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Glucocorticoid-Induced Cardioprotection: A Novel Role for Autophagy?

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1. Introduction

Glucocorticoids (GC) are commonly used as anti-inflammatory and immunosuppressive therapy by approximately 1% of the total adult population. Glucocorticoid therapy has also been used in non-autoimmune and non-inflammatory conditions such as acute myocardial infarction, angina, endocarditis as well as in invasive cardiology, coronary interventions and cardiopulmonary- bypass surgery. Despite ample evidence for GC's role as a natural, physiologic regulator of the immune system, little is known about the molecular events induced by GCs during a stress response. Autophagy is a survival mechanism which is upregulated in response to stress in the cell. It has been described as the cell's major adaptive strategy in response to a multitude of extracellular stresses, such as nutrient deprivation, mitochondrial damage, endoplasmic reticulum stress or infection. Conserved in all eukaryotes, it is mediated by a unique organelle, the autophagosome, which, under inclusion of cytoplasmic cargo, fuses with lysosomes in order to yield recyclable nutrient metabolites. Basal autophagic activity plays a vital role in maintaining homeostasis during cellular stress. Its malfunction has been implicated with human pathologies such as heart disease, neurological storage disease and cancer.

It is known that GC-triggered autophagy plays a role in cell death during development. However, recent landmark studies also indicate that autophagy operates as a hub, integrating cellular stress, metabolism and glucocorticoid mediated anti-inflammatory action. Importantly, recent lines of evidence suggest that glucocorticoids impact on key signalling components, which control the activity of the autophagic machinery.

In this review we will focus on the connections between these key signaling components and autophagy, describing their central roles as modulators of GC-induced protection during a cellular stress response.

2. Glucocorticoid generation and metabolism

The understanding of the physiological regulation of glucocorticoid activity has considerably improved over the last few decades. The generation of glucocorticoids from cholesterol, which occurs in the zonae fasciculata and reticularis of the adrenal cortex, is tightly regulated by the hypothalamic-pituitary-adrenal (HPA) axis where glucocorticoids regulate its own generation through negative feedback inhibition. Glucocorticoids are produced *de novo* under this control and are released into the blood as required, with a definite circadian rhythm producing peak concentrations in the early morning [1]. When secreted into the blood, most (90-95%) of glucocorticoids are sequestered to corticosteroid-binding globulin and albumin with the unbound fraction available to interact with their receptors [2]. Metabolic inactivation of glucocorticoids occurs predominantly in the liver, but also in the kidney with inactive metabolites excreted in the urine.

3. Molecular actions of glucocorticoids: Genomic and non-genomic pathways

The classical mode of glucocorticoid-induced gene expression, i.e. the genomic effect involves ligand-dependent activation and release from chaperone proteins (heat shock protein-90 and others), translocation of the receptor-complex to the nucleus where binding of the glucocorticoid receptor to glucocorticoid response element (GRE) in the promoter of the target genes will lead to transcriptional activation of the genes within hours [3, 4]. Activation of glucocorticoid-responsive genes occurs via interaction between the DNA-bound GR and transcriptional co-activator molecules such as CREB-binding protein, which have intrinsic histone acetyltransferase activity and cause acetylation of core histones. This tags histones to recruit chromatin remodeling engines and subsequent association of RNA polymerase II resulting in gene activation [5]. Increasing evidence suggests that glucocorticoids can also cause rapid activation of signaling molecules prior to altering gene expression. These so called non-genomic effects occur within minutes of glucocorticoid exposure and are not affected by inhibiting RNA transcription [5, 6].

Metabolic effects of glucocorticoids represent most of the adverse effects of glucocorticoid therapy and are mainly ascribed to the transcriptional activity of the glucocorticoid receptor, whereas the therapeutically beneficial anti-inflammatory actions are thought to be predominantly caused by the mechanism of transrepression where the activated GR can selectively repress the transcription of specific inflammatory genes without binding to DNA itself but by a number of pleiotropic actions at the promoters of inflammatory genes. Inflammatory genes are regulated by the actions of proinflammatory transcription factors such as nuclear factor- κ B (NF- κ B), activator protein -1 (AP-1), and signal transducer and transcription proteins. Activated GR binds to these transcription factors, either directly or indirectly, and recruits co-repressor proteins that blunt the ability of these transcription factors to switch on inflammatory genes [7]. Furthermore, many pro-inflammatory genes are repressed by GC at post-transcriptional level via mRNA destabilization or inhibition of

translation, however, this phenomenon cannot be accounted for by transrepression, therefore suggest the existence of an additional anti-inflammatory mechanism of GCs [8].

4. Ischemia/reperfusion-induced stress in the heart

Glucocorticoids play a key role in the response to stress in the heart where it can influence the regulation of blood pressure, inflammation, immune function and cellular energy metabolism [9-10]. These acute effects contribute to an adaptive response in the short term. Although the cardioprotective effects of glucocorticoids in the acute setting of ischemia/reperfusion have been experimentally demonstrated in animals [11-13] and humans [14], the molecular mechanisms still need to be fully elucidated.

Ischemia can be defined as an imbalance between the amount of oxygen, glucose and other substrates needed by the heart [15,16]. This leads to anaerobic metabolism and reduced contractile function. A biochemical imbalance occurs as the maintenance of the metabolism cannot be kept at a steady state due to inadequate coronary flow. A reduction in metabolite clearance also occurs during ischemia and intracellular pH levels drop as the acid by-products of glycolysis accumulate. The severity of ischemic injury depends on the duration of ischemia and subsequent reperfusion [15,16]. If, ischemia is maintained, reversible injury gradually transitions to irreversible injury and a myocardial infarct develops. Reperfusion with its reinforced oxygen and substrate availability is thus a prerequisite for myocardial salvage [16]. However, reperfusion after an ischemic period causes generation of free radicals and is associated with detrimental changes such as enzyme release, arrhythmias and intramyocardial haemorrhage which are known as reperfusion injuries [17]. Cardiomyocytes are highly dependent on a continuous supply of oxygen. During ischemia, cardiomyocyte capacity to generate sufficient ATP and creatine phosphate becomes depleted and multiple adaptive processes occur in response to these hypoxic environments created during ischemia. To reduce oxygen consumption, oxidative phosphorylation is limited and glycolysis is stimulated. This aids in ATP production, even under low oxygen supply [18]. Prolonged ischemia leads to cardiac failure which is characterized by the progressive death of myocytes [19]. Three major morphologies of cell death have been described, viz. apoptosis (type I), cell death associated with autophagy (type II) and necrosis (type III).

