates the effector caspases 3,6 and 7 responsible for the cleavage of important cellular substrates, giving the classic apoptotic phenotype (figure from [34]).

1.3 Acute myeloid leukemia

Acute myeloid leukemia (AML) is an aggressive hematological malignant disorder, characterized by accumulation of immature myeloid progenitor cells in the bone marrow and peripheral blood. The pathology is due to a block in differentiation in the early stage of hematopoiesis, combined with dysregulation of proliferation and apoptosis [35]. Since AML is a heterogeneous group of diseases the symptoms are diverse and nonspecific, but they are usually directly attributed to the leukemic infiltration of the bone marrow with concomitant cytopenia. Typical clinical signs are fatigue, hemorrhage, infections and fever due to a decrease in platelets, red and white blood cells [36]. The major hypothesis of leukemogenesis is known as "the two hit model", and was first presented by Gilliland in 2001. This hypothesis implies that two different mutations, in a transcription factor and a tyrosine kinase, will impair differentiation and confer survival and/or proliferative advantages, and are both necessary for AML development [37]. Acute promyelocytic leukemia (APL) is a subtype of AML, and accounts for more than 10% of all AML cases. It is characterized by accumulation of immature promyelocytes in the bone marrow and is highly associated with chromosomal translocation of the retinoic acid receptor alpha (RARα) on chromosome 17 [38]. RARs belongs to a family of nuclear hormone receptors which in complex with retinoic X receptor (RXR) acts as transcriptional repressors or activators [39]. In most chromosomal translocations (>98%), RARα is fused to the promyelocytic leukemia protein (PLM) gene on chromosome 15, resulting in the t(15;17) chromosomal translocation, generating the PML-RARα fusion protein. This fusion protein blocks differentiation by acting as a transcriptional repressor directly [40] or via recruitment of various partners [41, 42]. In addition, the fusion protein induces hypermethylation with silencing of genes necessary for promyelocytic differentiation [43, 44]. The prognosis of APL has changed dramatically the last three decades from being the worst subtype of AML to becoming the most favorable, all due to the first example of successful molecular targeted therapy.

1.3.1 **The successes story of APL treatment**

Before the mid-80s, when chemotherapy was the sole agent in acute promyelocytic leukemia (APL) treatment, this malignancy was considered highly fatal with only 40% 5-year disease free survival (DFS), despite a complete remission rate (CR) of 80% [45]. However, in 1985 it was discovered that *all-trans* retinoic acid (ATRA) induce differentiation of immature promyelocytes to terminally differenced granulocytes, which eventually undergo apoptosis [40, 46]. This effect is attributed to the direct binding of ATRA to the ligand-binding site of the RARα moiety of the PML-RARα fusion protein, initiating the release of transcriptional co-repressors and recruitment of co-activators [45]. Although ATRA therapy alone had promising effect with CR up to 85%, prolonged ATRA treatment lead to ATRA resistance, early relapse and retinoic acid syndrome (RAS). An international effort to optimize the ATRA regime was initiated, and since the early 1990s ATRA has been combined with conventional chemotherapy such as daunorubicine (DNR), resulting in CR up to 95% with 74% 5-year DFS [47]. During the 1990s one of the oldest drugs in medicine, namely arsenic (ATO; As2O3) further improved APL treatment. ATO targets the PLM moiety of the PML-RARα fusion protein and induce apoptosis of APL cells [48, 49]. During the last decade several clinical trials has shown promising results for the combination of ATRA/ATO in APL treatment [50].

1.3.2 **The NB4 cell line**

In 1991, an ATRA-maturation inducible cell line (NB4) were isolated from an acute promyelocytic leukemia (APL) patient in relapse [51]. After long-term ATRA treatment of the initially isolated NB4 cell line, a maturation resistant sub-line NB4-RA^r (NB4-Retinoic Acid resistant) was also isolated [52, 53]. The NB4 cell (and the RA^r sublines R1 and R2) bear the APL specific t(15;17) chromosomal translocation, and are currently the only human APL cell lines available. The NB4 cell lines are unique tools to investigate *in vitro* biological responses of APL, and have been the major cell line used in **Paper I** of this thesis. The NB4-RA^r is the only ATRA-resistant cell line with the t(15;17) chromosomal translocation (APL specific), and the

occurrence of this subpopulation in APL patients may explain why patients invariably experience relapse with resistance to retinoic acid (RA). However, *in vitro* maturation of these "resistant" cells is possible using cAMP-elevating agents or stable cAMP analogs when the cells have become maturation competent using RA beforehand, a situation described as RA priming and cAMP triggering [53]. Since cAMP stimulates ATRA-induced maturation of ATRA "resistant" APL blasts [53], cAMP agonists have been contemplated as adjuncts to APL therapy [54-56]. Together, this creates the basis for initiating **Paper I**.

1.4 cAMP signaling

Second messengers are small molecules transmitting extracellular signals (from hormones, growth factors and neurotransmitters) to the inside of the cell, where they orchestrate a network of signaling events leading to a myriad of cellular actions. 3'-5'-cyclic adenosine monophosphate (cAMP) was the first second messenger to be discovered more than 40 years ago [57], and has since then been the basis of research awarded with three Nobel prices (1971, 1992 and 1994). cAMP regulates a range of physiological processes, and are involved in almost every known cellular function such as metabolism, gene transcription, cell division, growth and differentiation, apoptosis, secretion and neurotransmission [58]. It is produced from ATP upon G-protein coupled receptor (GPCR) activation of adenylyl cyclase [59], giving an overall 1000-fold amplification of the initial signal [60]. The mammalian adenylyl cyclases are usually transmembrane receptors and are encoded by nine genes and constitute several splice variants [61]. The level of free cAMP in the cell is controlled by hydrolyzing phosphodiesterases (PDEs). The PDE superfamily currently constitute 11 families and more than 50 enzymes with different properties [62]. With so many different proteins involved in cAMP synthesis and degradation it is undisputed that regulation of cAMP in the cell is highly prioritized. Today, three receptors for cAMP are identified; cAMP dependent protein kinase A (PKA), the two isoforms of the exchange proteins directly activated by cAMP (Epac1/2) and cAMP ion channels (Fig. 1.4).

1.4.1 **Protein kinase A**

Most of the intracellular effects of cAMP are mediated via protein kinase A (PKA), which is one of the most studied protein kinases. Inactive PKA presents as a tetramer, consisting of a regulatory (R) dimer subunit and two catalytic monomer subunits (C). Two mammalian R isoforms have been identified [63], with the RI distributed freely in the cytoplasm and the RII being mostly membrane-bound via PKA-anchoring proteins (AKAPs). The regulatory isoforms are further subdivided into RIα, RIβ, RIIα and RIIβ which is encoded by separate genes, and show different tissue distribution. Four different C-subunits are described (α,β,γ and PrKX), which is further sub-divided based on post-translational modifications or splice variants [62]. Upon intracellular increase of cAMP, two molecules of cAMP binds to the A and B site of each R subunit, inducing a conformational change in the tetramer, leading to dissociation of the two C-subunits [64]. These catalytic subunits will subsequently phosphorylate a variety of both cytosolic and nuclear proteins (reviewed in [65]) (Fig. 1.4). An important regulation of PKA is the involvement of PKA-anchoring proteins (AKAPs), which allow specificity of PKA signaling by constraining it to different compartments of the cell, close to specific effectors and substrates [66], and inhibition of the catalytic subunits by protein kinase inhibitors (PKIs).

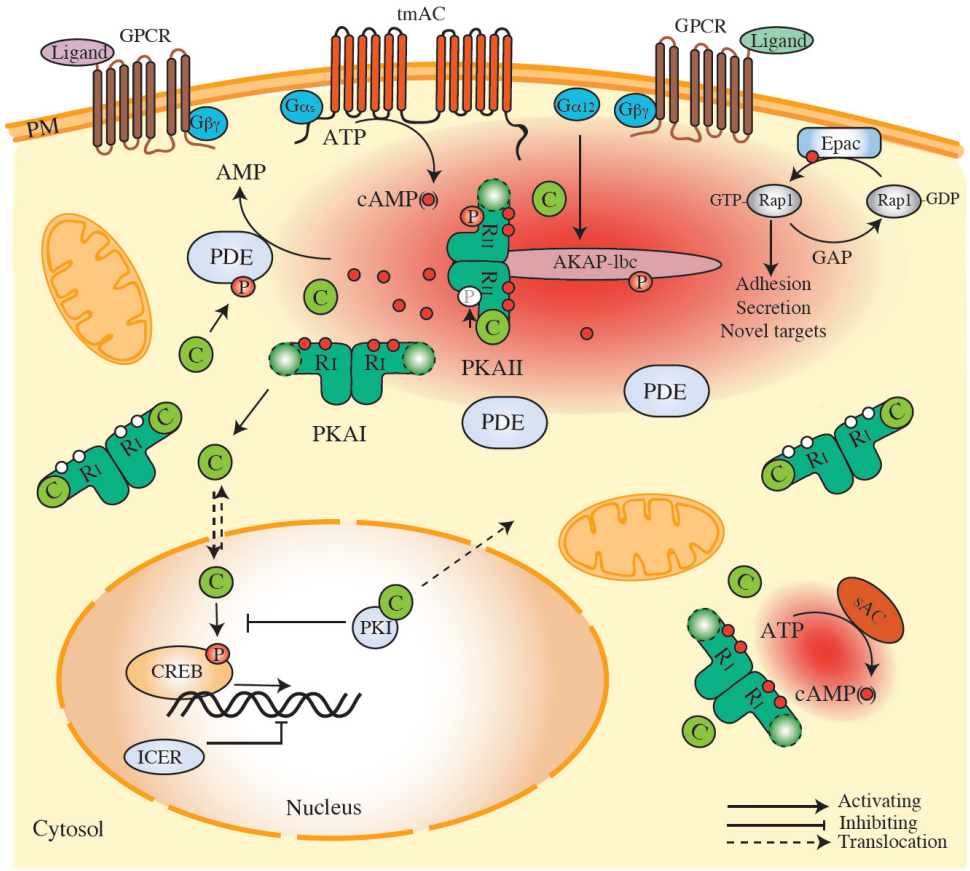


Figure 1.4 Overview of cAMP signaling. Most cAMP effects are mediated by PKA type I and II, as well as the newly recognized Epac 1/2. Ligand binding to G-protein coupled receptors (GPCR) leads to activation of heterotrimeric G-proteins. The dissociated Gαs activates transmembrane Adenylyl Cyclase to synthesize cAMP (red dots) from ATP. Soluble AC is depicted as sAC. Binding of two molecules of cAMP to the R subunit of PKA lowers the affinity for the C subunit which will dissociate and catalyze phosphorylation of cytosolic proteins or translocate to the nucleus where it phosphorylates nuclear targets like cAMP –response element binding protein (CREB). PKA-II usually presents in association with A-kinase anchor proteins (AKAP). When stimulated by cAMP, Epac activates Rap1,2 by exchanging the GDP to GTP. cAMP signaling is negatively regulated by both phosphodiesterase (PDE) and the protein kinase inhibitor (PKI) in the cytoplasm and nucleus (Figure from [62]).

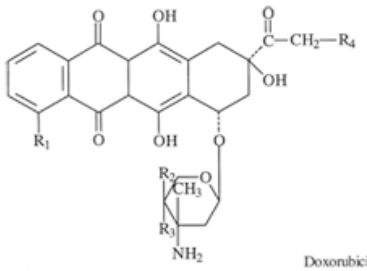
1.4.2 Modulation of intracellular cAMP using PGE_2, PDE inhibitors and cAMP analogs

Induction of intracellular cAMP production can be manipulated at any level in the cAMP production cascade. Receptor agonists such as prostaglandin E2 (PGE2) will activate adenylyl cyclace through $G\alpha_s$, and hence, increase the endogenous level of cAMP. Simultaneously, it is preferable to inhibit cAMP breakdown using phosphodiesterase (PDE) inhibitors such as isobuthylmethylxantine-IBMX (non-selective) or rolipram (PDE4 spesific). Since receptor mediated activation of cAMP can be attenuated by $G\alpha_s$ (stimulatory) to $G\alpha_i$ (inhibitory) switch [67] this can be overcome by activation of downstream targets such as cAMP receptors inside the cell using cAMP analogs. Since the discovery of cAMP in 1961, hundreds of synthetic analogs have been produced and tested for their therapeutic potential. Unfortunately, undesired side effects, metabolic instability, low cell penetration and the lack of tissue specificity were limiting factors for almost all of the first generation analogs. The N^6-modified cAMP analogs like N^6-benzoyl-cAMP (N^2-benz-cAMP) and N^6-monobuturyl-cAMP (N^6-MB-cAMP) have improved lipophilicity compared to cAMP, and are inefficient Epac activators while being full PKA activators [68]. 2´-O-methyl substitution of cAMP improved the selectivity for Epac 10-100 fold, and phenylthio substitution at position 8, particularly with a MeO- or Cl- at para-position, improved the selectivity even more. The combination of these two modifications resulted in the Epac specific agonist 8-para-chlorophenylthio-2´-O-Methyl-cAMP [68], commercially named 007. At present, there exists no single analog to discriminate between the type I and type II subtype of PKA, but this can be achieved by using specific analog pairs as done in **Paper I** of this thesis. While the above-mentioned cAMP analogs are referred to as activating analogs, there also exists inhibitory analogs, namely the Rp-cAMPS where the equatorial (Rp) oxygen is replaced by sulfur. These analogs are generally antagonistic or partially agonistic [69]. The use of synthetic cAMP analogs, targeted to specific proteins, are used as standard tools in current signal transduction research to study cell responses as well as mapping these responses to specific signaling pathways. An advantage of cAMP analogs is that they act within minutes, rather than hours or days, and can be easily

removed by washing the cells when applied in a cell culture system. In addition, the analog approach does not depend on artificially over expressed gene products, and can be used when transfection or microinjection is not applicable, e.g. in leukemia cells.

