Forkhead Box O in ischemia/reperfusion injury: a potential therapeutic target

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Abstract

Acute myocardial infarction (AMI) is a major cause of morbidity. AMI is caused by a blockage of one or multiple coronary arteries, leading to an ischemic (loss of blood flow) area of the heart. Restoring blood flow (reperfusion) to the ischemic area is the primary course of action when an AMI patient presents himself. Reperfusion of the infarcted area however causes injury to the previously ischemic area, meaning that the treatment itself is damaging to the myocardium. This damaging period is termed ischemia/reperfusion injury and the proposed processes propagating I/R injury will be described in this paper. Forkhead Box O (FOXO) family of transcription factors are regulators of many genes involved in processes like reactive oxygen species (ROS) scavenging, apoptosis, development and immunity. Almost all of the functions which can be exerted by FOXOs via their target genes can be related to I/R injury, as will be discussed in this paper. The primary goal of this paper is to give the reader better insight into the processes underlying I/R injury, and the functions and regulation of FOXOs. Can and should FOXOs, considering their numerous target genes, be targeted therapeutically to attenuate ischemia/reperfusion injury?

1. Introduction

1.0.1 Acute myocardial infarction

Acute myocardial infarction (AMI) is one of the major causes of morbidity in cardiovascular diseases. Infarctions are caused by the loss of blood perfusion as a result of an occluded coronary blood vessel. These occlusions are usually caused by the rupture of lipid filled atherosclerotic plaques, resulting in a thrombus, or better known, a blood clot in a coronary blood vessel (Arbab-Zadeh, Nakano [7]). As early as the mid seventies a majority of the infarcts was found to be directly related to atherosclerotic plaque presence and rupture (Davies, Woolf [8]). Atherosclerotic plaques are the result of lipid accumulation and consequent inflammation in the blood vessel wall, these plaques have a high chance to rupture and cause a blood clot. Considering the current high lipid dietary habits leading to obesity and diabetes mellitus, it is likely that acute coronary events will be a major strain on healthcare budgets in the near future. Since the heart is in essence a big muscle, constant blood flow to the myocardium is essential for proper function. When the affected area of the myocardium beneath the blocked vessel becomes ischemic (loss of blood flow), it results in metabolic changes due to lack of oxygen and nutrients (figure 1). Due to the very limited regenerative capacity of the heart, it is essential to limit the damage as much as possible as soon as possible. Persistent loss of perfusion will lead to an increase in infarcted area size and will cause adverse remodeling (i.e. thinning of the infarcted myocardial tissue and subsequent dilatation) over time (Mill, Stefanon [9]). Shortly, adverse remodeling is a result of cell death and scar formation in the infarcted

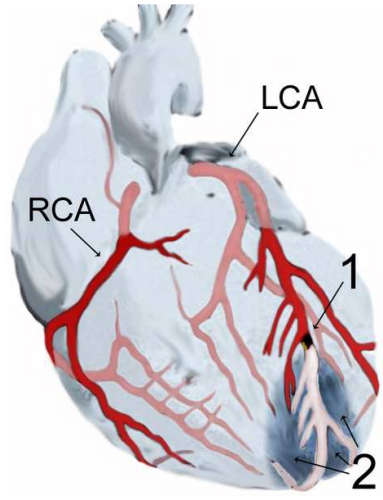

Figure 1. When a coronary artery is blocked (1) the area right beneath that blockage loses perfusion and becomes ischemic (2). This infarct area is prone to cell damage and cell death due to the loss of nutrients, oxygen and lack of metabolite clearance. LCA = left coronary artery, RCA = right coronary artery.

area. The weak spot of the myocardium thins and dilates with time and subsequently heart failure occurs due the inability of the affected area to contract.
With that in mind, it is essential to restore blood flow as soon as possible to the infarcted myocardium to preserve healthy tissue as much as possible. With a percutaneous coronary intervention for example, the occluded vessel is opened and the infarcted myocardium is reperfused (Keeley, Boura [10]). Clinical long-term outcomes and infarcted area size have been directly related to the speed of reperfusion, as can be expected. With an extended ischemic period, the infarcted area will be more prone to cell damage and subsequent cell death. Interestingly, it was found that the restoration of blood flow itself to the infarcted area can lead to cell death (Piper, Garcia-Dorado [11]). The influx of nutrients, oxygen and immune cells causes an adverse reaction in the already damaged tissue. Animal models show that ischemia/reperfusion injury accounts up to 50% of the infarct size (Yellon and Hausenloy [3]), which makes it essential to limit this damaging period during reperfusion. Later, the processes proposed as mediators of I/R injury will be explained in more detail.