5. The basic mechanisms of Autophagy

Autophagy, from greek *self eating*, is a conserved degradation and recycling system for long-lived proteins and other sub-cellular constituents. This degradation system is inherent to all eukaryotes and is mediated by a unique organelle, the autophagosome, which, under inclusion of cytoplasmic cargo, fuses with lysosomes in order to yield recyclable nutrient metabolites. Although already described in 1966 by de Duve and Wattiaux [20], Autophagy has received significant renewed attention in the last years. This new interest is primarily based on the recently gained understanding of the molecular components of the autophagic machinery. Genetic analyses in yeast identified more than 30 autophagy-related genes (ATG), and their corresponding proteins (Atg) participating in the autophagic pathway [21].

Multiple mechanisms exist for the mode of delivery of cytoplasmic material to the lysosome, giving rise to different types of autophagy. While microphagy is characterized by cytoplasm engulfment directly at the lysosomal surface by invagination of its membrane, macroautophagy involves the synthesis of double-membrane vesicles, which sequester portions of the cytoplasm [22]. Chaperone mediated autophagy (CMA) on the other hand involves selective motif tagged protein translocation directly through the lysosomal membrane [23]. However, shared by all three mechanisms is the final step of lysosomal cargo degradation by hydrolases, allowing the recycling of degraded material. Here we will focus on macroautophagy (herein referred to as autophagy), as it is the primary mechanism for cytoplasm-to-lysosome delivery.

The autophagic process can be divided into distinct steps, which include the induction, cargo packaging, vesicle nucleation, vesicle expansion and protein retrieval, docking and fusion and finally vesicle degradation [21]. In brief, the first event is the formation of the isolation membrane of the autophagosome. The Atg1 kinase complex governs these early steps in autophagosome formation. Central to this regulation is the nutrient sensor kinase mTOR (TORC). When mTOR is suppressed due to nutrient starvation, Atg1 kinase activity is triggered and affinity for Atg13 increases [24], which leads to the recruiting of other Atg proteins to initiate autophagosome formation. Cellular sources for this autophagosome formation step have been shown to be Golgi, ER, mitochondria and the plasma membrane [25]. During this process, two strongly interdependent conjugation systems are coordinating the events leading to the formation, elongation and sealing of the isolation membrane [26]. In the first conjugation system, Atg proteins 5, 7, 10 and 12 undergo a multimerization step with Atg16, leading to the formation of an Atg16 homotetramer, which assembles with four Atg12-Atg5 conjugates [27]. In the second conjugation system, the protein Atg8 is Atg4 dependently conjugated with phosphatidylethanolamine (PE) [28]. Reactive oxygen species play a role in controlling this step, as Atg4 oxidization enables autophagosome formation to proceed [29]. Next, two kinase complexes, PI3 kinase and Atg1, participate in the late stages of autophagosome formation. Atg6 (the mammalian orthologue, beclin-1) belongs to the PI3 kinase (PI3-K) class III complex. When Atg1 interacts with Atg13, progression towards a complete autophagosome takes place [27]. Cytoplasmic cargo is now confined. Docking and fusion with a lysosome will allow acidification of the autophagosome lumen, leading to the complete and rapid degradation of cargo into constituent components that are released into cytoplasmic space via permeases (Figure 1).

6. Autophagy as a protective response during stress in the heart

The terminally differentiated nature of cardiomyocytes demands a strong molecular reliance upon a functional autophagic degradation system. In cardiomyocytes autophagy has been described already in the late 1970's where it was emphasized as an important repair mechanism of sublethal injury [30]. Sybers and coworkers demonstrated the occurrence of myocyte autophagy in a fetal mouse heart that was kept for 1 h in organ culture. In addition they observed that Autophagy was accelerated by oxygen and glucose deprivation, but the hearts function could be restored following resupply of glucose and oxygen. However,

when the period of injury lasted for longer than four hours, necrotic cell death was induced [30]. To date, many models have produced clear evidence that upregulation of autophagy promotes cell survival under conditions of metabolic perturbations and energy deprivation [31-33]. In the ischaemic myocardium, autophagy is upregulated rapidly following 20 minutes of coronary artery occlusion, leading to an increased number in autophagosomes [34]. In isolated cardiomyocytes exposed to anoxia-reoxygenation it was shown that inhibition of autophagy leads to an increase in necrotic cell death, which was further increased by additional inhibition of apoptosis [35]. However, enhancing autophagic flux, as indicated by an increased rate of autophagosomal clearance, protects cardiac myocytes against ischaemic injury by reducing apoptosis [31]. Moreover, the homeostatic role of functional basal autophagic activity in the myocardium has been demonstrated by cardiac specific disruption of Atg5, manifesting in impaired contractility, hypertrophy, dilation and sarcomeric disarray [36-37].