1.5 Anthracyclines

The idea of using chemotherapy as treatment for cancer dates back to the first world war, when mustard gas used in chemical warfare was discovered to be a potent suppressor of hematopoiesis [70]. Since then many new drugs have been developed, and today more than 100 cytostatic drugs are used either alone or in combination. These drugs vary widely in their chemical composition, physiological properties, delivery, specificity and side effects. However, a common feature is their ability to affect cell division or DNA synthesis in fast-dividing cells. Anthracyclines are cytotoxic antibiotics and are among the most effective antitumor drugs ever developed, widely used in the treatment of hematological disorders as well as solid tumors. The two first anthracyclines, Daunorubicin (DNR) and Doxorubicin (DOX) were isolated in the 1960s from the actinobacteria *Streptomyces peucetius* [71, 72]. The anthracyclines consists of a tetracyclic ring structure connected to a sugar group. Although very similar in chemical structure, their physiological properties and clinical application may be diverse (Fig. 1.5).



	R_1	R_2	R_3	R_4
Doxorubicin	—OCH_3	—H	—OH	—OH
Daunorubicin	—OCH_3	—H	—OH	—H
Epirubicin	—OCH_3	—OH	—H	—OH
Idarubicin	—H	—H	—OH	—H

Figure 1.5 The molecular structures of daunorubicin and three similar anthracycline drugs (doxorubicin, epirubicin, idarubicin).

1.5.1 **The anti-tumor mechanism of anthracyclines**

After entering the cell through passive diffusion [73], anthracyclines bind the proteasome and the complex translocates into the nucleus of neoplastic and proliferating cells [74]. With higher affinity for the DNA molecule, anthracyclines intercalates between base pairs and thus inhibits macromolecular synthesis, which was the first explanation for the antitumor effect of anthracyclines [75]. Also, covalent modifications have been observed both *in vitro* and *in vivo* where the iron-complex of the drug participates to produce a covalent attachment to the G-bases of the DNA. This creates a interstrand cross linker [76], and may enhance chromatin aggregation. A more recently discovered anthracycline target is the chromosomal protein Topoisomerase II [77]. Being "Topoisomerase poisoners", anthracyclines stabilize an intermediate complex where the strands are cut and covalently linked to the enzyme. This hinders the relaxation of supercoiled DNA and block subsequent replication and transcription [78]. The final consequence is growth arrest in G1/G2 followed by programmed cell death [79]. Although numerous studies demonstrate anthracycline induced production of reactive oxygen species, evidence indicate that oxidative stress is unlikely to explain the anti-tumor effect [80, 81], but are of greater importance when describing the cardiotoxic side effects mediated by anthracyclines [82-87].

1.6 Reactive oxygen species (ROS)

Free radicals are characterized by the presence of one or more unpaired electrons, and thus are extremely reactive compared to their electron paired counterparts. There are several different radicals, but those derived from oxygen are of most concern in biological systems, and are collectively known as *reactive oxygen species* (ROS). ROS are produced as necessary intermediates in a variety of normal biochemical reactions were they act as intracellular signaling molecules [88]. Under physiologic conditions, the level of ROS is kept low and in balance by biochemical antioxidant

systems, when this critical balance is disrupted, oxidative stress occurs as a consequence of excess ROS. The mitochondrial respiratory chain is a major source of ROS, with 1-2% of the consumed oxygen being converted to superoxide [89], and even increase during hypoxia [90]. A common type of ROS is the superoxide radical, which is efficiently converted into hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). H_2O_2 may be converted into water by the enzymes catalase or glutathione peroxidase, or produce the *highly reactive* hydroxyl radical ($OH^{\cdot}$) via the Fe^{2+}-catalyzed Fenton reaction (for review see [91]). Unlike superoxide and hydrogen peroxide, hydroxyl radicals cannot be eliminated by enzymes. Therefore, hydroxyl radicals are highly toxic compounds, reacting with any substance in its vicinity such as lipids, nucleic acids and proteins [92]. Whenever free radicals are generated in living cells, the cellular response depends upon the cell type, in addition to intracellular localization, amplitude, life span and the type of reactive species [93].

1.7 Cardiotoxic side effects of anthracyclines

As mentioned previously, anthracyclines are among the most effective drugs used in oncological practice. Unfortunately, their clinical use is hampered by side effects in healthy tissue, most notably in the form of chronic cardiomyopathy and congestive heart failure (CHF) [81]. The risk of toxic cardiomyopathy is restricting the cumulative dose of these drugs, and therefore may reduce their therapeutic potential. Anthracycline induced cardiotoxicity is divided into subcategories depending on the time of manifestation. *Acute or subacute cardiotoxicity* are rare and occur during, immediately after or within a week of drug administration. The injuries may be transient electrophysiological abnormalities, pericarditis-myocarditis syndrome or acute left ventricular failure [94-97]. *Early chronic cardiotoxicity* is more common and usually presents within a year. It usually presents as dilated cardiomyopathy in adults and restricted cardiomyopathy in pediatric patients [98-100]. *Delayed cardiotoxicity* was described in the early 1990s among survivors of childhood cancer [101, 102]. These cancer survivors may have normal cardiac function for longer

periods, but experience ventricular dysfunction [102-104], heart failure and arrhythmias [101, 102, 105] years to decades after completion of chemotherapy.

1.7.1 **The cardiotoxic mechanism**

It is of general agreement that the mechanism of anthracycline induced cardiotoxicity is different from those mediating their antitumor effect. This is a very important concept enabling strategies to prevent cardiotoxicity without diminishing the antitumor effect. As with the antitumor effect of anthracyclines, the cardiotoxicity of these drugs have been the subject of considerable controversy and numerous pathways have been proposed and studied. However, intramyocardial production of reactive oxygen species (ROS) is generally accepted as a strong candidate. This was indeed documented during the mid-70s with *in vitro* studies showing ROS production from both doxorubicine (DOX) and daunorubicine (DNR) [106, 107]. Concurrently, Myers *et al.* showed amelioration of anthracycline induced cardiotoxicity by Vitamin E (alpha- tocopherol) without interfering with its effectiveness as an anti-tumor agent [82]. This enforced oxidative stress as the new theory explaining anthracycline induced cardiotoxicity, and was subsequently strengthened by the cardioprotective effect of the iron-chelator dextrazoxane [83], as well as several studies with transgenic animals overexpressing physiological antioxidants [84-87]. The chemical structure of anthracyclines is the basis for its ability to induce ROS formation, with the quinone moiety notorious for undergoing redox cycling. As seen from figure 1.6, the quinone form of anthracyclines is reduced to the unstable semiquinone form by P450 reductase, particularly in myocardial cells with high levels of flavin reductases [108]. This semiquinone is rapidly oxidized back to its original form, simultaneously creating superoxide anions. The latter can dismutate to form hydrogen peroxide (H_2O_2) and then hydroxyl radical, or it can react with nitric oxide to form peroxynitrite ($ONOO^-$). The notorious consequence of this cascade includes peroxidation of lipids and oxidative damage to proteins and DNA [109]. One can ask the question why the heart as an organ would be so vulnerable to free radicals, and the answer may be attributed to its highly oxidative metabolism and low amount of antioxidant defense [110]. In addition, since anthracyclines selectively down-regulate

glutathione peroxidase [110], cardiomyocytes encounter high levels of hydrogen peroxide. Cardiomyocytes are rich in mitochondria, giving rise to 50% of the total cell mass which makes the them both a source and a target of ROS [109]. Finally, anthracyclines seem to be more retained within cardiomyocytes than other cell types [111], maybe due to its high affinity for cardiolipin, a phospholipid mainly present in the mitochondrial membranes of the heart [112].

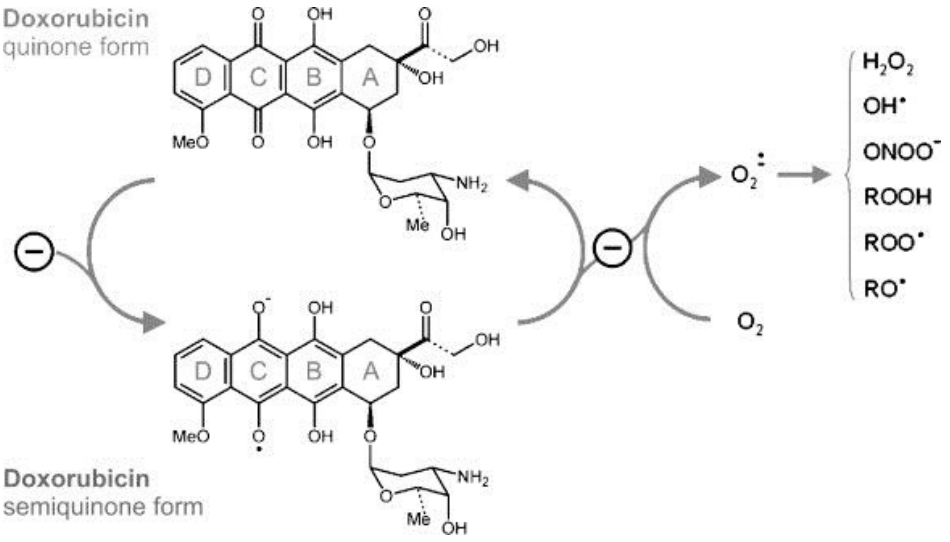


Figure 1.6 The chemical structure of doxorubicin is the basis for its ability to induce the formation of ROS and is similar for daunorubicine, the anthracycline used in this thesis. Doxorubicin (and daunorubicine) consists of a tetracyclic ring containing adjacent quinone-hydroquinone groups in rings C–B, coupled with the sugar daunosamine attached by a glycosidic linkage to the ring A. One-electron reduction of the quinone moiety results in the formation of a semiquinone radical that creates a superoxide anion when regenerated back to its the parent quinone. This initiates a reaction cascade with the formation of other reactive oxygen and nitrogen species (ROS, RNS) (figure from [109]).

1.7.2 **Strategies to prevent cardiotoxicity**

Since the manifestation of the cardiotoxic side effects is highly correlated to the total (cumulative) dose of the anthracycline given, the rationale was first and foremost to limit the total administration dose of the anthracyclines. Today there are maximum

recommended cumulative doses for all anthracyclines, such as 450 and 900 mg/m2 for doxorubicin (DOX) and daunorubicin (DNR), respectively [113]. However, there is always an associated cardiotoxic risk when using anthracyclines. Some patients tolerate cumulative doses twice as large as recommended, while others experience cardiac injury at standard doses [114]. Another strategy to prevent anthracycline induced cardiotoxicity is altering the administration schedule, since cardiotoxicity is related to peak anthracycline doses. Several reports suggests that continuous infusion, compared to a single bolus injection of the drug, reduce the risk of cardiotoxicity [115, 116], while others found no relationship between administration schedule and cardiotoxicity [117]. Anthracycline analogues have been synthesized and tested for their ability to replace the conventional DOX and DNR, but none of them has shown convincing results in being more cytotoxic and less cardiotoxic [118]. However, some of these analogues, such as epirubicin and idarubicin, have shown decreased cardiotoxicity in preclinical and clinical studies [119, 120]. The introduction of liposomal anthracyclines changed tissue distribution away from sensitive organs such as the heart, as they cannot pass areas with tight capillary junctions. In addition, the drug release is slower and therefore high peak concentrations can be avoided [121]. Another possibility is combining anthracycline treatment with cardioprotectants. Several agents with antioxidant properties such as Probucol [122], Amifostine [123], Carvedilol [124] and Sildenafil [125] have shown promising cardioprotective effects *in vitro* and *in vivo*. However, the iron-chelator Dexrazoxane is the only agent with proven cardioprotective effect, defined by reduced signs of congestive heart failure (CHF) in cancer patients receiving chemotherapy [126]. Finally, prevention or attenuation of anthracycline mediated cardiotoxicity have been demonstrated in animal studies by increasing endogenous antioxidants or introducing exogenous antioxidants. Although Vitamin E, Vitamin A and carotenoids individually demonstrate cardioprotective effects [82, 127-130], Stahl and Sies stated that a cocktail of antioxidants in naturally occurring compounds have far more profound effects due to synergistic actions of the individual compounds [131]. In **Paper II** we investigated the cardioprotective effect of dietary supplementation with the antioxidant-rich red palm oil.