1.0.2 Transcription factors, an introduction to FOXO

Gene regulation in cells is highly dependent on a complex transcription activation machinery consisting of various protein partners. Transcription factors can activate or inhibit transcription by binding a DNA consensus sequence, after which other proteins are recruited or inhibited in the transcription machinery . The Forkhead box O (FOXO) family of proteins is one such transcription factor family and is found to be evolutionary conserved from the nematode *Caenorhabditis elegans* to mammals. The Forkhead box O (FOXO) family members are potent transcription factors involved in cell cycle control, protection against reactive oxygen species (ROS), tumorigenesis, autophagy, tissue development and DNA damage repair (Carlsson and Mahlapuu [12], Burgering [13], Calnan and Brunet [14], de Keizer, Burgering [15]). The FOXO protein consists of a DNA binding domain (Forkhead domain), transactivation domain (TAD), nuclear localization domain (NLS) and a nuclear export domain (NES), to exert its function (Calnan and Brunet [14]). As the function would suggest, localization of the FOXO protein is of essential importance since transcription activation is regulated by binding to DNA. FOXOs can be regulated by various post translational modifications (PTMs) like acetylation, ubiquitination and phosphorylation. These regulation steps will be explained in more detail in a later paragraph. The numerous functions of the FOXO transcription factors suggests they could have a function during I/R injury.
The main goal of this paper is to gain a broader understanding of the possible I/R injury attenuating or promoting actions of FOXOs after AMI. Can FOXOs be used as targets, in a positive or negative way, to attenuate I/R injury? A detailed explanation of FOXO regulation and function will be given, this will be related to the processes occurring during I/R injury. What are the main functions of FOXOs and how can these functions be related to the processes taking place during I/R injury? Are FOXOs beneficial, damaging or even both, during I/R injury? Understanding FOXO function in processes like cell death and immune cell regulation will give better insight in possible therapeutic actions focused on FOXOs shortly after AMI.

1.1 Ischemia/reperfusion injury

The exact pathophysiology of I/R injury is unclear, but several mechanisms have been proposed that mediate the damage caused by restored blood flow. Most important of these proposed process are impaired metabolism due to the sudden influx of metabolites and the inability to process these

metabolites properly by the damaged tissue; reactive oxygen species (ROS) production caused by sudden influx of oxygen and damage to mitochondria; rapid change in pH; activation of necrosis and apoptosis pathways; and inflammation due to rapid influx of mainly neutrophils as a response to an abundance of chemokines (figure 2) (Prasad, Stone [16], Eltzschig and Eckle [2]).

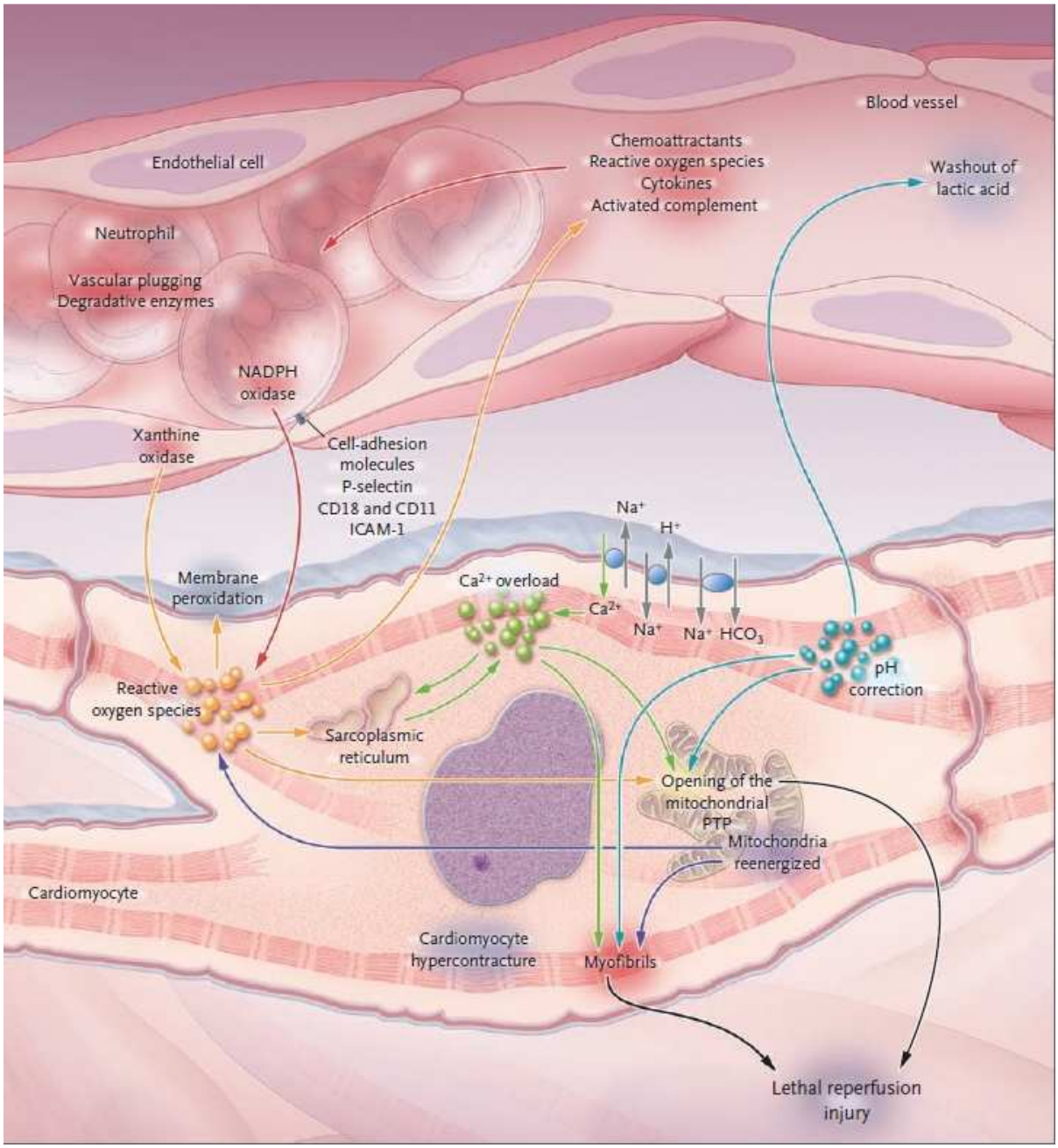

Figure 2. During reperfusion of the ischemic myocardium, several key processes are involved in causing ischemia/reperfusion injury. The reperfused myocardium is subjected to higher reactive oxygen species generation; calcium overload causing hypercontracture; rapid correction in pH due to the efflux of metabolites like lactic acid; and influx of immune cells, mainly neutrophils, in the acute stage, due to accumulation of cytokines. These processes combined lead to cell death by activation of intrinsic apoptosis and by opening of mitochondrial permeability transition pores. Yellon and Hausenloy [3]