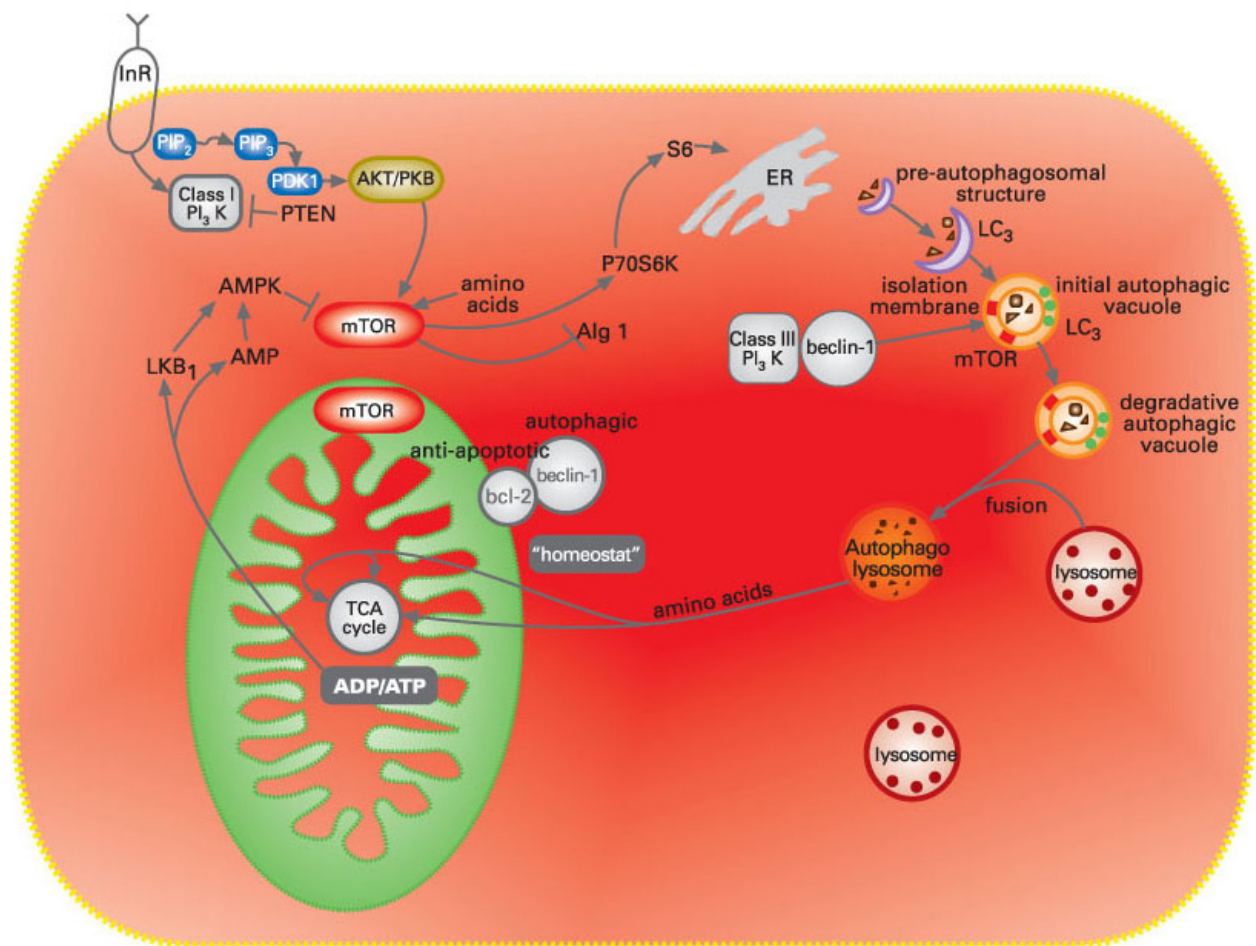


Figure 1. Schematic representation of the autophagic process, indicating the signalling network from induction to permease efflux with amino acid release, providing substrates for the TCA cycle.

7. Detrimental effects of Autophagy in the heart

Literature suggests that autophagy is uniquely controlled during ischemia and reperfusion. A large body of evidence indicates that upregulation of autophagy particularly during the reperfusion phase is detrimental and exacerbates myocyte death. Energy sensing mediated by the 5'-AMP-activated protein kinase (AMPK) appears to be central to this control mechanism. In glucose-deprived cardiac myocytes, autophagy resulting from ischemia has been shown to be accompanied by AMPK activation, and was inhibited by dominant negative AMPK, suggesting an AMPK dependent mechanism [34]. AMPK is rapidly activated during myocardial ischemia, and leads to an increase in glucose uptake and oxidation as well as fatty acid oxidation [38]. Autophagy is enhanced after reperfusion, which is accompanied by an inactivation of AMPK and an increase in beclin-1 [34]. As AMPK switches off ATP-dependent processes, [39] its inactivation at reperfusion may contribute to an unfavorable metabolic environment. Moreover, data indicate that energy sensing mediated by AMPK is also differentially controlled depending on the severity of the ischaemic insult [40]. These reports are strengthened by recent data derived from cultured myoblasts, where a differential induction of cell death was observed, which was dependent on the severity and duration of the ischaemic insult [33]. Only mild ischaemic injury induced autophagy and apoptosis, while severe injury led to primarily necrotic cell death.

8. Autophagy and myocardial metabolism

The total cellular ATP amount in the cardiac myocyte is consumed in less than one minute [41] indicating the very high metabolic demand of the myocardium, and at the same time highlighting the existence of an extremely efficient system of energy conversion. In ischaemic conditions, energy metabolism is disrupted to a level where energy production cannot meet the myocardial energy demand. However, there is a clear role for autophagy in ischemia to influence the cell's energy profile, indicative to maintain metabolic supply-demand homeostasis [33]. ATP levels decrease rapidly with ischemia and recover rapidly after reperfusion [42]. These dynamics of ATP depletion become highly relevant when considering the molecular overlap between autophagy, apoptosis and necrosis [32; 43-44, Figure 2]. It has been demonstrated that an ATP depletion of >50% is needed in order to change the mode of cell death from apoptotic to necrotic [45]. *Vice versa*, a progressive replacement of necrosis with apoptosis has been described, when intracellular ATP becomes available again [46]. Recent evidence strongly indicates the previously underestimated metabolic role of autophagy in generating metabolite substrates by shifting the cellular energetic balance [33; 47-49], suggesting that intracellular ATP availability may be controlled to a significant degree by the autophagic flux (Figure 2). These data strongly suggest that not only the magnitude of autophagic activity but also the cell's metabolic profile and microenvironment are crucial in controlling a favorable cellular response other than necrosis, and delaying apoptosis [32] (Figure 3).