1.8 Red Palm Oil

Red Palm Oil (RPO) comes from the fruit of the oil palm (*Elasis guineensis*) and has been used as a nutritional source as well as medicine for more than 5000 years. Throughout history, RPO has been the primary source of dietary fat, and until modern medicine, also the choice of remedy for almost every illness in Africa and south-east Asia. It is a balanced oil with 50% saturated fatty acids in addition to 40% and 10% mono- and polyunsaturated fatty acids, respectively. More importantly, it contains a spectrum of vitamins and antioxidants such as carotenoids (vitamin A), tocopherols and tocotrienols (Vitamin E) [132]. RPO can be described as "a powerhouse of nutrition", and is of high value in the treatment and prevention of malnutrition and vitamin deficiency. Being a good mix of fat and vitamins, RPO provides children with the daily recommended amount of Vitamin A from just one teaspoon. Further, RPO is regarded as a potent anti-cancer food due to its high content of antioxidants, especially tocotrienols. Tocotrienols are one of the most potent anticancer agents of all natural compounds [133] and has shown anti-proliferative and pro-apoptotic effect for skin, stomach, prostate, breast and other forms of cancer (for a review see [134]). During the past two decades, the effect of RPO on the cardiovascular system has been intensely investigated. Surprisingly, RPO is cardioprotective despite being the source of large amounts of saturated fat. Dietary RPO have shown to reverse the process of atherosclerosis [135], improve cholesterol levels [136, 137] and protects against ischemia induced stress [138]. In addition, it is an undisputed fact that countries with particularly high consumption of red palm oil, such as Malaysia, Indonesia and Papa New Guinea, are among the countries with the lowest incidents of heart disease.

1.9 Myocardial ischemia and reperfusion

Myocardial ischemia is a state where a coronary occlusion hinders the normal arterial blood supply to parts of the myocardium, which impairs normal oxidative metabolism [139]. The crucial initiating event leading to an occlusion is endothelial dysfunction. Each of the primary risk factors for coronary artery disease (hypercholesterolemia, hypertension and free radicals due to smoking) leads to endothelial injury, and hence,

entry of lipoprotein molecules and subsequent invasion of macromolecules into the intima of the artery wall [140]. The eventual result is formation of an atherosclerotic plaque, ready to rupture and initiate the formation of a potentially fatal thrombus. Following a cascade of ischemic events the final consequence is irreversible tissue damage as a result of apoptosis and necrosis [139].

1.9.1 The underlying mechanism of acute myocardial ischemia

The heart is fully dependent on aerobic metabolism to make energy in the form of adenosine triphosphate (ATP) by mitochondrial oxidative phosphorylation. During ischemia, the myocardium switches to anaerobic metabolism via glycolysis in order to produce ATP. However, glycolytic production of ATP is not sufficient to cover the energy demand of the ischemic myocardium and to sustain the hearts contractile function [141, 142]. The two main consequences of ischemia are lack of adequate amounts of oxygen and nutrients, and reduced washout of metabolites such as lactate, protons, NADH2 and CO2 [139] (Fig. 1.7 A). The increase in anaerobic glycolysis leads to cellular acidosis, and together with accumulation of other metabolic waste products, this will inhibit glycolysis and further reduce the levels of ATP [143]. The reduced energy level will subsequently inhibit ATP-powered ion pumps and lead to intracellular ionic alterations [144]. Excess internal sodium increase the osmotic pressure and cause cell swelling and rupture [145], while calcium overload will induce electrical and mechanical abnormalities in cardiac tissue [146]. Simultaneously, accumulation of free fatty acid metabolites, together with acidosis induced lysosomal activation, may cause membrane injury [144]. The presence of residual oxygen during ischemia will produce and accumulate reactive oxygen species (ROS), which in turn may damage the cell membrane and further depolarize the mitochondria [147]. Taken together, the final events inducing myocardial infarction is mitochondrial damage due to calcium overload, general membrane damage and proteolysis, all leading to myocardial cell death via apoptosis, necrosis (and autophagy).

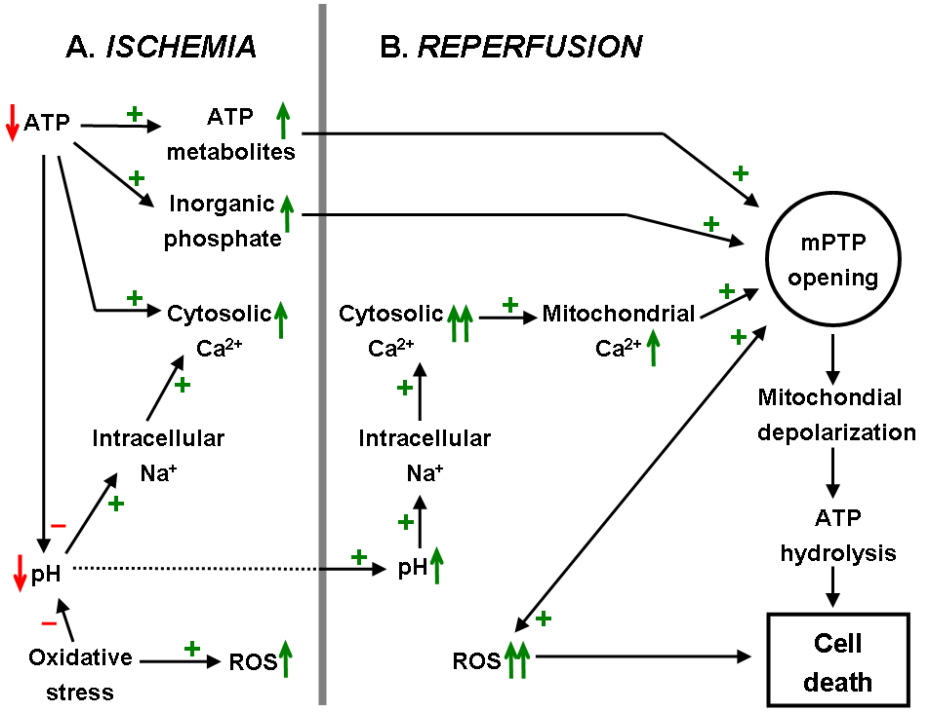


Figure 1.7 The main events of ischemia (A) and reperfusion (B) leading to myocardial infarct**: (A)** Reduced oxygen supply during ischemia will depress mitochondrial metabolism and results in reduced production of ATP while incomplete residual mitochondrial respiration will produces reactive oxygen species (ROS). With reduced respiration comes loss off membrane potential leading to reversal of the ATP synthase and hence hydrolysis of ATP into ADP and inorganic phosphate. Reduced wash out will accumulation lactate, protons and CO_2 and lead to cellular acidosis and proteolysis. Depletion of ATP inhibit ATP dependent ion pumps resulting in increased cytosolic [Ca^{2+}] due to Na^+/H^+ exchange and reversal of Na^+/Ca^{2+} exchanger. The ischemic cascade culminates in cell death either by necrosis or apoptosis. (B) Reperfusion gives a sudden oxygen burst, which increases ROS production. Removal of extracellular H^+ induce Na^+/H^+ exchange and further increase cytosolic [Ca^{2+}] due to Na^+/Ca^{2+} exchange. Subsequent mitochondrial [Ca^{2+}] overload will together with ATP metabolites and increased ROS trigger opening of the mitochondrial permeability transition pore (mPTP). mPTP opening will depolarize mitochondria, and hence, induce additional ROS production and accelerate mPTP opening, finally culminating in cell death (figure modified from [148]).

1.9.2 **Lethal reperfusion induced injury**

Reperfusion is the restoration of blood flow to the ischemic area, and is a prerequisite to salvage affected myocardial tissue after an ischemic insult. Clinically, reperfusion is achieved by thrombolytic treatment, percutaneous coronary intervention (PCI) or

coronary artery bypass surgery. Paradoxically, reperfusion *per se* may contribute to the total infarcted area [141], a term described as *lethal reperfusion injury,* and is defined as injury to the tissue arising when the blood supply returns to the myocardium after a period of ischemia [149]. Other myocardial reperfusion-induced injuries are; 1) temporal depression of function known as myocardial stunning, 2) reduced perfusion due to microvascular damage and 3) reperfusion arrhythmias [150]. The existence of lethal reperfusion injury was for a long time highly debated, but today its existence is accepted as a contributor to the final infarct size after a prolonged period of ischemia followed by reperfusion. Proof of its existence, is the presence of viable cells after an ischemic event, which lose viability during the first hours of reperfusion [151]. In addition, administration of pharmacological agents at the immediate onset of reperfusion reduces the extent of cell death after an ischemic episode [18, 152-154]. The main event linking reperfusion injury to cell death is the opening of the mitochondrial permeability transition pore (mPTP) (Fig. 1.7 B). This is a Ca^{2+} and ROS dependent process [155], that is inhibited by the acidic environment during ischemia [156, 157]. Opening of the mPTP leads to influx of solutes and water, followed by swelling of the mitochondrial matrix. Subsequently, the outer mitochondrial membrane burst, and various pro-apoptotic substances leak into the cytosol and initiates apoptosis [158]. Since the discovery of the mPTP, research has focused on inhibition of the pore at reperfusion, a process shown to be cardioprotective by reducing the extent of lethal reperfusion injury [158-160].

1.10 Modulation of ischemia-reperfusion induced injury

The achievements in cardiology regarding early reperfusion strategies have greatly improved the survival of patients with acute coronary syndromes. However, a limitation in the current clinical reperfusion strategies, such as thrombolysis, percutaneous coronary intervention (PCI) or coronary artery bypass surgery, is that they do not reduce the cellular consequences of lethal reperfusion induced injury. During the last decade, our understanding of the mechanisms underlying reperfusion induced injury has been substantially enhanced, and animal studies have revealed that

lethal reperfusion injury contributes to nearly half of the total infarct size (Fig. 1.8) [161]. Different ways to limit or delay cardiomyocyte cell death have emerged in the laboratory and are evaluated as clinical candidates to further improve the outcome in patients suffering from acute myocardial ischemia (AMI). Since mPTP opening seems to be the "point of no return" in lethal reperfusion injury, a substantial amount of research has focused on manipulating the intracellular milieu away from what facilitates mPTP opening during early reperfusion, such as maintaining acidosis [162], reducing Ca^{2+} overload [163] and scavenging ROS [164]. Other cardioprotective strategies include pharmacologic interventions and sub lethal ischemic conditioning. Many of these cardioprotective interventions have common features on their way to inhibit mPTP opening, involving the reperfusion injury salvage kinase (RISK) pathway.

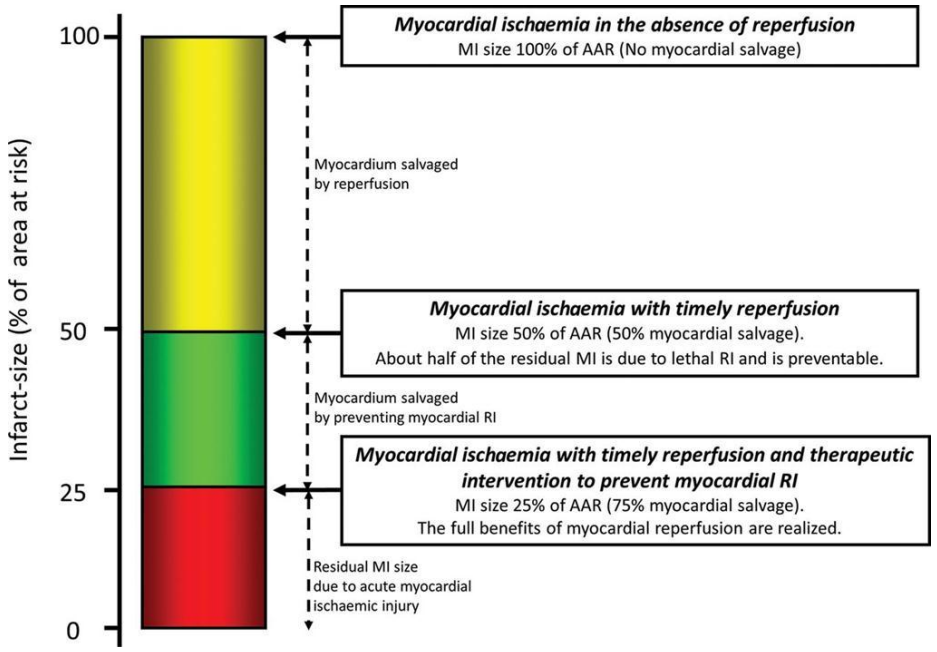


Figure 1.8 The figure illustrates the necessity of reperfusion to salvage the myocardium and also the benefits of a therapeutic intervention to prevent lethal reperfusion injury. Nearly 50% of the total infarct size is due to lethal reperfusion injury presenting it as an important target for additional cardioprotection (figure from [161]).