1.1.1 Metabolic dysfunction during ischemia

Metabolic dysfunction during ischemia is caused by lack of oxygen and perfusion, leading to a switch to non-aerobic glycolysis and resulting lactic acid accumulation. When the ischemic myocardium is reperfused, the accumulated lactic acid is cleared, resulting in a sudden shift in pH causing a shock in the tissue. This phenomenon was termed "pH paradox", describing the paradoxical worsening of cell viability when the acidotic pH of the ischemic period is normalized by reperfusion. Lemasters *et al.* show by using rat neonatal cardiomyocytes, that this pH paradox is indeed directly related to pH and not to the re-oxygenation of the cells. Cells brought to a pH of 6.2 were re-oxygenated at pH 6.2 or pH 7.4, the change in pH accounted for 60% of cell death while re-oxygenation at pH 6.2 had no effect on cell viability (Lemasters, Bond [17]). So counter intuitively, restoring the pH to normal levels to stop the damage caused by the acidic environment, results in damage to the cells.

1.1.2 Deficiencies in ROS clearing during I/R injury

Reactive oxygen species are oxygen-containing molecules, of which the superoxide and hydroxyl radical forms are extremely reactive due to the presence of unpaired electrons. Together with the less reactive yet easily diffusible hydrogen peroxide (H2O2), these ROS are common byproducts of oxygen metabolism (Kevin, Novalija [1]). Under normal situations, mitochondria leak small amounts of superoxide at complexes I and III of the electron transport chain during ATP production. Leakage of these radicals is compensated with clearance of ROS by anti-oxidant enzymes, among others, mitochondrial manganese superoxide dismutase (mnSOD), so that the presence of damaging radicals like superoxide in the cytosol is limited (figure 3). Under ischemia however changes occur in the complexes mediating the electron transport chain and anti-oxidants like mnSOD lose efficiency in processing leaking superoxide molecules. It is as of yet unclear how the down-regulation of these

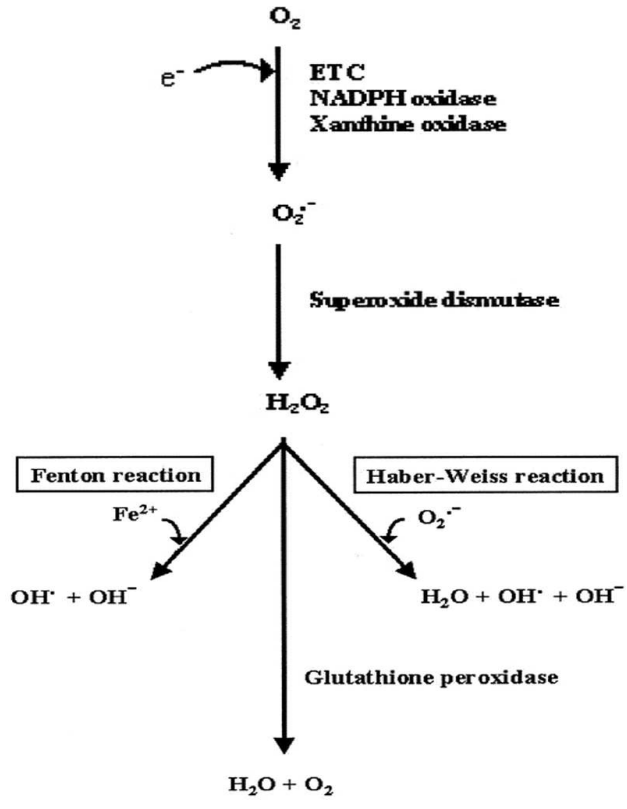

Figure 3. Reactive oxygen species are the result of multiple sources and consist of the highly reactive superoxide and hydroxyl, and the highly diffusible hydrogen peroxide (H2O2). The production of ROS is balanced by enzymes which work in a less efficient manner during pathological conditions like ischemia/reperfusion injury. Kevin, Novalija [1]

anti-oxidants during I/R injury occurs (Arduini, Mezzetti [18], Jassem and Heaton [19]). Arduini *et al*. have shown that the inefficient action of the anti-oxidant enzymes caused by the ischemic episode continues during reperfusion (Arduini, Mezzetti [18]). When reperfused, an influx of oxygen causes a switch from anaerobic to aerobic energy production. The potentially damaged mitochondria during ischemia combined with inefficient clearance of ROS by anti-oxidant enzymes, causes an excess of ROS production and leakage. The accumulated ROS are highly reactive and are able to cause damage to the genomic and mitochondrial DNA, proteins and lipids. Besides this, ROS stimulate prolonged mitochondrial pore formation (Yellon and Hausenloy [3], Pagliaro, Moro [20]), which is an important mediator of cell death during I/R injury.