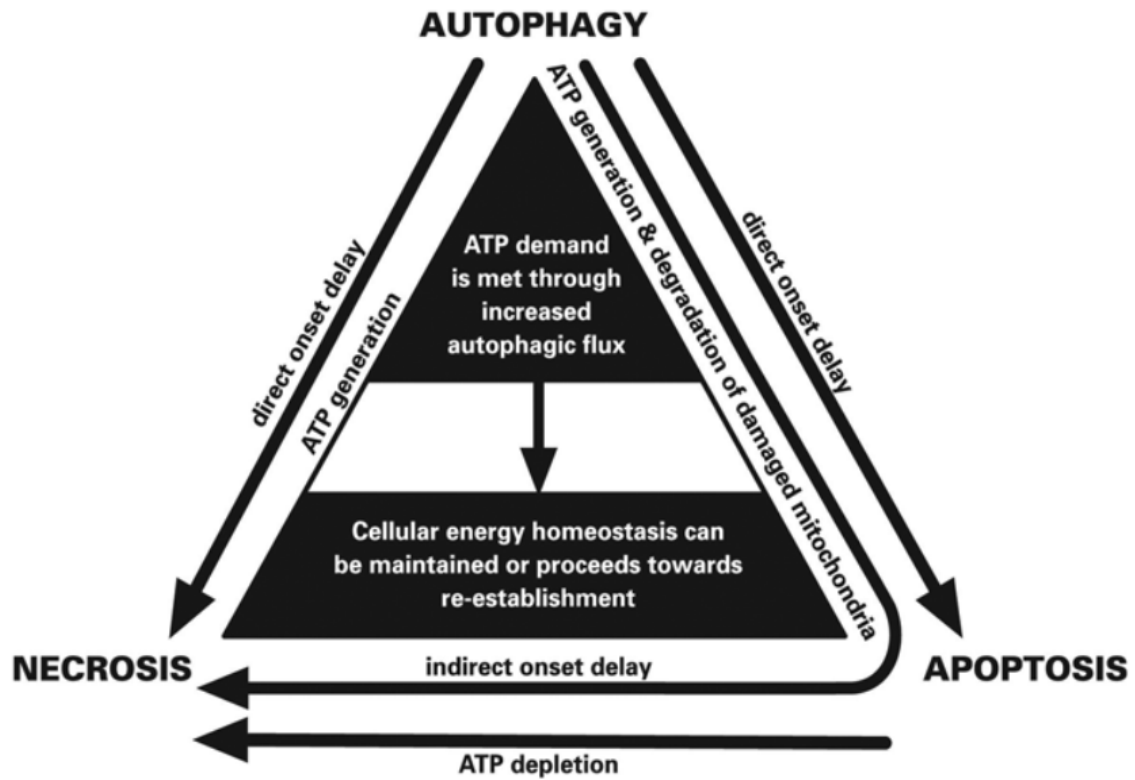


Figure 2. Recent evidence strongly indicates the previously underestimated metabolic role of autophagy in generating metabolite substrates and ATP by shifting the cellular energetic balance, with a direct and indirect effect on apoptosis and necrosis.

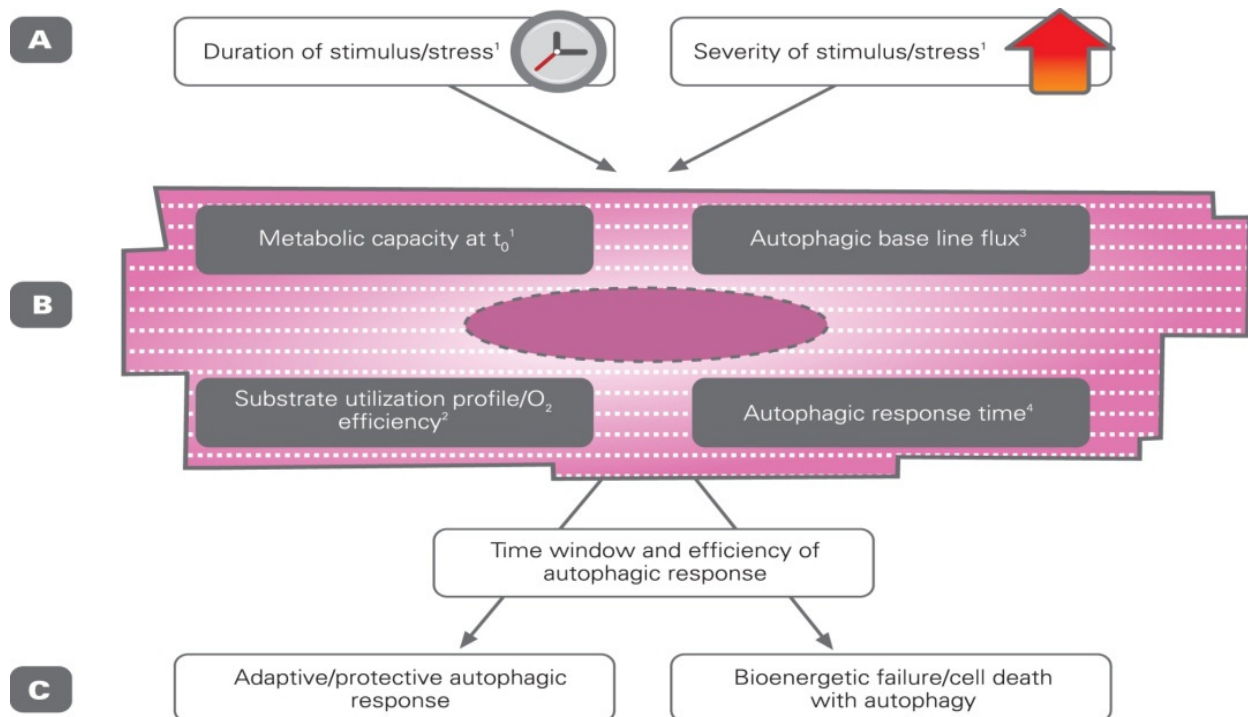


Figure 3. Whether autophagy may manifest in cytoprotection or type II programmed cell death is dependent on the severity and duration of the ischaemic insult, as well as autophagic flux- and metabolic parameters.

The above results also stress the significance of the severity and duration of the ischaemic event, to allow a sufficient induction of the autophagic machinery to take place. In fact, a direct relationship between autophagic flux and myocardial function has recently been proposed [50], indicating the strong need to measure autophagic activity accurately. Further characterization of the autophagic flux in clear context with myocardial injury will help to answer questions when autophagy functions as a primarily destructive pathway, manifesting in type II programmed cell death or when autophagy functions in a cytoprotective manner.