1.10.1 **Reperfusion Injury Salvage Kinase Pathway (RISK)**

The RISK pathway is a "mind-made" and currently expanding signaling cascade, describing the molecular events mediating cardioprotection at the time of reperfusion. It was named by Hausenloy and Yellon in 2004, and involves activation of particular anti-apoptotic protein kinases such as PI3K-Akt and MEK1/2-Erk1/2 [153]. Later, many other kinases have been included in the RISK pathway such as protein kinase A, C and G (PKA, PKC, PKG) as well as ribosomal protein s6 kinase (p70s6k) and eNOS [165] (Fig. 1.9). A variety of agents have the ability to convey cardioprotection by activating the RISK signaling pathway (for review see [165]). Different RISK agonists mediate a signaling cascade from the cell membrane to the mitochondria, converging on the glycogen synthase kinase 3β (GSK3β) [166]. Inhibition of the mitochondrial permeability transition pore (mPTP) represents the end-effector which may involve activation of the mitochondrial ATP-sensitive potassium channel (mitoK_{ATP}). A more recently described signaling pathway is the survivor activating factor enhancement (SAFE) pathway introduced by Lecour in 2009 [167]. This signaling cascade involves the innate immune system and activation of the JAK/STAT-3 dependent signaling pathway by cytokines such as IL-6 and TNFα.

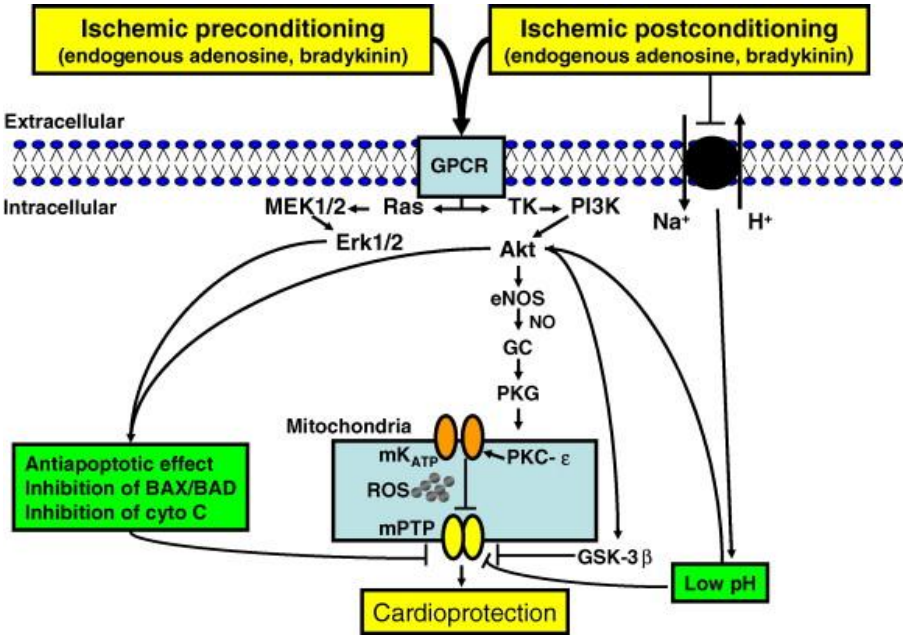


Figure 1.9 A simplified overview of the components linking IPC and IPost to cardioprotection. Autacoid activation of G-protein coupled receptors (GPCR) activates the reperfusion injury salvage kinase (RISK) pathway either via PI3K/Akt or MEK/ERK signaling cascade. The different signaling pathways converge on the mitochondria, with inhibition of the mitochondrial permeability transition pore (mPTP). Inhibition of mPTP may be direct, or via activation of the mitochondrial ATP-sensitive potassium (mitoK$_{ATP}$) and increased ROS production (figure from [168]).

1.11 Cardioprotective therapies

1.11.1 **Ischemic conditioning**

Ischemic conditioning (IC) entails cycles of alternate sub lethal ischemia and reperfusion, applied to the heart either before (*pre*conditioning) [169] or after (*post*conditioning) [170] a lethal period of myocardial ischemia (index ischemia). Since its discovery in 1986 [169], the cardioprotective effect of Ischemic Preconditioning (IPC) has been demonstrated in all species tested, including humans,

as well as non-cardiac tissues such as brain, liver, gut, bladder and skin [171]. A limitation of IPC as a clinical intervention is the prerequisite to be administered *prior* to the index ischemia, making it relevant only in planned cardiac procedures such as coronary artery bypass graft surgery. On the other hand, Ischemic Postconditioning (IPost) is applied to the heart at reperfusion to modulate the outcome of acute myocardial infarct, and therefore represents a more clinical relevant procedure. During both the pre- and post-conditioning phase several autacoids such as adenosine, bradykinin, opioids, norephinerine and cytokines are released and initiate a variety of signaling pathways within the same or nearby cells [172]. Regarding IPC, it is self-evident that signaling during the preconditioning phase is necessary to transduce signals from the cell surface to intracellular targets for condition initiated cardioprotection [173]. Intriguingly, the signaling pathways recruited at the first few minutes of reperfusion are equally important for IPC mediated cardioprotection [174]. In fact, the signaling pathways recruited at the onset of reperfusion during both IPC and IPost are highly similar, involving cell surface receptors, protein kinases, redox signaling and finally inhibition of mPTP (for review see [168]) (Fig. 1.9).

1.11.2 **Pharmacologic therapy (insulin, GSK3βi and CRF)**

The invasive nature of both IPC and IPost involves series of inflations and deflations of a PCI balloon at the occluded area in a infarct-related artery. This may destabilize, and hence, loosen parts of the atherosclerotic plaques/thrombus which may cause additional occlusion downstream. As an alternative, pharmacologic intervention using a variety of different agents, has shown to be cardioprotective in pre-clinical studies [165], with activation of similar signaling pathways as observed for IPC and IPost [173]. Pharmacologic therapy may therefore be suitable alternatives to the mechanical IPC and IPost interventions. In **Paper III** and **IV** we explore the cardioprotective effect of insulin, corticotropin releasing factor (CRF) and a direct GSK3β inhibitor.

Insulin reperfusion therapy

Insulin is a peptide hormone produced in the pancreas and is central in the regulation of carbohydrate and fat metabolism in the body. The insulin receptor (INSR) is a heterotetrameric glycoprotein situated in the plasma membrane. The two extracellular α-subunits make up the insulin binding domain, while the two intracellular β-subunits constitute the receptor tyrosine kinase (RTK) domain. Upon insulin binding the INSR form a dimer and the RTK is auto-phosphorylated, causing substrate binding and subsequent activation of signaling pathways such as RISK [175]. Administration of the "metabolic cocktail" compromising glucose, insulin and potassium (GIK) reduced mortality in patients with myocardial infarction undergoing reperfusion [176]. In 2001, Jonassen *et al.* demonstrated in the *ex vivo* rat heart, that insulin was the important cardioprotective component of the cocktail [177]. When present from the onset of reperfusion, insulin mediate cardioprotection via the PI3K/Akt/p70s6k pathway [152]. Later, insulin has shown to inhibit mPTP opening [178] via NO signaling or GSK3β inhibition [159]. In the **Paper IV** we explore the possible additive effect of combining insulin treatment with other cardioprotective strategies.

Inhibition of Glycogen synthase kinase 3 (GSK3β)

Glycogen synthase kinase 3 (GSK3) is an evolutionary conserved protein kinase with homologs in every eukaryotic specie examined. There are two isoforms of the enzyme, namely GSK3α and GSK3β, both exerting catalytic activity towards a number of intracellular substrates [179]. The two isoforms have 98% identity in their central catalytic domain [180], however, the β isoform has reported to have a generally higher catalytic activity than the α isoform [179]. GSK3 was first identified as a regulator of glycogen metabolism where it phosphorylates and thereby inhibits glycogen synthase, the rate-limiting enzyme in glycogen metabolism [181]. Although the original name has stuck, the scope of GSK3 regulation extends way beyond intermediary metabolism, as the enzyme has emerged to become an important component of fundamental processes including cell fate determination, metabolism, transcriptional control, and, in mammals, oncogenesis and neurological diseases [182]. Unlike other kinases, GSK3 is constitutively active in resting cells, while

becoming inactivated in response to cellular stimuli [183]. Since GSK3β negatively regulates downstream signaling mechanisms, phosphorylation, and hence, inactivation of GSK3β stimulates many cellular functions by removing the negative constraint imposed by GSK3β. It is now 20 years since insulin was shown to be involved in GSK3 inhibition [184]. Today we know that inhibition of GSK3 requires phosphorylation of an N-terminal serine residue, and that protein kinase B (PKB/Akt) is responsible for insulin mediated inhibition of GSK3. The inhibitory N-terminal serine can also be targeted by other kinases such as p70 ribosomal S6 kinase-1 (p70S6K1) and most of the downstream kinases of mitogen-activated protein kinase (MAPK) (Fig. 1.10). In addition, inhibitory GSK3 phosphorylation has also been demonstrated with cAMP elevating agents or cAMP analogs [185] (reviewed in [186]). In the **Paper IV,** we evaluate the combination of GSK3β inhibition with other cardioprotective strategies.

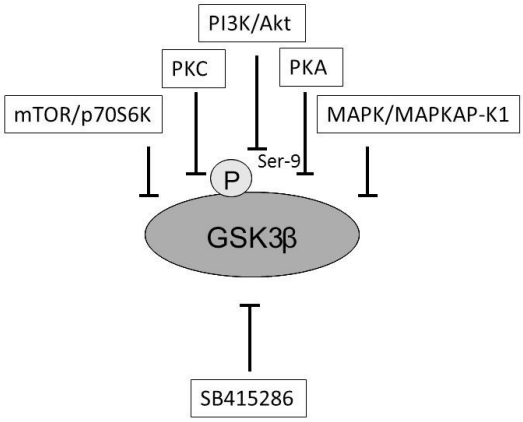


Figure 1.10 Many upstream signaling kinases such as mTOR/p70s6K, protein kinase C (PKC), phosphatidylinositol 3- kinase (PI3K)/Akt and MAPK/MAPKAP-K1 are reported as inhibitors of GSK3β through phosphorylation at Ser-9. In addition, the commercially available GSK3β inhibitor (SB415286) used in **Paper IV** is depicted.

Cardioprotective effect of Corticotropin releasing factor (CRF)

Corticotropin releasing factor (CRF) is a small hormone and neurotransmitter produced in the hypothalamus, where it plays a central role in behavioral and autonomic responses to stress, by stimulating the release of adrenocorticotropic hormone (ACTH) and b-endorphin from the pituitary [187]. The CRF family of peptides mediate their response via CRF receptors type -1 (CRFR1) and type- 2 (CRFR2). Human and rodents express both receptors, however, with more splice variants of CRFR2 such as α, β and γ in humans and α and β in rodents [188]. The CRFR2 are situated in the peripheral vascular system with distinct cardiovascular responses, such as regulating vagal and sympathetic activity [189, 190]. In addition, CRF have shown to have direct cardiac effects in mice [191] , rats [192] and guinea pigs [193], probable via the highly expressed CRFR2. Previous results show that CRF are cardioprotective against a simulated ischemic event in rat neonatal cardiomyocytes [194], and the cardioprotective effect of preconditioned media was abolished in the presence of a CRF antagonist, indicating that CRF is released into the media during mild stress and act on other cells to stimulate protection [194]. In **Paper III** of this thesis we showed that the cardioprotective effect of CRF was mediated via CRFR2 both in cardiomyocytes as well as *ex vivo* rat heart, a mechanism that was dependent on PKA and PKC mediated signaling [195].