1.1.3 Cell death and the immune system during I/R injury

The last two major events during ischemia/reperfusion injury are activation of apoptosis, necrosis and autophagy related cell death pathways; and immune cell accumulation in the reperfused necrotic tissue. Since cell death and the immune reaction are closely related in this scenario, these two processes will be considered parallel to each other. Apoptosis is a strictly controlled cell death mechanism which is activated when cells become irreversibly damaged. Simply put, cells commit suicide to protect the organism from long-term detrimental effects which can be caused by damaged cells. During apoptosis the cell condenses, after which apoptotic bodies start forming, resulting in fragmentation of the cell (Kerr, Wyllie [21]). These cell fragments are phagocytosed and cleared by

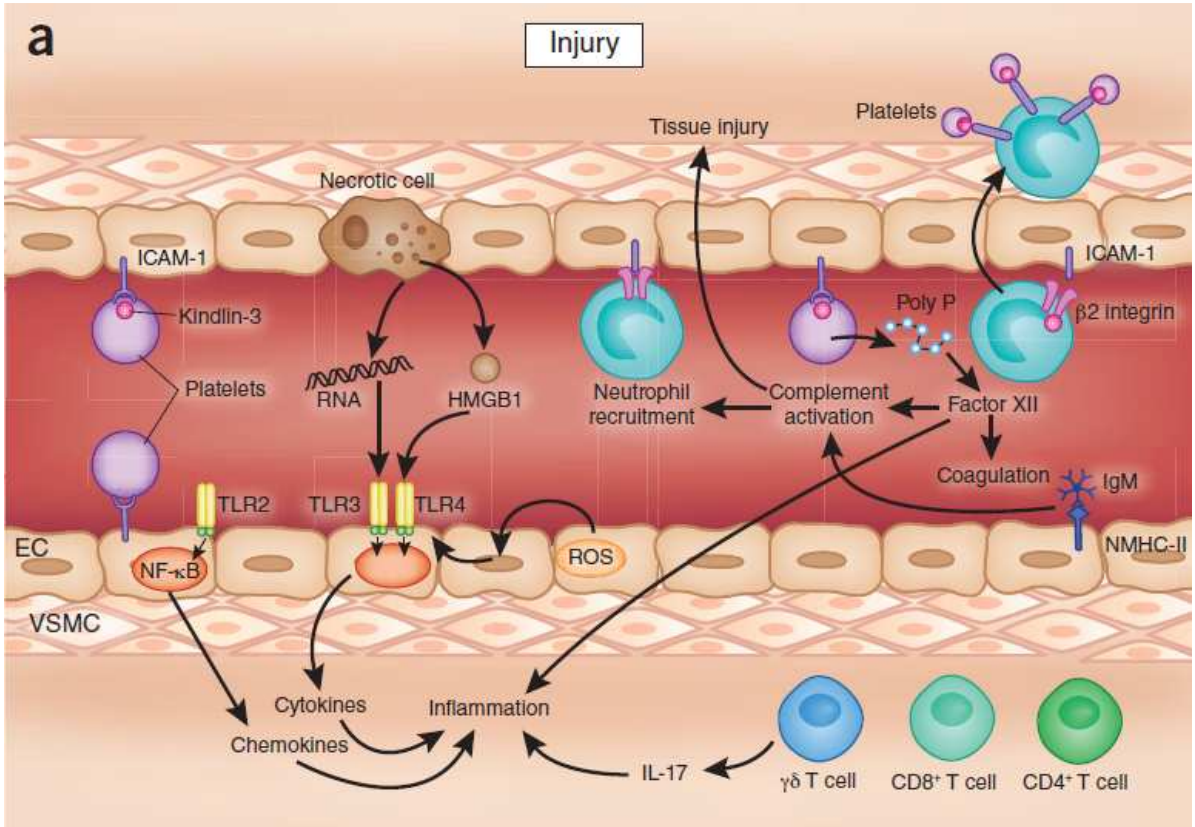

Figure 4. During the ischemic period factors like NF-kB, which is stabilized during hypoxia, and ROS up-regulate Toll-like receptor, which can interact with immune cells. This, combined with cytokines and adhesion molecules which are up-regulated during the ischemic period, leads to a massive recruitment and influx of immune cells during reperfusion causing ischemia/reperfusion injury. Eltzschig and Eckle [2]

other cells.
A central role in apoptosis activation in I/R injury has been proposed for mitochondria, with focus on the mitochondrial permeability transition pores (mPTP). In normal situations mPTP formation and opening is strictly controlled and transient mPTP opening is thought to regulate reversible cellular signaling via calcium release (Duchen, Leyssens [22], Petronilli, Miotto [23]). Parallel to this, transient NAD+ and ROS release from the mitochondria is also thought to be regulated by mPTP formation and opening (Di Lisa 2001, Li 2012). The exact regulation of the transient opening and closing of these mPTP is unclear, but it is likely that it is an important aspect of intra-cellular signaling judging by the importance of calcium ions in several signaling pathways. During an I/R episode the sudden increase in ROS and change in pH causes long-lasting mPTP opening, leading to detrimental effects on cell viability. The formation of pores on the mitochondrial membrane leads to a collapse of membrane potential and consequently, depletion of ATP and NAD+ because oxidative phosphorylation is halted. The lack of energy production and parallel to this the mPTP opening, lead to release of pro-apoptotic molecules like cytochrome C, and subsequent induction of apoptosis.