9. Autophagy and glucocorticoids-relationship and metabolic response

The multifaceted relationship between autophagy and glucocorticoids is already indicated in embryogenesis. Recent reports not only demonstrate an important metabolic role for autophagy during embryogenesis and postnatal development [51,52], but also indicate a likely molecular link between autophagic programmed cell death and steroids during development [53]. Embryos from the *atg5^{-/-}* knockout mouse model die perinatally due to energy depletion, leading to a reduced plasma- and tissue amino acid concentration [54]. Moreover, increased apoptosis is displayed in various embryonic tissues derived from such embryos, supporting a role for autophagy in the removal of apoptotic bodies or in delaying the onset of apoptotic cell death [54]. Targeted disruption of beclin 1 in mice also leads to early death in embryogenesis [55]. Many examples of autophagy as a mode of programmed cell death during embryogenesis exist, suggesting that an important role for autophagic cell death in development. It has been shown that autophagic cell death requires the genes *ATG7* as well as beclin 1 and can be induced by caspase-8 inhibition [56]. In addition, embryonic fibroblasts from *Bax/Bak* double knockout mice undergo autophagic cell death, which can be suppressed by inhibitors of autophagy and which is dependent on *ATG5*. These data suggest a role for *Bcl-2* family proteins controlling also non-apoptotic cell death in addition to regulating apoptosis [57]. Especially in lower eukaryotes, the rise in steroid titers can elicit a transcription regulatory hierarchy that results in synchronous autophagic cell death [53]. Such steroid triggered programmed autophagic cell death has been observed in larval salivary gland cells [53] as well as motoneurons [58]. These findings suggest that steroids can play a governing role in very specific scenarios, controlling autophagic activity and the duration of increased flux, which in turn can control the synchronous induction of cell death. It is however not known, whether a robust increase in cortisol release in humans following psychological stress, trauma, sepsis or starvation can elicit similar effects on autophagic activity during embryogenesis. Such studies deserve a great deal of attention.

Although limited data are available, similarities exist between the role of the autophagic pathway as a response mechanism to metabolic perturbations and glucocorticoids in the regulation of metabolic responses. Chronic excessive activation of glucocorticoid receptors leads to major cellular metabolic rearrangements such as insulin resistance, glucose intolerance and dyslipidaemia. Obesity and metabolic syndrome, which are characterized by a nutrient overload, have been associated with a hyperactivation of tissue mTOR,

indicating a blunted autophagic response [59]. The systemic glucocorticoid excess is associated with an increase in cardiovascular risk factors [60]. One of the major causes of impact on these risk factors is thought to be the glucocorticoid mediated intravascular volume overload [60]. As the access of glucocorticoids to their receptors is controlled by the isozymes of 11- β -hydroxysteroid dehydrogenase in a tissue specific manner, makes manipulation of this pathway an attractive therapeutic target. By selective isozyme inhibitors, the glucocorticoid activity can be modulated locally, keeping systemic glucocorticoid concentrations within homeostatic range.

Also in the acute setting a relationship exists between glucocorticoid availability and autophagy induction. In the treatment of acute lymphoblastic leukemia, glucocorticoids are used as crucial therapeutic agent, due to their effect on inducing G1 phase cell cycle arrest and apoptosis. Recently it was shown that dexamethasone treatment induces cell death and involves the induction of autophagy before the onset of apoptosis [61]. Moreover, another level of interaction has been demonstrated as the role of autophagy in innate immunity has recently become clear. Both the ATG16L1 risk allele as well as ATG5 are selectively important for the function of the Paneth cell, a specialized epithelial cell in the small intestine [62]. Through genome-wide association screenings it was shown that the autophagic pathway plays a fundamental role in the predisposition to the inflammatory bowel condition Chron's disease [63]. Taken together, these data indicate the dynamic relationship between glucocorticoid-induced metabolic perturbations, autophagy induction, inflammation and cell death susceptibility. Further investigations are likely to provide new insights into this complex relationship to treat cardiovascular disease more effectively by exploiting the modulation of the autophagic machinery in context with controlling local glucocorticoid activity.

10. The role of the mitogen-activated protein kinases (MAPKs) during ischemia/reperfusion-induced stress in the heart

Great efforts have been made to disentangle the intricate relationship between signalling pathways and the stress response of the heart during ischemia/reperfusion-induced injury. Analysis is complicated due to the fact that several pathways can be activated simultaneously with differential effects. It has become however evident that the MAPKs are major mediators of I/R-induced injury. Recent data pin point the MAPK's as one of the crossroads between autophagy and glucocorticoid signalling events.

Three major classes of MAPKs (Figure 4), which include the extracellular signal regulated protein kinase (ERK)/p42/44, c-Jun NH2-terminal protein kinase (JNK)/stress activated protein kinase (SAPK) and p38 MAPK families have been identified [64,65]. The ERK pathway has been depicted as a pro-survival pathway and is activated by a variety of mitogens and phorbol esters [66,67]. The JNK and p38 MAPK pathways are regarded as pro-apoptotic pathways and are mainly activated by environmental stress and inflammatory cytokines [67,68].