2. SUMMARY OF RESULTS

2.1 Paper I

Cyclic AMP can promote APL progression and protect myeloid leukemia cells against anthracycline-induced apoptosis

Recently, cAMP has been advocated to improve acute promyelocytic leukemia (APL) therapy since it enhance *all-trans* retinoic acid (ATRA) induced differentiation of APL cells [53]. This study was aimed to evaluate the influence of cAMP on anthracycline induced apoptosis of APL cells, since anthracyclines, such as daunorubicin (DNR), are a component of classic APL therapy. To our surprise, we found that elevated levels of cAMP antagonized DNR induced death of APL patient blasts, ATRA sensitive and resistant NB4 cells, most acute myeloid leukemia (AML) patient blasts, and some AML cell lines. Using receptor specific cAMP analogs we could determine the mechanism to be dependent on cytoplasmic PKA-I while independent of perinuclear PKA-II, an observation that was verified using $NB4_{RII\alpha,\beta}$ knockdown cells. Pro-apoptotic Bad was strongly phosphorylated at PKA site Ser118 in cAMP-stimulated NB4 cells, whether exposed to DNR or not, which implies that cAMP-stimulation can release Bcl-2/-Xl bound to Bad and thereby protect against DNR. Another PKA substrate, the cAMP-responsive element binding protein (CREB), associated with therapy resistance in AML, was highly phosphorylated at Ser133 by elevated cAMP *(for a summarized overview see Fig. 1).* The protective effect of cAMP was also demonstrated in a hypoxic mimetic environment of leukemic bone marrow, and finally in immunodeficient NSG mice transplanted with NB4 cells. Animals given clinically relevant cAMP agonists had shorter life span and more rapid terminal weight loss than their corresponding counterparts. We conclude that cAMP agonists can accelerate the APL progression both in the absence and presence of DNR, and therefore suggests caution in using cAMP elevating drugs in combination with anthracyclines in APL patients.

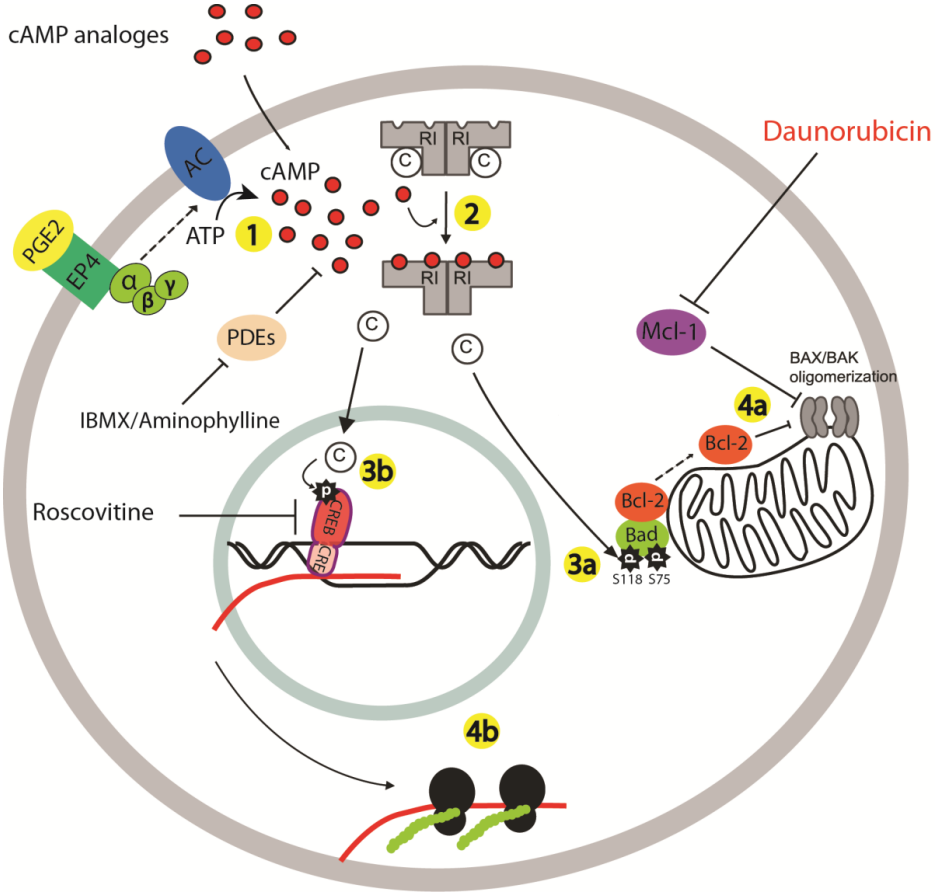


Figure 2.1. Overview of the pathway(s) involved in cAMP promoted protection against daunorubicin (DNR) induced apoptosis. (1) Increased levels of cAMP were achieved by prostaglandin E2 induced activation of adenylyl cyclase together with inhibitors of cAMP phosphodiesterases (IBMX/Aminophylline), or by introducing synthetic cAMP analogs. (2) Two molecules of cAMP binds to the A and B site of each R subunit, inducing a conformational change in the tetramer, leading to the dissociation of the two C-subunits which can phosphorylate a variety of both cytosolic and nuclear proteins. In unstimulated cells, pro-apoptotic Bad may complex with Bcl-2/Bcl-Xl and thereby block Bcl2 pro-survival functions. (3a) cAMP stimulation increased S118 phosphorylation of Bad which liberates Bcl-2/Bcl-Xl sequestered by Bad, and may compensate for DNR mediated down regulation of anti-apoptotic Mcl-1 in these cells. Since cAMP also showed Bad-independent protective effect, a search for additional survival-associated potential PKA targets revealed (3b) increased phosphorylation of the PKA specific Ser133 of CREB in NB4 cells. The broadly acting cyclin-dependent protein kinase (CDK) 7/9 inhibitor roscovitine (RCV) attenuated the protective effect of cAMP stimulators against DNR without affecting the phosphorylation of Bad. This supportes the existence of protective actions of cAMP involving CDK-dependent events, possibly mediated via PKA activation of CREB induced transcription.

2.2 Paper II

Dietary red palm oil protects the heart against the cytotoxic effects of anthracycline

Anthracycline induced cardiotoxicity is directly correlated to the cumulative dose and may hamper the therapeutic effect of anthracyclines. Therefore, a current working hypothesis is that high enough anthracycline doses can be administered in combination with cardioprotectors, such as red palm oil (RPO). Male Wistar rats fed a standard rat chow (SRC) diet (control) or SRC supplemented with RPO (200ul/day), were treated with 2mg/kg^{-1} daunorubicine (DNR) (or saline as control) on alternated days for a 12 period. After completed treatment protocol, *ex vivo* heart function was evaluated using the Working Heart perfusion apparatus. At the end of perfusion protocol, hearts were freeze clamped and the tissue analyzed for mRNA or protein changes (RT-PCR and WB) of antioxidant systems and stress signaling proteins. We found that RPO diet supplementation improved cardiac function after treatment with DNR, with increased aortic output (25%) and coronary flow (26%). Also, RPO diet supplementation counteracts DNR mediated down regulation of superoxide dismutase 1 (SOD1) and nitric oxide synthase 1 (NOS1) mRNA. For SOD1 this was also mirrored at protein level. Finally, RPO prevented DNR induced activation of the stress related kinases p38 and JNK, and up regulated the pro-survival kinase ERK. Based on our results, circumstantial evidence indicate that RPO mediated antioxidant therapy may reduce the harmful consequence of anthracycline induced cardiotoxicity.

2.3 Paper III

Activation of corticotropin releasing factor receptor type 2 in the heart by corticotropin releasing factor offers cytoprotection against ischemic injury via PKA and PKC dependent signaling.

There is currently no clinical therapy for lethal reperfusion induced injury, which makes it an important target for residual cardioprotection. This study aimed to verify the possible cardioprotective effect of acute administration of Corticotropin releasing factor (CRF) in neonatal cardiac cells and *ex vivo* rat hearts, and further delineate the signaling pathway involved. CRF significantly reduced infarct size to 35.3 ± 3.1% from 52.1 ± 3.1% in control hearts. *In vitro*, CRF was cytoprotective when administered prior to a lethal simulated ischemic event, reducing apoptotic and necrotic cell death by 18%. CRF was not protective when administered at the point of hypoxic reoxygenation or ischemic reperfusion. CRF induced cardioprotection was mediated via CRF receptor type 2 (CRFR2) since cardioprotection was abrogated in the presence of the CRFR2 inhibitor astressin-2B. The ERK1/2 inhibitor PD98059 failed to inhibit cardioprotection in the *ex vivo* heart while inhibitors of both protein kinase A and protein kinase C abrogated CRF-mediated protection both *ex vivo* and *in vitro*. To summarize, acute pre-treatment with CRF peptide protects the heart from a lethal ischemic insult, reducing cell death *in vitro* and infarct size *ex vivo*. Consistent with previous studies using urocortins [196, 197], CRF mediates its protective effect against ischemic stress through CRFR2 activation. Finally, we suggest the involvement of both PKC and PKA while excluding ERK1/2 dependent signaling.

2.4 Paper IV

Abrogated Cardioprotection and Blunted Akt Phosphorylation when Combining Ischemic Postconditioning with Pharmacological Reperfusion Therapy

Several pre-clinical studies suggest strategies to reduce reperfusion induced cell death, and this study aimed to investigate whether the combination of cardioprotective treatments could afford additional effect when compared to the treatments given alone. We used the *ex vivo* Langendorff perfused rat heart model to modulate cell injury and evaluate infarct size induced by ischemia-reperfusion. Insulin and a direct GSK3β-inhibitor (GSK3βi) administered at immediate reperfusion reduced infarct size by approximately 50%. Combination of the two treatments did not have additive cardioprotective effect compared to the treatments given alone. Further, the cardioprotective effect of insulin and/or GSK3βi was lost in the presence of the ROS-scavenger MPG, indicating a ROS dependent signaling pathway for cardioprotection. Our data also suggests that cardioprotection induced by insulin administration at reperfusion, is mediated via mitoK_{ATP} and PKC dependent signaling. Surprisingly, the combination of ischemic postconditioning (IPost) with either of the two pharmacologic reperfusion therapies (Insulin or GSK3βi), abrogated cardioprotection imposed by the therapies given alone. Loss of cardioprotection was unaffected by MPG and was associated with blunted levels of phosphorylated Akt.

3. AIMS OF THE STUDY

The overall aim of this thesis was to participate in the search for ways to directly or indirectly minimize anthracycline mediated cardiotoxicity and also to reduce the degree of ischemia-reperfusion induced injury.

Paper I: Evaluate the influence of cAMP on anthracycline induced apoptosis of acute promyelocytic leukemia (APL) cells, since cAMP has been advocated as adjuvant to classical APL therapy.

Paper II: Study the possible cardioprotective effect of Red Palm Oil against anthracycline mediated cardiotoxicity.

Paper III: Delineate the cardioprotective effect of corticotropin releasing factor (CRF) against ischemia-reperfusion induced injury.

Paper IV: Investigate the combination of known protective therapies against ischemia-reperfusion induced injury.

4. METHODOLOGICAL CONSIDERATIONS

This chapter will describe the main methods used in the thesis, with special emphasis on the favorable or unfavorable aspects for the given methods. For a detailed description of materials and methods the reader is referred to the articles.

4.1 Cell culture experiments

4.1.1 **Immortalized cell lines, primary cells and patient material**

Immortalized cell lines is an invaluable resource as they are easy to handle, are a limitless self-replicating source and have relatively high degree of homogeneity. Several characterized acute myeloid leukemia (AML) cell lines are regarded as typical for different AML subtypes, and are often used as *in vitro* models for AML disease. The NB4 cell line used in **Paper I** was originally isolated from bone marrow of an acute promyelocytic leukemia (APL) patient in relapse [51], and is currently the only APL specific cell line available. The NB4 cells harbor the t(15;17) translocation and differentiates into granulocytes upon ATRA-treatment [51], and undergoes apoptosis in response to arsenic trioxide (ATO) and anthracyclines [198, 199]. The NB4-LR1 and NB4-LR2 sublines used in **Paper I,** represents the two retinoic acid "resistant" cell lines, where LR1 but not LR2 has the ability to mature in the presence of cAMP. The letter "L" stands for Prof.**L**anotte and represents the laboratory from which the NB4 subline has been isolated. The HL60 cell line was also isolated from a patient with APL, but is a less differentiated variant compared to NB4. It presents without the APL specific t(15;17) translocation, and is now recognized to be an AML cell line [200], but still has the APL phenotype in response to ATRA [201] . Mv4-11 is an AML cell line that in contrast to NB4 and HL60 expresses wt p53, which have shown to be important for cAMP mediated cell survival [202]. Even though cell lines are important tools for biochemical research, one should never neglect the artificial aspect of cell lines represented by a homogenous group of immortalized cells. Since they can grow continuously in culture, they are prone to genotypic and phenotypic changes. Subcultures can arise with the most rapidly growing clones within the

culture being selected [203]. Primary cells can be more attractive since they are genetically stable and have characteristics very close to the tissue of origin [203]. In **Paper III** we isolated neonatal mouse cardiomyocytes, and within 2 days a confluent monolayer of spontaneously beating cardiomyocytes was formed and ready for experiment. Also, human primary cells/patient material can be used to determine accuracy of extrapolating human data from an animal model, as done in **Paper I**. Although there are advantages of using primary cell lines, their limitations are slow doubling time and restricted passages. The strength of a paper will increase when including more than one model. In **Paper I** we have studied several AML cell lines, included patient material from 10 AML/APL patients and also an *in vivo* mouse model. In **Paper III** we have performed experiments on primary mouse cardiomyocytes as well as whole heart preparation (Langendorff) from rat.