Besides the apoptotic pathway, a portion of the cellular population becomes necrotic due the aforementioned changes in cellular homeostasis. The transition to necrosis can have several reasons, among which, the positioning of the cell deeper in the tissue farther away from (micro)vessels; depletion of factors like ATP which are necessary for the ATP dependent apoptosis pathway; and incomplete progression of a cell in the apoptosis pathway due to accumulation of damage. As opposed to apoptosis, which is a controlled cellular death pathway, necrotic cells swell and burst as a reaction to the many changes during an I/R episode. These necrotic cells are extremely immunogenic due to the cellular debris released after death. The accumulation of chemokines during the I/R episode attracts neutrophils massively and neutrophil infiltration into the cardiac tissue is expedited by the heightened expression of cell attachment proteins. The debris and damage causes a sterile immune reaction, in which no bacterial or viral particles are present, yet the severity of the reaction is the same (Chen and Nunez [24]). Many toll-like receptors present on T-cells are activated by cell debris and oxidative stress, which may also happen during I/R injury (Eltzschig and Eckle [2]). During the ischemic period cells are damaged and are ready to be taken up by immune cells. This cleansing period is necessary to clear the toxic environment for future healing. The accumulation of chemokines and cytokines however attracts extreme amounts of immune cells, neutrophils in particular, when reperfused, which cause havoc in the already damaged (figure 4).

1.2 FOXO regulation

The FOXO family consists of FOXO1, FOXO3, FOXO4 and FOXO6 and all are spatiotemporally expressed, to a more or lesser extent, in most tissues of the body (table 1) (Furuyama, Nakazawa [25], Biggs, Cavenee [26], Hoekman, Jacobs [27]). The differential expression might make it possible to target specific FOXOs depending on the tissue of interest. If one of the FOXO family members is found to be mainly expressed in cardiac tissue, systemic administration of a particular drug to inhibit or induce that isoform should have the strongest effect in the myocardium. The highly conserved regulation of the FOXO family however will pose a problem in this regard, since targeting one single isoform could be difficult or even impossible.

FOXO activity is strictly controlled by localization since DNA interaction is required for trans-activation of target genes. Localization is regulated by several external stimuli like insulin signaling, growth factor signaling, oxidative stress, nutrients and cytokines. Via various post-translational modifications (PTMs) like phosphorylation, acetylation and ubiquitination, FOXO localization and activity can be regulated. Perhaps the best known upstream pathway of FOXO regulation is the one mediated via the insulin receptor, which is activated by insulin in fed conditions. Upon insulin signaling Akt is activated by phosphoinositide 3-kinase, which causes FOXO to be phosphorylated and subsequently transported out of the nucleus. The phosphorylation is proposed to be enhanced by acetylation of FOXO by cAMP response element-binding protein (CREB)-binding protein (CBP) and the associated p300 (CBP/p300). The acetylation and phosphorylation of FOXOs creates docking sites for 14-3-3 adaptor proteins which translocate FOXOs out of the nucleus, causing inhibition (figure 5). The inhibitory phosphorylation of FOXOs by Akt can occur at different threonine and serine residues depending on the FOXO isoform (Brunet, Bonni [28], Kops, de Ruiter [29], Greer and Brunet [4]). It is clear however that phosphorylation by Akt always leads to the cytosolic accumulation of FOXO. During the ischemic period, cardiac tissue is cut off from the blood supply and subsequently nutrient deprivation occurs. The lack of insulin signaling causes a down-regulation of active Akt, which will subsequently lead to less phosphorylation by Akt of FOXOs. FOXOs will no longer be translocated out of the nucleus and will exert their transcription activation function. In ischemic hind limb in rats, total phospho-ser256-FOXO1 levels were unchanged while total protein levels

Gene name	*Alternate names*	*Mouse name*	*Expression pattern*
FOXO1a	FKHR	fkhr1, Foxo1a	Ubiquitous. Highest in He, Sp, Ad, Ki, Br
FOXO1b	FKHR pseudogene 1 (FKHRP1)		
FOXO3a	FKHRL1, AF6q21, FOXO2	fkhr2, Foxo3a	Ubiquitous. Highest in He, Br, Sp, Lu, Ki, Ad, Ov
FOXO3b	FKHRL1 pseudogene 1 (FKHRL1P1)		
FOXO4	AFX, AFX1, MLLT7	afx, Afxh, Foxo4, Mllt7	Ubiquitous. Highest in He, Br, Sp, Lu
FOXO5	zFKHR		
FOXO6	FOXO6	Foxo6	Br, Th, Ki

He, heart; Sp, spleen; Ad, adipose tissue; Ki, kidney; Br, brain; Lu, lung; Ov, ovaries; Th, thymus

Table 1. The FOXO family of proteins is spatiotemporally expressed in many tissues and plays a critical role in tissue development and regulation. Adapted from Greer and Brunet [4]

were significantly higher (Milkiewicz, Roudier [30]). From this can be concluded that active, un-phosphorylated FOXO1 levels are higher under ischemic conditions.

Parallel to Akt phosphorylation, FOXOs can be phosphorylated by JNK at threonine residues and a newly discovered serine residue (Essers, Weijzen [31], de Keizer, Packer [32]). Activation of the JNK pathway is dependent on several stressors like cytokines, UV radiation, oxidative stress and I/R injury (de Keizer, Packer [32], Portbury, Ronnebaum [33], Sugden and Clerk [34]). JNK activity was seen to be up-regulated specifically in ischemia/reperfusion injury as a response to stress dependent MAPK cascades. Stress dependent JNK activation should lead to FOXO phosphorylation and subsequent sequestration of FOXOs to the nucleus. So unlike phosphorylation by Akt, FOXO phosphorylation by JNK is a signal to accumulate in the nucleus with subsequent activation of target gene transcription by FOXOs.
Lastly, AMP-activated protein kinase (AMPK) is also proposed as an oxidative stress activated kinase which seems to serve an important role in cardioprotection during I/R injury. Mice with mutant forms of AMPK were found to have greater infarct sizes due to I/R injury compared to their wildtype littermates (Wang, Gao [35]). While the exact mechanism of AMPK action during I/R injury is unknown as of yet, AMPK kinase is considered a cardioprotective kinase which exerts its action possibly via adiponectin during I/R injury (Dyck [36]). Greer *et al.* show that AMPK can phosphorylate and regulate FOXO3 activity in fibroblasts. This phosphorylation is found to be AMP dependent and