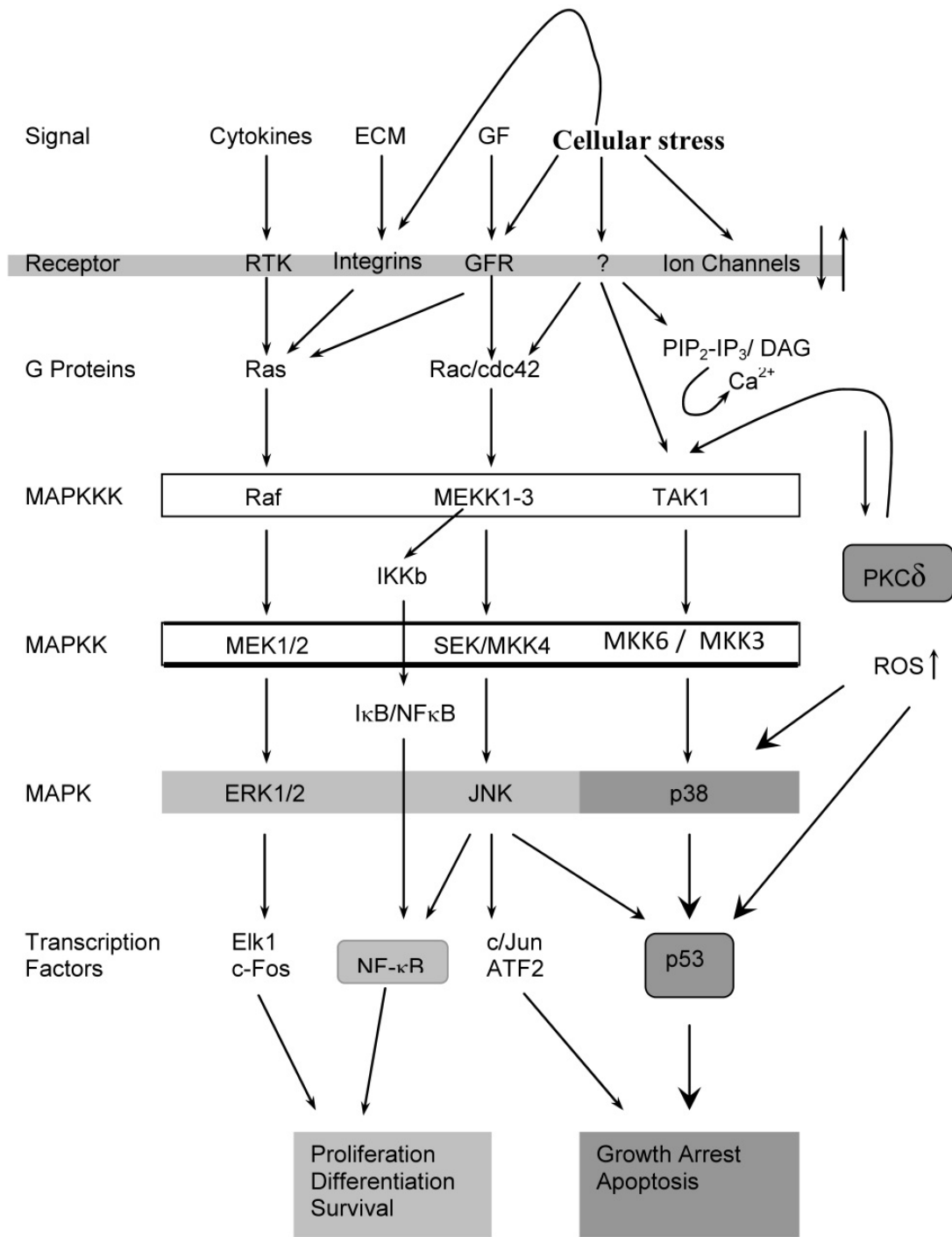


Figure 4. MAPK activation in stress-induced signalling in the heart. A variety of stress signals can activate the MAPKs directly or indirectly. MAPKs comprise a family of tyrosine/threonine kinases. Receptor activation initiates a cascade of phosphorylation events involving sequential activation of G proteins, MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and finally MAPK. Activated MAPK, in turn, is responsible for the phosphorylation and activation of various other regulatory proteins and transcription factors, which induce the expression of genes involved in the regulation of cell proliferation and apoptosis. ERK kinases mediate cell survival and proliferation, whereas JNK and p38 induce growth arrest and apoptosis (modified from Wernig and Xu, 2002).

Studies using chemical inhibitors have led to the conclusion that activation of the p38-MAPK promotes cardiac myocyte death during extended periods of ischemia [69-71]. In a cultured neonatal rat cardiac myocyte model, inhibition of p38-MAPK protects against ischaemic injury by decreasing LDH release [69,70]. In addition, Barancik and co-workers (2000) reported that a specific inhibitor of p38-MAPK, SB203580, protected pig myocardium against ischaemic injury in an *in vivo* model by reducing infarct size [71]. Several studies indicated that p38-MAPK plays a pivotal role in promoting myocardial apoptosis [69,72-73]. Ma and co-workers (1999) demonstrated that in isolated perfused rabbit hearts, ischemia alone caused a moderate but transient increase in p38-MAPK activity [72]. Ten minutes' reperfusion further activated p38-MAPK, which remained elevated throughout reperfusion (20 minutes). Administration of SB203580 before ischemia and during reperfusion completely inhibited p38 MAPK activation and exerted significant cardioprotective effects, characterized by decreased myocardial apoptosis and improved post-ischaemic function, as well as attenuated myocardial necrotic injury. In contrast, administering SB203580 10 minutes after reperfusion (a time point when maximal MAPK activation had already been achieved), failed to convey significant cardioprotection. Mackay and Mochly-Rosen (1999) indicated that in neonatal rat cardiac myocytes, two distinct phases of p38 activation were observed during ischemia: the first phase began within 10 minutes and lasted less than 1 hour, and the second began after 2 hours and lasted throughout the ischaemic period [69]. They demonstrated that SB203580 also protected cardiac myocytes against ischemia by reducing activation of caspase-3, a key event in apoptosis. However, the protective effect was seen even when the inhibitor was present during only the second, sustained phase of p38 MAPK activation. Subsequent studies by Yue and co-workers (2000), exposing rat neonatal cardiomyocytes to ischemia showed a rapid and transient activation of p38-MAPK and JNK [73]. On reoxygenation, further activation of SAPKs was noted. With pretreatment of the cells with SB203580 apoptotic cells were reduced, suggesting p38 MAPK activation mediates apoptosis in rat cardiac myocytes subjected to ischemia/reoxygenation. In addition, Yue and co-workers (2000) also showed that SB203580 improved cardiac contractile function in rat isolated ischaemic hearts. Inhibition of p38 MAPK activation, therefore, correlated with cardioprotection against ischemia/reperfusion injury in cardiac myocytes as well as in isolated hearts [73].

Zechner and co-workers (1998) also reported that overexpression of MKK6 (Figure 4), an upstream activator of p38 MAPK, resulted in protection of cardiac myocytes from apoptosis induced either by anisomycin or MEKK1, an upstream activator of the JNK pathway [74]. In addition, expression of MKK6 elicited a hypertrophic response, which was enhanced by co-infection of p38 β [75]. Therefore, a distinct isoform of p38 MAPK, p38 β , may participate in mediating cell survival. In contrast, over expression of MKK3 in mouse cardiomyocytes led to apoptosis, which was increased by co-infection of p38 α [75]. Therefore differential activation of p38-MAPK isoforms may exert opposing effects: p38 α is implicated in cell death, while p38 β may mediate myocardial survival.