4.1.2 Isolation of neonatal mouse cardiomyocytes and simulated ischemia

To evaluate any anti-apoptotic action of corticotropin releasing factor (CRF) against ischemia-reperfusion induced injury, neonatal mouse cardiomyocytes was isolated and subjected to simulated ischemia. Isolated cardiomyocytes from heart tissue are, as expected, fully differentiated and morphologically similar to the cells of the donor organ, but presents without interstitial tissue and the presence of other cell types which may complicate the measurements in intact tissue. In addition, it enables the study of single cell populations subjected to identical defined conditions such as media constituents, humidity and partial pressure. The disadvantage of using primary cells is the risk of contamination and also the loss of their natural environment with regards to neurological input, nutrients and physical contact (cell-cell and cell-matrix). The cells may therefore change during isolation which may affect their reliability as a physiological cell model. With regards to simulated ischemia-reoxygenation, control of incubation conditions is easy. However, compared to the intact organ, cell-culture presents without the interaction between cardiomyocytes, endothelium and the intracellular space.

4.1.3 **Drug doses and evaluation of apoptosis**

For a cell-culture experiment to be clinically relevant, it is important utilize drug concentrations that are comparable to what patients experience during therapy. Minotti *et al.* reported that anthracyclines given in clinical relevant doses induce DNA breaks via inhibition of Topoisomerase II, while ROS induced breakage was only relevant as supra-clinical doses [81]. In addition, the anthracycline dose also influence apoptotic cell morphology, since the classical features of apoptosis such as membrane blebbing and chromatin condensation are absent at high doses, a phenomenon described as "frozen" cell death [204]. The peak plasma concentrations of daunorubicine (DNR) in patients is approximately 5 μM, but most often in the range of 1-2 μM [80]. In **Paper I**, we used both a high (5μM) and also lower doses (0.1μM and 0.5μM) of DNR, which are comparable to the measured levels in patient plasma. However, different cell lines show varying ability to accumulate drugs intracellularly [205], and therefore the intracellular drug concentrations can be difficult to predict. Idarubicin (IDA) is a more lipophilic drug compared to DNR, and accumulates more easily inside cells. It is considered 4-8 times more efficient in inducing apoptosis compared to doxorubicine (DOX) [206]. The IDA doses used in **Paper I** ranged between 0.01-0.5 μM. *In vitro* determination of cell viability **(Paper I)** was assessed by light microscopy to visualize membrane budding/blebbing, and differential interference contrast microscopy to visualize chromatin condensation in response to Hoechst staining. This is a well-established routine assay to evaluate apoptosis. Evaluation of apoptotic cell death in **Paper III** was assessed using a commercial single-stranded DNA (ssDNA) apoptosis ELISA (Enzyme-Linked ImmunoSorbent Assay detection) kit (Chemicon) according to the manufacturer's instructions

4.2 Animal work

4.2.1 **Animal strain and anastetichs**

In **Paper I** we established an *in vivo* leukemic model by transplanting male NOD-*scid* IL2rγ^{null} (NSG) mice with NB4 cells (acute promyelocytic leukemia (APL) cells). The NSG mouse is the best model to choose for cancer zenograft since it is the most immunodeficient mouse (lacking mature T cells and B cells, functional natural killer cells and are deficient in cytokine signaling) and therefore easily engraft human cells and tissues. In **Paper II, III and IV** hearts from male Wistar rats were used in the *ex vivo* isolated heart perfusions. Wistar rats are a frequently used and all-purpose animal model for biomedical research. In most published studies, including our own, we utilize young and healthy animals which may not be the best representation of the cancer patient (**Paper II**) or elderly CVD patient (**Paper III and IV**), which often presents with a complicated medical history and treatment regime. As an anesthetic agent we used sodium pentobarbital (i.p injection) commonly used for animal research, due to practicality, low cost and little to no influence on the study design. Although being a cardio depressant [207], it does not influence the final infarct size [208] as is documented for other volatile anesthetics such as isoflurane [209].

4.2.2 **The isolated Langendorff and Working heart perfusion model**

The isolated Langendorff and Working Heart perfused preparations are *ex vivo* setups where the heart is excised from the animal and mounted by aorta to the perfusion apparatus. These highly reproducible preparations allow studies of cardio-specific effects without the confounding influence of the circulation or neurological factors. However, this also makes the model less clinical relevant than an *in vivo* preparation. While oxidation of fatty acids is the main energy source for the *in vivo* heart, glucose is usually the only substrate present in the perfusion fluid for the *ex vivo* preparation.

The Langendorff heart preparation

In the Langendorff perfusion setup, first described by Oscar Langendorff in 1895 [210], the heart is retrogradely perfused with oxygenated Krebs-Henseleit buffer (KHB) (37ºC) at a constant hydrostatic pressure (80mmHg) (or constant flow rate). In both settings the aortic valve is forced shut, directing the fluid into the coronary ostia, perfusing the entire ventricular mass of the heart and finally draining into the right atrium. This effluent can be collected and measured (coronary flow; CF). Due to lack of serum proteins and blood cells, the KHB have reduced oncotic pressure and oxygen-carrying capacity, which increase CF and the risk of edema in isolated hearts compared to the *in vivo* setting [211]. Due to the *ex vivo* nature of the setup, the heart function will deteriorate throughout the protocol at a rate of 5-10%/h depending on different factors such as the skills of the operator, specie, perfusion fluid and animal age [212]. A water-filled latex balloon connected to a pressure transducer is inserted into the left ventricle, via the left atrium, enabling functional data such as heart rate (HR), left ventricular developed pressure (LVDP) and contractility (dP/dT) to be recorded. Great care must be taken when inserting and securing the balloon to minimalize the amount of mechanical stress imposed on the heart. Finally, maintaining the heart at physiologic temperature is highly necessary as both hypo- and hyper-thermia can influence contractility, heart rate as well as the final infarct size [213]. Prior to the experiments, a set of inclusion parameters were defined and experiments were aborted if they failed to meet these criteria. Our inclusion criteria were as followed; at the end of stabilization - LVDP > 80 mmHg and CF; 8-16 ml/min and at the end of reperfusion - LVDP > 30 mmHg or CF > 4 ml / min.

The Working heart preparation

Although the Langendorff perfused isolated heart is beating, it is considered as “nonworking” since no perfusate is ejected from the left ventricle. Some major modifications were made to the model, and the isolated working heart preparation was presented by Neely and Morgan in the 1960s [214]. It is a more complex preparation in which the perfusate is delivered to the left ventricle via the cannulated

left atrium and ejected in the normal direction via the left ventricle and out the cannulated aorta. This preparation is a left ventricular ejecting heart, thereby a heart performing work, giving the ability to measure pump function with different filling pressures and afterloads. The preload of the preparation is determined by the height of the atrial perfusion reservoir relative to the heart, but is usually set to 20 cm for rat heart preparations. The left ventricle then pumps the perfusate through the aorta against a hydrostatic pressure (afterload), which is determined by the height of the fluid column above the aortic cannula and is usually set to 60-100cm for a rat heart. In the ventricular ejection phase a portion of the perfusate enters the coronary ostia, and thereby perfuses the heart muscle. This coronary flow can be collected and measured as described for the Langendorff preparation.

These two perfusion models (reterograde and working) are optimized for investigation of distinct end-points. While the Langendorff setup is a perfect model for determination of infarct size, the working heart perfusion is more suitable for evaluation of cardiac function [215].

4.3 Examination of signaling pathways

4.3.1 **Western Blotting**

When doing Western Blot (WB) analysis, proteins are separated by size on a polyacrylamide gel and transferred onto a membrane. The primary antibody binds to the protein of interest or a posttranslational modification such as phosphorylation. The secondary antibody binds the primary antibody, and is conjugated to a reporter group which produces a detectible signal when the appropriate substrate is added. WB is regarded as a semi-quantitative analysis due to its indirect measurements as well as being a multi-step procedure, the latter increasing the possibility of variation. As a result, relative quantities of samples on the same blot can be compared. WB results are illustrated by presenting representative blots as done in **Paper I** that can be supplemented with the densitometrical readings of all blots as done in **Paper II, III and IV**. Phosphorylated proteins were normalized to the total level of the

corresponding protein to determine phosphorylation mediated regulation. To ensure equal loading on the gels, housekeeping proteins such as Actin or GAPDH were visualized simultaneously as the proteins of interest.

In **Paper II, III and IV,** phosphorylated proteins and stress induced proteins were analyzed in heart tissue. To minimalize the amount of handling induced stress and de-phosphorylation, hearts were allowed to stabilize for 20 min before introducing regional ischemia (**Paper III and IV**), snap frozen in liquid nitrogen at the end of perfusion protocol and lysed in in the presence of protease inhibitors.

4.3.2 Intracellular Phospho-flow Cytometry

Flow cytometry is a technique to count and analyze cells in a fluid stream as they pass by an electronic detection apparatus. Phospho-flow cytometry combines the ability of monoclonal antibodies (mAb) to recognize post-translational modifications, such as phosphorylation, and the single-cell analysis of flow cytometry. It is therefore a fantastic tool for analyzing signaling pathways, because it can identify cellular subpopulations and simultaneously analyze biochemical processes within single cells. Constantly, major advances are made to the method, expanding the number of simultaneous multiparametric analysis of physical and/or chemical characteristics. Compared to more conventional techniques, phospho-flow cytometry can analyze multiple parameters in single cells in a heterogeneous and small cell population at a rapid pace.

In **Paper I** we used phopho-spesific antibodies to investigate the signaling cascade in NB4 cells subjected to daunorubicin (DNR) and cAMP. After stimulation, the cells were fixed in paraformaldehyde (PFA) and permealized in methanol as suggested by Krutzik and Nolan 2003 [216]. Methanol dehydrates the cells and denatures the proteins which may destroy epitopes recognized by the monoclonal antibodies (mAbs). However, this process is reduced when the methanol is applied ice cold. In addition, methanol makes nuclear proteins available which is necessary when looking at e.g. the different Stat proteins. Otherwise, it is important to be consistent with

regards to time and temperature during fixation, permeabilization and mAb staining, since variations may affect measurements of phosphorylation induction [216]. Also, it is important to know that the amount of mAb is dependent of volume and not cell number, as absolute intensities change almost linearly with mAb concentration [216].

4.3.3 qRT-PCR

Quantitative real time polymerase chain reaction (qRT-PCR) is a method based on standard polymerase chain reaction (PCR), and is used to amplify and simultaneously quantify a targeted DNA sequence. The results can be presented as an absolute number of copies or a relative value when normalized to a reference gene. qRT-PCR can produce accurate quantitative data, is sensitive, requires low amounts of RNA template, but unfortunately requires expensive equipment and reagents. Normalization of gene expression data is used to correct sample-to-sample variation. Real-time results are usually normalized to a "housekeeping gene", coding a protein required for basic cellular function with a relatively constant expression in all cells of an organism. The qRT-PCR experiments in this thesis **(Paper II)** are in concordance with other cardiac qRT-PCR studies [217], and use glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene. In a study on cardiac stem cells, GAPDH was the most consistent housekeeping gene under normoxic conditions, while beta-actin was the most consistent during hypoxia [218]. However, a recent study on the stability of different "housekeeping genes" showed that the anti-cancer drug irinotecan upregulate the level of GAPDH in the rat colon. They encourage the use of normalization couples, but presented ubiquitin C as the most favorable gene if restricted to only one normalization gene (in a rat model of irinotecan-induced mucositis) [219]. We should therefore in future experiments consider to normalize the values to more than one "housekeeping gene".