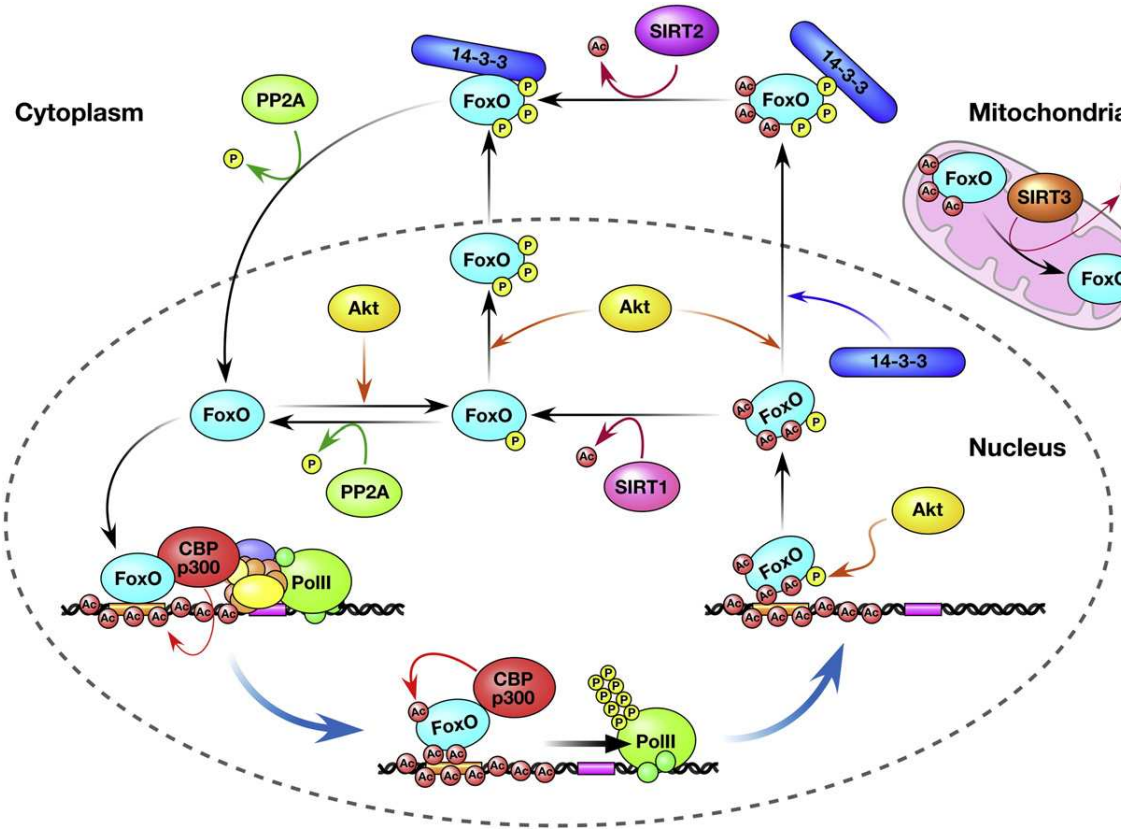

Figure 5. Schematic representation of FOXO regulation. FOXOs are regulated by post-translational modifications like phosphorylation and acetylation. After binding to DNA, FOXOs are acetylated by CBP/p300 to exert their transcription activation function. Subsequent phosphorylation by Akt, which is up-regulated by insulin signaling, causes FOXO translocation to the cytosol. 14-3-3 proteins are proposed as transporters which bind to FOXOs when phosphorylated. Daitoku, Sakamaki [6]

can take place at six serine residues and one threonine residue (Greer, Oskoui [37]). Interestingly, while AMPK can phosphorylate and activate all FOXO family members, it seems to show a preference for FOXO3 phosphorylation, which is present in cardiac tissue. While FOXO phosphorylation by Akt and JNK has a direct effect on FOXO localization, the effect of AMPK phosphorylation seems to be more complex. The localization is not affected, though target gene transcription does seem to be up-regulated upon FOXO phosphorylation by AMPK (Greer, Oskoui [37]). Sengupta *et al.* show that the relation between AMPK and FOXOs is also present in cardiomyocytes. When treated with H2O2 to simulate oxidative stress, neonatal rat cardiomyocytes show an induction of FOXO1 and FOXO3 nuclear localization as a result of phosphorylation by AMPK (Sengupta, Molkentin [38]). Active AMPK in turn is higher under oxidative stress. An increased FOXO action was shown to be cardioprotective due to decreased cell death, lower ROS, higher anti-apoptotic proteins and increased anti-oxidants. FOXO importance was further proven *in vivo* in mice with a cardiomyocyte specific knock-out of both FOXO1 and FOXO3. Mice were subjected to acute I/R injury and FOXO1/FOXO3 deficient mice were shown to have a bigger infarct size compared to their wildtype littermates (Sengupta, Molkentin [38]).
Taken together, during an I/R episode FOXO activity is up-regulated because Akt activity is decreased since insulin signaling is lost. Alongside this regulatory response, JNK and AMPK phosphorylate FOXOs after being triggered by ROS, which is abundantly present during I/R injury, and cause FOXO localization to the nucleus and increased activity respectively. From this, one can conclude that during I/R injury, when there is a lack of insulin and a prior ischemic period followed by exceeding ROS production during reperfusion, FOXOs can be important players in cellular homeostasis.