To determine whether p38 MAPK activation was isoform selective, rat neonatal cardiomyocytes were infected with adenovirus encoding wild-type p38 α or p38 β [70]. They

showed that transfected p38 α and p38 β were differentially activated during sustained ischemia, with p38 α remaining activated but p38 β deactivated. Furthermore, cells expressing a dominant negative p38 α , which prevented ischemia-induced p38 MAPK activation, were resistant to sustained ischaemic injury. Therefore, activation of p38 α MAPK isoform is detrimental during ischemia.

11. MAPK inactivation by phosphatases

Dephosphorylation of either the threonine or tyrosine residue within the MAPK activation loop TxY motif alone can result in their enzymatic inactivation. In intact cells, dephosphorylation and inactivation of MAPK occur, within minutes to several hours depending on the cell type and activating stimulus. In endothelial cells, exposure to serum leads to ERK activation that is sustained at high levels for over 2 h. In contrast, different patterns can be observed in the a PC12 cell line where EGF-stimulated ERK activation is transient, with inactivation initiated within 5 min and nearly complete within 15-30 min, whereas this MAPK displays prolonged activation for several hours on stimulation with NGF [76]. It is believed that different patterns of ERK activation elicited by EGF and NGF underlie their differential effects to drive either cellular proliferation or differentiation, respectively [77]. Using PC12 cells as a model system to identify key phosphatases suppressing ERK activation, biochemical studies revealed that early rapid inactivation of these MAPKs reflects, in part, threonine dephosphorylation by the serine/threonine protein phosphatase PP2A [76].

In addition to threonine dephosphorylation, these studies also indicated that tyrosine-specific protein phosphatases (PTPs) also contribute to ERK inactivation [76]. Currently, 50 or more PTPs have been characterized [78-80], and although the PC12 cell PTPs were not identified molecularly [76], recent studies in other cell types have identified a possible role for three related PTP gene family members [81-84]. Notwithstanding the importance of these early reports on PP2A and tyrosine-specific PTPs inactivating ERKs, little is known about their general importance in terminating MAPK signaling, of the molecular mechanisms that may control phosphatase catalytic activity, or of their specificity for inactivating different MAPK isoforms.

In contrast to these protein phosphatase classes, there has been significant and rapid progress in our understanding of the role played by a subclass of PTP that possess activity for dephosphorylating both phosphotyrosine and phosphothreonine residues, known as the dual specificity phosphatases (DSPs).

The first mammalian DSP was identified as the mouse immediate early gene 3CH134 or its human orthologue CL100, which is induced rapidly after exposure to growth factors, heat shock, or oxidative stress [85-87]. Recombinant CL100/3CH134 was shown to dephosphorylate threonine and tyrosine residues of ERK, which was paralleled by its inactivation. These studies showed that CL100/3CH134 was specific for dephosphorylation of ERK when compared to a number of other unrelated phosphoproteins [76]. A correlation between 3CH134 levels and ERK inactivation was also found in mammalian cells, leading to its renaming as MAPK phosphatase-1 (MKP-1) [88]. Despite this important early work, the

relevance of MKP-1 in ERK inactivation remains to be elucidated. Firstly, ERK activity is apparently normal after deletion of the MKP-1 gene in mice [89]. Secondly, it has also become evident that MKP-1 is at least as effective in inactivating JNK and p38 when compared to the ERKs [90-91]. Thirdly, newly identified members of the DSP gene family appear highly selective for ERK and may represent the true physiological regulators of this MAPK isoform. Since the initial cloning of MKP-1, eight additional mammalian DSP gene family members have been identified and characterized, which include MKP-2, MKP-3, MKP-4, MKP-5, MKP-X, PAC1, M3/6 and B59. These DSPs all appear to be effective in mediating inactivation of MAPKs.

The following model for MAPK inactivation by DSP is suggested (Figure 5). Stimulation by growth factors, cytokines, cellular stresses or some active oncogenes leads to rapid transcription of one or a subset of DSP genes. Increased DSP transcription may reflect activation of specific MAPK, although alternative pathways are not excluded. After translation of the DSP mRNA into protein, the catalytically inactive DSP translocates to a specific subcellular compartment within either the nucleus or the cytosol. Upon encountering its target MAPK, the DSP binds tightly through its amino terminus, which in turn triggers activation of the phosphatase catalytic domain. If the bound MAPK is already activated, then this will result in its rapid inactivation. Conversely, if the MAPK is not