5. DISCUSSION

5.1 Evaluation of cAMP as adjuvant in APL treatment

A major concern with conventional cancer therapy is that only subgroups of patients respond favorably to a given treatment, and that side effects often limit the dose efficiency of the treatment. These two issues are addressed in **Paper I** and **Paper II**, respectively. Over the years, new treatment modalities with more targeted therapy are evaluated, and one successful approach is the use of *all-trans* retinoic acid (ATRA) in acute promyelocytic leukemia (APL), and has changed the prognosis from the most rapidly fatal leukemia two decades ago to the most curable leukemia subtype today. Current first-line APL therapy is based on differentiation-associated maturation, and hence, elimination of the leukemic cells by ATRA, which is combined with an anthracycline (Daunorubicine-DNR) to eliminate residual cells [220]. However, the occurrence of ATRA resistant cell populations in APL patients may explain why *in vivo* treatments invariably lead to relapse with resistance to ATRA [52]. The ATRA-maturation inducible cell line NB4 [51] and the maturation resistant sublines NB4-RA^r (R1 and R2) [52, 53] were isolated from an APL patient in relapse. Although the NB4-RA^r cell lines are retinoic acid (RA) maturation resistant, the R1 subline responds to RA by other means (proliferation at low doses) [53], meaning that the RA signaling is not defective. Interestingly, the maturation resistant cell line (R1) can actually undergo terminal maturation when ATRA is combined with cAMP elevating agents [53]. The authors describe this as an RA-dependent priming step making the cells competent to undergo maturation, followed by cAMP-dependent triggering of the primed cells to undergo terminal maturation [53]. It is clear that both these processes are necessary for maturation since the RA resistant R2 subline lack the RA-dependent component, making it unresponsive to cAMP triggered maturation [53]. In addition, subtle changes in cAMP levels using antagonizing cAMP analogs ((RP)-8Cl-cAMPS) disturbed maturation of RA-sensitive NB4 cells [53]. This emphasizes the crosstalk between RA and cAMP as a key component in ATRA mediated therapy, where endogenous cAMP seems sufficient for sensitive NB4 cells to mature. An

important paper by Ruchaud *et al.* implies that uncoupled priming and triggering may explain APL relapse with resistance to ATRA and advocates cAMP as an adjunct to current APL therapy to improve further the long-term survival of these patients [53]. Thus, recent studies demonstrate cAMP enhanced ATRA effect on survival of syngenic PML-RARA APL mice and mice transplanted with NB4 cells [54-56], and also retarded the APL progression in a patient [55]. However, none of these studies incorporate anthracyclines in their experiments, and therefore no study has evaluated the impact of cAMP on the anti-leukemic effect of anthracyclines which is currently an important component of first-line APL therapy. In **Paper I** we found that cAMP *abrogate* the anti-leukemic effect of daunorubicin (DNR) in acute promyelocytic leukemia (APL) cells. These findings suggest awareness when cAMP stimulation is considered combined with ATRA to boost APL cell differentiation since the expected beneficial effect of cAMP on APL cell maturation may be outweighed by enhanced survival of ATRA-resistant APL blasts. Awareness should also be undertaken when patients who experience increased cAMP levels are treated with anthracyclines, such as patients with inflammatory pulmonary diseases who are treated with phosphodiesterase inhibitors.

5.1.1 The plethora of cAMP signaling

cAMP is a remarkable regulator of fundamental cell processes, including cell proliferation, differentiation and apoptosis [221]. cAMP activity is mainly mediated via cAMP-dependent protein kinase A type I and II (PKA I and PKA II) or cAMP-stimulated exchange factor Epac 1 and Epac 2 [221]. The versatility of cAMP signaling is highly represented in hematopoietic cells, with induction of apoptosis in thymocytes [222, 223] and the myeloid leukemia (AML) cell line IPC-81[224], while protecting mature neutrophilic granulocytes against TNFα induced death [225]. In addition, cAMP synergizes with glucocorticoids and PKC signaling in inducing apoptosis in immature murine T cells [226, 227], while it cooperates with retinoic acid in the differentiation of various leukemia cells, such as NB4 [53, 228, 229]. The findings in **Paper I** present another example of the diverse nature of cAMP signaling. We found that cAMP abrogate the anti-leukemic effect of daunorubicin (DNR) in

acute promyelocytic leukemia (APL) cells. Protection was dependent on the generally cytoplasmic PKA-I rather than perinuclear PKA-II, and was independent of Epac. PKA I dependency is also described for cAMP induced apoptosis in the AML cell line IPC-81 [230], while recent findings by Nguyen *et al.* demonstrate that activation of both PKA type I and PKA type II is required for ATRA-induced maturation of the APL cell line NB4 [231]. cAMP is also involved in heart physiology and pathology. In cardiomyocytes, cAMP is the main second messenger, orchestrating the signals in sympathetic and parasympathetic systems, mediated via G_s-and G_i-protein respectively. More recently, cAMP was found to be involved in the regulation of cardiomyocyte cell death. Increased endogenous cAMP protects neonatal rat cardiomyocytes against NO-induced apoptosis, a mechanism involving both PKA/CREB and Epac/Akt-dependent pathways [232]. Also, cAMP reduces the mortality after acute myocardial infarct (AMI) in transgenic mice overexpressing adenylate cyclase VI, by attenuating adverse left ventricular (LV) remodeling and preserving LV contractile function [233]. It has been documented a nearly twofold rise in cAMP levels during myocardial ischemia [234]. Paradoxically, Lochner *et al.* showed cyclic increase in tissue cAMP during the classical multi-cycle preconditioning protocol [235], and others have shown that brief periods of increased tissue cAMP, as a result of β-adrenergic [236] or ischemic preconditioning [234, 237], are cardioprotective against a subsequent ischemic period [237-240]. This cardioprotection is abolished in the presence of the β-adrenergic blocker alprenolol, indicating that temporally dependent increase in tissue cAMP during ischemic preconditioning is essential for its cardioprotection [237, 240]. The cAMP receptor, PKA, is suggested to be important in the cardioprotective mechanisms of ischemic preconditioning [239, 241]. This is supported by Sanada *et al.* showing that pharmacological preconditioning using a PKA directed cAMP analogue protects the *in vivo* myocardium against ischemia-reperfusion injury [239], which cannot be achieved using the Epac directed cAMP analog [242]. In **Paper III** of this thesis, we showed that acute pre-ischemic treatment with corticotropin releasing factor (CRF) reduced neonatal cardiomyocyte death *in vitro* and infarct size in intact *ex vivo* rat hearts exposed to lethal ischemic induced stress. The CRF mediated cyto-

/cardioprotection was mediated through CRFR2 and involved activation of PKC and PKA dependent signaling pathways. This involvement of PKA is in contrast to the mechanism of urocortin (UCN) peptides against ischemia-reperfusion injury [196, 197]. Recently, it has been shown that UCN evoke inotropic and lusitropic effects in the *ex vivo* rat heart through Epac activation [243], indicating a link between CRFR2 and Epac in the heart.

5.2 The clinical picture of anthracycline mediated cardiotoxicity

Acute and subacute anthracycline induced cardiotoxicity is relatively rare and occur in 1-2% of patients [244], while the types of chronic cardiotoxicity is clinically significant and requires long-term therapy [114, 245-249]. A highly quoted study by Von Hoff and colleagues from 1979, gave early recommendations for a maximum dose of 550mg/m^2 doxorubicin (DOX) [250], based on relatively low incidence of heart failure up to that level. Since then, it has become evident that the predicted incidents of anthracycline induced cardiotoxic events was underestimated, as suggested by the authors themselves [250]. A large retrospective analysis of three Phase III trials, published by Swain in 2003, found the level of cardiotoxic incidents to be higher than previously described [251]. They observed an estimated cumulative percentage of patients with DOX-related congestive heart failure (CHF) to be 5%, 26% and 48% at cumulative doses of 400 mg/m^2, 550 mg/m^2, 700 mg/m^2 respectively [251]. In addition, they estimated a significant risk of cardiotoxicity at relatively low anthracycline doses (200-250mg/m^2) [251], an observation supported by others [252]. The introduction of the anthracyclines has played an important role in the improvement of cancer therapy and has created a population of childhood cancer survivors almost reaching 350000 in the US alone (in 2005) [253]. For many oncologists the risk of cardiotoxicity has, and for some still are, a pale problem compared to the cancer itself. Although presenting as a secondary priority, cardiotoxicity must never be ignored. Therefore, research devoted to prevention and early diagnosis of anthracycline induced cardiotoxicity is very important. Today,

ultrasound or MUltiple Gated Acquisition scan (MUGA) are used to evaluate left ventricle ejection fraction before, during and after chemotherapeutic treatment, to follow heart function and to early diagnose cardiomyopathy [254]. Unfortunately, limited studies with long-term follow up of heart function have been conducted, which is necessary since anthracyclines also are used to treat curable tumors which enable the patients to live for decades thereafter. A study of 201 survivors of childhood cancer, found 38% and 63% to have abnormal cardiac function, using noninvasive imaging, when exposed to 495mg/m^2 and 500mg/m^2 DOX, respectively [102]. In another study, 229 adults treated with DOX during childhood cancer was diagnosed with symptomatic (10%) and asymptomatic (6%) heart failure more than 15 years after treatment [255]. To sum up; anthracycline has contributed to an excellent survival rate of childhood cancer patients. Unfortunately, for some the price is paid later with cardiac failure, and is an increasing cause of heart transplantation in young adults [256].

5.3 Cardiac antioxidant therapy

The molecular mechanisms of the anthracycline mediated anti-cancer effect and toxicity in healthy tissue are mediated at two fundamental levels, by interference with DNA and increased oxidative stress. However, it is fairly accepted that the cardiotoxic effect of anthracyclines are attributed to the latter, with elevated levels of ROS and interference with mitochondrial function. In addition, a review by Gewirtz *et al.* suggests that the anti-cancer effect of anthracycline at clinical doses is mediated by DNA interference and not increased ROS [80]. Still, many oncologists believe that ROS induction is an important component of anthracycline mediated removal of cancer cells, and therefore do not recommend antioxidant diet/supplementation during chemotherapy since it can interfere with the anti-cancer effect of the anthracycline. In a systematic review of randomized, controlled clinical trials, none of the studies reported any evidence of significant decrease in efficiency of chemotherapy when combined with antioxidant supplementation [257]. In fact, most studies reported decreased general toxicity in healthy tissue when chemotherapy was

accompanied with antioxidant supplementation [257, 258]. Numerous antioxidants and ROS scavengers have been *in vitro* and *in vivo* assayed for the prevention of cardiotoxicity (for review see [259, 260]). As previously mentioned, Stahl and Sies [131] stated that a cocktail of antioxidants in naturally occurring compounds have far more profound effects due to synergistic actions of the individual compounds. **Paper II** of this thesis report that Red Palm Oil stabilize important antioxidant systems in cardiac tissue otherwise down regulated by daunorubicin (anthracycline) [131]. While the majority of preclinical studies indicate a potential benefit of certain dietary agents, some studies cannot find any benefit of the same antioxidants. For that reason the authors "*stress the difficulty to extract unequivocal conclusions from such a wide number of studies using many different concentrations of antioxidants, in different experimental models, and especially with many different doses of the drug*" [260]. The lack of high quality clinical studies does not enable firm conclusions to be made. Studies with long-term follow-up, which can identify late-onset cardiotoxicity, evaluating both cardiotoxicity and anti-tumor effect are necessary. Today, only one compound, the iron-chelator Dexrazoxane, show consistent cardioprotective effect in preclinical [261] and clinical studies [262], without any influence on the anti-tumor effect of the drug. The American Society of Clinical Oncology recommends dexrazoxane as a cardioprotector for patients with metastatic breast cancer who will benefit from a cumulative dose of doxorubicin (DOX) exceeding 300mg/m^2. The same is recommended for other adult patients, with caution of possible, currently unknown, anti-anthracycline effects in mind [263]. As mentioned above, we have shown (**Paper II**) that daunorubicin significantly down regulate cardiac levels of important antioxidant systems. Consumption of endogenous cardiac antioxidants, such as oxidation of glutathione, are also demonstrated during ischemia-reperfusion e.g. in patients undergoing coronary bypass surgery with aortic cross-clamping [264]. Several studies, with varying results, have evaluated the cardioprotective effect of different antioxidants against ischemia-reperfusion induced injury in patients undergoing planned cardiac surgery [264]. The use of *N*-acetylcysteine (NAC) to enhance intracellular glutathione has shown promising results in small-scale trials to

improve patient outcome after acute myocardial infarction or surgery induced ischemia [265].

5.4 Modulation of myocardial ischemia-reperfusion injury

In 1972 it was established that myocardial reperfusion of the occluded coronary artery would reduce myocardial infarct size [266]. Today, reperfusion strategies using thrombolytic treatment and percutaneous coronary intervention (PCI) represents the cornerstones of current therapy for patients suffering from acute myocardial infarction (AMI). However, in-hospital mortality of this patient group are still 6-14% [267], urging the need for novel interventions to further reduce infarct size and improve survival rates of these patients. Although reperfusion is a necessity for myocardial salvage, the price to pay is accompanying reperfusion injury (Fig. 1.8). There is currently no clinical therapy for lethal reperfusion injury, which makes it an important target for residual cardioprotection. Lethal reperfusion injury represents a hot area of ongoing research and has led to the discovery of several pre-clinical therapies (mechanical and pharmaceutical), with potential of being administered simultaneously as classical reperfusion strategies to further reduce the final infarct size.