1.3 HIF-1 and FOXOs

HIF-1 is expressed in almost all mammalian cells and is a key factor in sensing oxygen concentration and proper cellular reaction during low oxygen situations. HIF-1α, which is the oxygen sensitive subunit, forms a dimer with the constituvely expressed HIF-1β subunit in the nucleus (Wang and Semenza [39], Niecknig, Tug [40]). The HIF-1 dimer acts as a potent transcription factor which is shown to activate more than 300 genes in endothelial cells (Manalo, Rowan [41]). Under normoxic conditions the HIF-1α subunit is ubiquitinated and degraded rapidly by mainly oxygen dependent prolyl hydroxylases (PHDs). PHDs work in an oxygen and ROS dependent manner, constantly targeting the HIF-1α subunit for degradation thereby inhibiting HIF-1 transactivating function. Under hypoxic conditions however, PHDs are less active which causes HIF-1α to escape degradation, translocate to the nucleus and form an active dimer with the HIF-1β subunit. A secondary regulation is achieved via inhibition of CBP/p300 binding to the C-terminal transactivation domain (CAD) of HIF-1α under normoxic conditions. The asparagyl residue in the CAD of HIF-1α is hydroxylated by factor-inhibiting HIF (FIH), thereby blocking the binding of CBP/p300 to HIF-1 (Lisy and Peet [42]). HIF-1 needs the acetylase and co-activating function of CBP/p300 to activate target genes. Under hypoxia the oxygen dependent activity of the FIH-1 enzyme is limited and HIF-1α escapes hydroxylation, thereby it is able to bind CBP/p300 and become active.

HIF-1 is shown to activate several pro-apoptotic genes under hypoxia and it is also involved in stabilization of p53 (Greijer and van der Wall [43]). Both the activation of pro-apoptotic genes and the stabilization of p53 leads to apoptosis. The pro-apoptotic action of HIF-1 might be a result of severe or extended ischemia and ROS production, since transiently activated HIF-1 is commonly regarded as a pro-survival factor. Bakker *et al.* show in their 2007 study that FOXO3a and HIF-1 are partners in a feedback loop regulating apoptosis and growth during hypoxia and reperfusion. It was shown that FOXO3a transcription increases in a HIF-1 dependent manner under hypoxia and that FOXO3a in its turn activates *CITED2* transcription (Bakker, Harris [44]). CITED2 inhibits HIF-1 and its apoptotic signaling, causing a negative feedback loop from FOXO3 to HIF-1 via CITED2. CITED2 blocks the binding of CBP/p300 to HIF-1, thereby blocking its activation. Samarin *et al.* propose an hypoxia dependent increase in connective tissue growth factor (CTGF) release from endothelial cells via HIF-1 and FOXO3a (Samarin, Wessel [45]). CTGF is important for endothelial cell migration and growth and should as such serve an essential role during I/R injury by stimulating endothelial cells to form new capillaries (Shi-Wen, Leask [46]). When endothelial cells are put in low oxygen tension or when hypoxia is simulated with a chemical agent, transcription and translation of FOXO3a increases in an HIF-1 dependent manner. Interestingly, HIF-1 also seems to promote FOXO3a nuclear translocation (Samarin, Wessel [45]). How HIF-1 stimulates FOXO3a nuclear localization however is unclear since the researchers do not comment about the direct effect of HIF-1 in FOXO3a nuclear localization. Dimova *et al.* on the other hand show that FOXO4 directly decreases HIF-1 protein levels under hypoxia by causing proteasomal degradation of HIF-1 (Dimova, Samoylenko [47]). It is also stated however that FOXO3a has no such effect, which might mean that while FOXO4 inhibits HIF-1 in muscle, there is no inhibiting action of FOXO3a on HIF-1 in heart. Ferber *et al.* however show that FOXO3a does have an inhibiting effect on HIF-1α. Under hypoxia, HIF-1α stabilization via ROS and PHDs is blocked when FOXO3a is activated. This phenomenon is shown to be c-Myc dependent since c-Myc re-expression rescues HIF-1α stability (Ferber, Peck [48]). The researchers however do state that FOXO3a inhibition of HIF-1α seems to be cell lineage dependent. c-Myc dependent inhibition of HIF-1α by FOXO3a was observed in FOXO3a.A3-ER transduced epithelial cells (RPF-E), but not in DL23 cells.