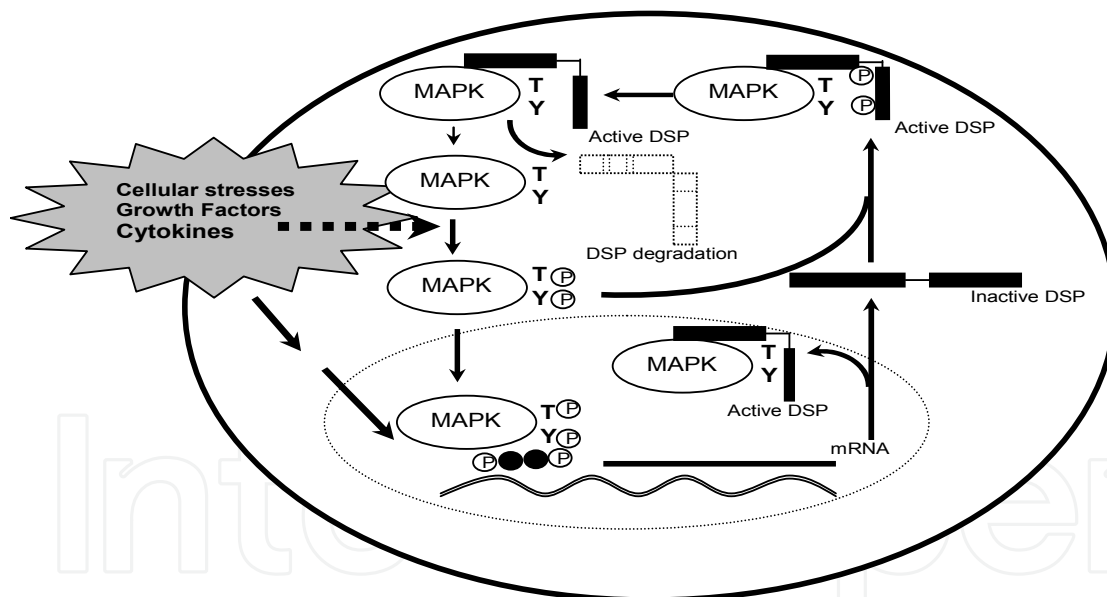


Figure 5. Cell exposure to growth factors, cytokines and cell stresses leads to induction of a subset of DSP genes. Increased expression is likely to reflect activation of transcription factors (black circles) via both MAPK-dependent and independent pathways. Newly synthesized DSPs translocate to specific subcellular compartments as dictated by anchorage and/or localization motifs not yet identified. Specific binding to target MAPKs through regions within the DSP amino terminus then triggers activation of the phosphatase catalytic domain. Bound MAPKs are in turn inactivated by dephosphorylation on threonine and tyrosine residues localized within the “activation loop” motif of TxY. Inactive MAPKs then dissociate, leaving the DSP free to bind and inactivate another MAPK molecule. In the absence of continued DSP gene transcription and protein synthesis, rapid degradation may limit their duration of activity in cells (Camps et al., 1999).

active, then its tight interaction with an active DSP is expected to block any possibility of kinase activation by a subsequent stimulus. MAPKs that fail to bind the DSP within its amino terminus remain active or susceptible to activation after extracellular stimulation. Depending on their cellular localization, these regulatory effects allow for selected inhibition of MAPK activities in specific subcellular compartments. Some DSPs have been shown to possess short half-lives [76], suggesting that in the absence of continued gene transcription and protein synthesis, their rapid turnover limits their duration of action in cells. Overall, tight control of DSP gene induction, combined with their differential binding and catalytic activation by a specific repertoire of MAPKs, provides a sophisticated mechanism for rapid targeted inactivation of selected MAPK activities.

12. MAP kinase phosphatase-1 (MKP-1): A mediator of the beneficial effects of glucocorticoids during ischemia/reperfusion-induced stress in the heart

It is noteworthy that many genes that are positively regulated at post-transcriptional level by p38-MAPK, are negatively regulated at the same level by glucocorticoids. Lasa and co-workers investigated the effects of GCs on the p38-MAPK pathway and have shown that dexamethasone destabilized cyclooxygenase-2 (COX-2) mRNA by inhibiting the function, but not the expression of p38-MAPK [92]. The inhibition of p38-MAPK was then shown to be mediated by MKP-1. We and others have also demonstrated that dexamethasone induces the expression of MKP-1 which potently inactivated p38-MAPK [93-94].

Wu and Bennet (2005) demonstrated that MKP-1 promotes cell survival in fibroblasts through the attenuation of stress responsive MAPK-mediated apoptosis [95]. Upregulation of MKP-1 has also been shown to be associated with cardioprotection by long-chain polyunsaturated fatty acids [93]. It has also been reported that transgenic mice overexpressing MKP-1 were protected, whereas knock-out mice show greater injury after ischemia/reperfusion [96]. The exact mechanism of the beneficial effects of glucocorticoids on the heart during ischemia/reperfusion-induced stress still remain to be established. In view of the significant contribution of apoptosis, necrosis and autophagy during ischemia/reperfusion-induced stress, it is expected that GC-induced cardioprotection to be associated with reduced apoptosis and necrosis. Indeed, Fan and co-workers have demonstrated that dexamethasone, administered intraperitoneally or added directly to the perfusate, significantly improved post-ischemic functional recovery and reduced infarct size compared to untreated controls [94]. These were associated with upregulation of MKP-1 protein expression [94]. Furthermore, it was also shown by us that upregulation of MKP-1 during simulated ischemia/reperfusion is associated with an attenuation of apoptosis in neonatal cardiomyocytes [93].

13. Conclusions

It has been suggested that upregulation of MKP-1 during ischemia/reperfusion-induced stress attenuates myocardial injury [93,94]. MKP-1, found predominantly in the nucleus, is a

dual specific phosphatase which dephosphorylates phosphotyrosine and phosphothreonine-containing protein kinases such as the MAPKs. MAPKs are known to be involved in intracellular signalling pathways that regulate gene expression in response to a variety of extracellular signals. MAPKs are activated during ischemia/reperfusion-induced stress in the heart. It was also demonstrated that glucocorticoids act via MKP-1 induction and subsequent p38-MAPK inhibition to induce cardioprotection during ischemia/reperfusion-induced stress [94].

MAPKs have been found to be involved in autophagic, apoptotic and necrotic cell death during stress responses of the heart [97-102]. Autophagy is foremost a survival mechanism which is activated in cells subjected to nutrient or growth factor deprivation. However, when the cellular stress continues, cell death may occur via autophagy, or becomes associated with features of apoptotic or necrotic cell death [103]. Apoptosis is essential for removal of specifically targeted cells, through the process of apoptotic body formation and phagocytosis [104]. Necrosis is a pathological cellular response requiring no ATP. Necrotic cells are morphologically characterized by disrupted membranes, cytoplasm and mitochondrial swelling, disintegration of organelles and complete cell lysis [105]. Cell death following ischemia/reperfusion-induced stress is thought to manifest in morphological features indicative for all three, apoptotic, necrotic and autophagic cell death [106].

MKP-1 has been shown to be involved in the regulation of apoptosis [107] and it was also very recently demonstrated that MKP-1 may lead to autophagy induction in cancer cells [108]. We have recently demonstrated that inhibition of MKP-1 and subsequent increased p38-MAPK phosphorylation during ischemia/reperfusion-induced stress is associated with attenuated autophagy and increased apoptosis and necrosis in the heart (unpublished data). We thus propose the following mechanism of GC-induced protection in the heart: During ischemia/reperfusion-induced stress in the heart, p38 MAPK is activated, GCs sustain/upregulate autophagy via an increase in MKP-1 and subsequent dephosphorylation of p38 MAPK which ultimately protects the heart from apoptosis and necrosis, driven by the effects of autophagy on the metabolic balance sheet of the heart.

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