5.4.1 **"From Bench to Bedside"**

Unfortunately, it is often difficult to transfer promising pre-clinical strategies into clinical interventions which actually improve the outcome of AMI patients. The reason for this may be attributed to confounding factors involving patient selection, reperfusion strategy (thrombolytic treatment, PCI or coronary artery bypass surgery), administration time of the novel reperfusion intervention as well as varying determinants of infarct size such as ischemic time, area at risk, collateral flow and the endpoints evaluated for cardioprotection (reviewed in [268]). While coronary heart disease (CHD), is caused by or associated with, risk-factors and co-morbidities such as aging, hypertension, diabetes and left ventricular hypertrophy [269], most pre-clinical studies, including our own, evaluate the effect of novel cardioprotective

strategies using healthy and juvenile animals. In fact, atrial tissue from elderly [270] and diabetic patients [271] was resistant to ischemic preconditioning (IPC) in the *in vitro* hypoxia-reoxygenation model. Further, diabetic patients has twice as high glycogen synthase kinase 3β (GSK3β) activity compared to non-diabetic patients, and since we (**Paper IV**) and others [159] have demonstrated an important role for GSK3β inhibition during cardioprotection, it is possible that this can explain why the protective effect of IPC and IPost are lost in diabetic animal models [272, 273]. Another major confounding factor is concomitant patient medication either directed against the ongoing MI or associated risk factors and co-morbidities. Several independent studies have demonstrated that different pharmaceuticals may actually be cardioprotective themselves (β-blockers and ACE inhibitors) or they can abrogate the otherwise cardioprotective effect (anti-diabetic sulfonylurea and chronic use of statins) (reviewed in [274]). **Paper IV** of this thesis contributes to the literature by demonstrating the latter. We found that the combination of IPost with pharmacological treatment strategies, here represented by insulin or GSK3β inhibition, completely abrogated cardioprotection afforded by the separate treatment given alone. Loss of cardioprotection was associated with reduced phosphorylation of the pro-survival kinase Akt. The molecular explanation for this observation is still not resolved. It does, however, imply that drugs which can modulate Akt, GSK3β or other components of the RISK pathway may interfere with IPost mediated cardioprotection. Hence, **Paper IV** provide with suggestions as to why clinical trials using IPost have shown varying result.

5.5 Similarities of anthracycline and ischemia-reperfusion mediated cell injury

The myocardium is generally considered a postmitotic organ, excluding any cardiomyocyte turnover in the normal or diseased organ. With the idea of terminally differentiated cardiac cells came also the controversy of cardiomyocyte cell death [275], as this would eventually lead to the loss of myocardial mass. Today, scientists are suggesting that death and regeneration are part of the normal homeostasis of the

heart [276], and we know that cell death is involved in both ischemia-reperfusion induced injury [125, 277, 278] as well as anthracycline induced cardiotoxicity [279]. As described in the introduction, anthracycline mediated cardiotoxicity is believed to be distinct from its anti-tumor effect, and is likely to be a result of ROS mediated cardiomyocyte damage. Concomitantly, ROS is an important component of ischemia-reperfusion mediated cell death, and it is therefore relevant to believe that free radical injury to the myocardium induced by anthracyclines is similar to that induced by ischemia-reperfusion. This is supported by studies demonstrating pharmacological cardioprotection against both ischemia-reperfusion injury as well as anthracycline induced cardiotoxicity [280, 281]. More interestingly, the most effective experimental treatment against ischemia-reperfusion injury, ischemic preconditioning (IPC), are also protective against anthracycline (epirubicin) induced cardiotoxicity [282]. Conversely, the only approved clinical cardioprotector used to attenuate anthracycline mediated cardiotoxicity, the iron-chelator Dexrazoxane, are protective against ischemia-reperfusion in the *ex vivo* rat heart [283].

5.5.1 **Increased intracellular ROS**

Induction of myocardial oxidative stress is the most frequently proposed mechanism of anthracycline mediated cardiotoxicity and is described in the cytoplasm, mitochondria and sarcoplasmic reticulum [92]. Redox cycling of anthracyclines are based on the quinone moiety of the molecule and also their ability to form anthracycline-iron complexes [92]. Increased cardiac ROS may injure cell membranes and nuclear/mitochondrial DNA leading to changed protein pattern, disturbed cell signaling and altered membrane permeability, all culminating in increased Ca^{2+} and apoptosis [109]. It is also of general agreement that increased ROS production is involved in myocardial reperfusion injury [150, 284, 285]. Under normal conditions, $O_2^{-}\cdot$ would be converted to H_2O_2 by superoxide dismutase. However, due to acidosis during ischemia, ferric and ferrous ions released from metalloproteins, catalyze the generation of highly reactive hydroxyl radical from both $O_2\cdot^{-}$ and H_2O_2 (Fenton reaction) [286]. Paradoxically, we (**Paper IV**) and others have demonstrated that transient and low concentrations of RNS/ROS are required at

immediate reperfusion to mediate cardioprotection elicited by ischemic conditioning [287-289]. In summary, this thesis illustrates the myriad roles of ROS in a biological system. Harmful in one setting (anthracycline induced cardiotoxicity), protective in another (ischemic pre- and post-conditioning), and also being necessary for steady state signaling. It is obvious that strict regulation is a prerequisite to handle the diverse effects of ROS, which involves time-, concentration- and location dependencies of different reactive oxygen species.

5.5.2 **Mitochondrial related death/survival pathways**

The effects of oxidative stress on the mitochondrial intrinsic pathway are well described, but the involvement of the extrinsic pathway is not yet clarified. Most of the ROS induced cellular events described in the above section contribute to cell death via apoptosis and necrosis, and is believed to be the primary mechanism of anthracycline induced cardiomyopathy [290]. Anthracyclines create Ca^{2+} flux aberration, and hence, increased mitochondrial [Ca^{2+}]. This leads to dissipation of the transmembrane potential, mitochondrial swelling and leakage of apoptotic signaling molecules, a process involving opening of the mitochondrial permeability transition pore (mPTP) [290]. Similarly, (as described in section 1.9.2) the main event linking ischemia-reperfusion to cell death is also opening of the mPTP. This is a Ca^{2+} and ROS dependent process [155], inhibited by the acidic environment during ischemia while manifested at immediate reperfusion [156, 157]. Attenuation of mPTP opening during early reperfusion has gained increased focus, and many cardioprotective interventions activate important anti-apoptotic protein kinases such as PI3K-Akt and MEK1/2-Erk1/2 (RISK pathway) [153]. Inhibition of mPTP by ischemic conditioning involves mitoK_{ATP}, PKC and ROS signaling but has previously not been linked to insulin and GSK3βi mediated cardioprotection. However, our results in **Paper IV** indicate dependency on PKC and mitoK_{ATP} mediated signaling in cardioprotection afforded by post-ischemic insulin treatment. It is possible that the administration time (*pre* or *post* ischemia) explain the opposing findings presented in **Paper IV** compared to others [159, 291]. Recently, inhibition of glycogen synthase kinase 3β (GSK3β) has been suggested as the key event linking cardioprotection to inhibition of mPTP

[159], and the involvement of Akt phosphorylation and GSK3β inhibition during cardioprotection are described in **Paper IV**. Similarly, anthracyclines seems to inhibit Akt phosphorylation and thereby increase the activity of GSK3β [292]. In addition, upstream kinase activation of PI3K-Akt with subsequent GSK3β inhibition, are protective against anthracycline induced cardiomyocyte apoptosis [292].

6. CONCLUSION

The overall aim of this thesis was to participate in the search for ways to directly or indirectly minimize anthracycline mediated cardiotoxicity, and also to reduce the amount of ischemia-reperfusion injury. We therefore addressed and answered the following specific aims:

Paper I – Evaluate the influence of cAMP on anthracycline induced apoptosis of acute promyelocytic leukemia (APL) cells, since cAMP has been advocated as adjuvant to classical APL therapy.

We found that cAMP abrogates anthracycline induced APL cell death in a PKA type I dependent manner. The mechanism was associated with inhibition of pro-apoptotic Bad and activation of the acute myeloid leukemia (AML) oncogene CREB. *In vitro* findings were mirrored *in vivo* (NSG mice with orthotopic NB4 cell leukemia) were cAMP induced more rapid APL progression. Together these data suggests that the beneficial pro-differentiating effects of cAMP should be weighed against the non-beneficial pro-survival effect found in this study.

Paper II - Study the possible cardioprotective effect of Red Palm Oil against anthracycline mediated cardiotoxicity

Red palm oil (RPO) diet supplementation improved cardiac output in hearts from animals treated with anthracycline, and also stabilized important antioxidant systems such as SOD 1, otherwise decreased by anthracycline treatment. RPO also inhibited anthracycline induced stress kinases such as p38 and JNK and up regulated the pro-survival kinase ERK.

Paper III - Delineate the cardioprotective effect of corticotropin releasing factor (CRF) against ischemia-reperfusion injury

CRF offered cytoprotection when administered prior to a simulated ischemic insult and also reduced infarct size in the *ex vivo* perfused rat heart. CRF had no protective effect when administered at the time of reoxygenation/reperfusion. The protective effect of CRF was mediated via CRFR2 and was dependent on PKC and PKA signaling.

Paper IV - Investigate the combination of known protective therapies against ischemia-reperfusion injury

In this study we found that combination of two pharmacologic reperfusion therapies (Insulin or GSK3β-inhibition) did not lead to additive cardioprotection compared to the two treatments given alone. However, combining ischemic postconditioning with either of the two pharmacologic therapies (Insulin or GSK3β-inhibitor) completely abrogated cardioprotection afforded by the separate therapies. This loss of cardioprotection was associated with blunted Akt phosphorylation.

6.1 Concluding Remarks

This thesis has indirectly (**Paper I**) and directly (**Paper II, III, IV**) focused on the heart as the primary organ for protection. In **Paper I** we have participated in the search for ways to improve current acute promyelocytic leukemia (APL) treatment. The introduction of novel agents to current treatment regimens may reduce, and hopefully someday replace, the use of anthracyclines, and thereby alleviate some of the cardiotoxic side effects imposed by these agents. In **Paper II** we demonstrate the potential of a natural antioxidant source, the red palm oil (RPO), as a cardioprotector against anthracycline induced cardiotoxicity. RPO is also cardioprotective against ischemia-reperfusion induced injury and has highly anti-cancer potential due to the high level of tocotrienols. **Paper III and IV** contribute with pharmacologic and mechanical treatments to reduce ischemia-reperfusion induced injury. In **Paper III** we show that the novel agent Corticotropin releasing factor (CRF) exerts cardioprotection, and demonstrated that the time of administration (*pre* or *post* ischemia) may give distinct outcome. Finally, **Paper IV** highlights the importance of the finely tuned cellular signaling involved in cardioprotection, as the combination of cardioprotective treatments may counteract each other, and address the struggle of pre-clinical studies to successfully translate into the clinic. As a final remark, my thesis emphasizes the similarities of anthracycline and ischemia-reperfusion induced cell injury. This encourages a more tight collaboration of oncologists and cardiologists in the clinic, as well as between pre-clinical researchers from these to experimental fields.

7. FUTURE PERSPECTIVES

7.1 Paper I

cAMP has been suggested as adjuvant to current acute promyelocytic leukemia (APL) therapy due to its pro-differentiating effects on ATRA sensitive and in-sensitive cells. In **Paper I** we have demonstrated that cAMP attenuate daunorubicin induced apoptosis in acute myeloid leukemia (AML) and APL patient cells and the APL cell line NB4. The next step would be to combine ATRA with daunorubicine (DNR), in the presence and absence of cAMP, to evaluate the net effect of cAMP stimulation regarding increased differentiation versus reduced apoptosis.

Both Bad and CREB phosphorylation are under current investigation as therapeutic targets in AML and other malignancies. CREB is an AML proto-oncogene, and overexpression is associated with a poor outcome in the patients. Small molecules that inhibit binding of CREB to its partners have been identified, and was effective at micromolar concentrations, without off-target inhibition of transcriptional machinery [293]. Therefore, *in vivo* experiments with clinical relevant inhibitors of Bad and CREB should be tested for their ability to inhibit cAMP induced protection of leukemic cells.

7.2 Paper II

Paper II demonstrate that diet-supplementation with red palm oil (RPO) during chemotherapy protect the rat heart against daunorubicin induced cardiotoxicity, with improved aortic output and coronary flow. However, in a follow-up study we would lengthen the perfusion protocol in order to achieve a clearer difference between the groups regarding the hemodynamic parameters. More importantly, in order to investigate if RPO exert any adverse effect in regards to the anti-cancer effect of the anthracycline, we would like to evaluate the cardioprotective effects of RPO in a leukemia rat model.

7.3 Paper III

Since we strive to perform clinical relevant basic research, we were disappointed when corticotropin releasing factor (CRF) did not have any cardioprotective effect when administered at reperfusion/reoxygenation. However, there is a possibility that CRF could exert protection if applied in another concentration. This further warrants a set of dose-response experiments with CRF administration at the moment of reperfusion.

7.4 Paper IV

Previous studies have shown that insulin has to be present from the onset of reperfusion to be cardioprotective, and that cardioprotection is lost when insulin administration was delayed 15 min into reperfusion. Similarly, the mechanical intervention of IPost has to be performed at early reperfusion. It is likely that the loss of protection, when combining IPost with insulin therapy (or GSK3i), is due to abrogation of, or interference with, the pro-survival signaling mechanisms activated by these treatments when administered separately. Intriguingly, the lack of cardioprotection was associated with blunted Akt phosphorylation at early reperfusion. In the continuation of this study, we would like to investigate if increased phosphatase activity and/or reduced receptor signaling is involved in this phenomenon.

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