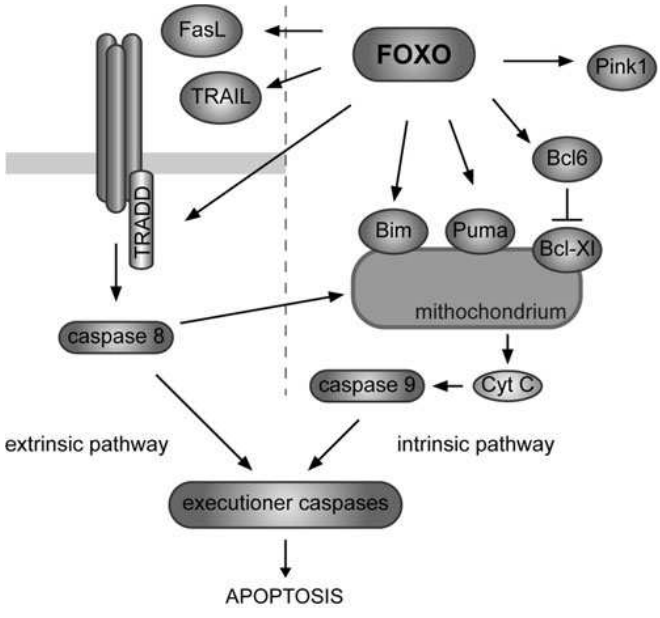

Figure 6. FOXOs are proposed to be central players in both the mitochondria mediated and the FAS pathway mediated apoptosis initiation. Traditionally, FOXOs are considered to be pro-apoptotic via activation of apoptosis inducing target genes.
van der Vos and Coffer [5]

To summarize, HIF-1 and FOXO seem to have a complex relation in which both factors can up-regulate and inhibit each other directly or via other protein partners. This complex relationship looks to be cell lineage and FOXO isoform dependent. It is not hard to imagine that FOXO regulation by HIF-1 and *vice versa* could be spatiotemporal as is proposed to

be the case during other FOXO regulatory mechanisms. Whether influencing FOXOs via therapeutic intervention might have direct effect on the two major HIF-1 actions during I/R injury, namely apoptosis and angiogenesis, is an interesting topic to consider.

1.4 FOXO target genes, potential cardioprotective effectors

Describing every target gene of a FOXOs would be well beyond the scope of this paper and as such, only the target genes possibly involved in protection against I/R injury will be described in this paragraph. While FOXO target genes have been extensively described in mainly tumor cells, FOXO action remains highly tissue and context specific (Calnan and Brunet [14]). Several studies have proposed FOXO target genes as effectors in cardiac and endothelial tissue protection.
The first detrimental process during I/R injury to consider is ROS production. Since excessive ROS production and the subsequent cellular damage is considered one of the major propagators of I/R injury, it is interesting to see how FOXO activation can protect the heart during I/R injury from ROS. As stated previously, during I/R injury one of the proteins that is dysregulated is mnSOD. This enzyme is one of the key elements in ROS scavenging and protection against ROS damage. In human colon carcinoma cells, activation of FOXO3a directly regulates mnSOD expression and protein abundance. When cells are treated with H2O2 to simulate oxidative stress, FOXO3a activation increases ROS scavenging via mnSOD (Kops, Dansen [49]). While several other studies have documented the ROS scavenging capabilities of FOXO target genes, this function is not yet very well described in cardiac tissue. Guo *et al.* show that when FOXO3a is inhibited by adaptor protein p66Shc, mnSOD is decreased and subsequently ROS scavenging and oxidative stress resistance is lowered (Guo, Gertsberg [50]). Sengupta *et al.* show in an extensive study the effects of FOXO1 and FOXO3a double knock-out (KO) in mice hearts. In double KO mice, the expression of *SOD2* and *catalase*, two essential FOXO activated anti-oxidants, were drastically lowered compared to wildtype littermates during I/R injury (Sengupta, Molkentin [38]). Subsequently more oxidative DNA damage was observed as could be expected due to the lack of the two anti-oxidant enzymes. Tan *et al.* also report on the ROS scavenging activity of FOXOs in cardiomyocytes. ROS seem to be elevated in an insulin sensitive manner, this is shown to be the result of FOXO3a inhibition by insulin signaling via Akt (Tan, Wang [51]). FOXO3a controls *catalase* up-regulation directly as a response to ROS and as such FOXO3a signal for ROS scavenging in cardiomyocytes by activating *catalase* transcription.

A second detrimental process during I/R injury is loss of cardiomyocytes due to necrosis and apoptosis. FOXOs are traditionally considered to induce apoptosis by activating transcription of pro-apoptotic genes (figure 6). In carcinogenesis for example, FOXOs have been named as anti-tumorigenic factors due to their ability to induce apoptosis and stopping tumor cell growth. Sunters *et al.* showed that FOXO3a induces the transcription of pro-apoptotic gene *Bim*. Silencing FOXO3a showed lower levels of FOXO3a and BIM, and subsequently less apoptosis was observed in breast cancer cells (Sunters, Fernandez de Mattos [52]). Similar results were reported for neuroblastoma cells in which FOXO3a induces mitochondria mediated apoptosis via induction of *Bim and Noxa* (Obexer, Geiger [53]). Silencing *Bim* or *Noxa* ablated FOXO3a mediated apoptosis by up to 60%, showing that *Bim* is very important in FOXO pro-apoptosis signaling. Parallel to the mitochondria mediated apoptosis, FOXOs are reported to induce apoptosis via the pro-apoptotic Fas ligand (van der Vos and Coffer [5], Zhang, Tang [54]). In I/R injury treated rat myocardium, when treated with the naturally occurring proteolytic enzyme Bromelain, phosphorylated Akt and phosphorylated FOXO levels were increased. This resulted in a significantly lower apoptotic cardiomyocyte rate and

decreased infarct size. While this *in vivo* study did not focus on apoptosis inducing FOXO target genes, it is clear that FOXOs are pro-apoptotic in rat cardiomyocytes (Juhasz, Thirunavukkarasu [55]). The previously named studies portray FOXOs as apoptosis inducers, which would mean that cardiomyocyte survival is hampered during I/R injury. Though few, some studies ascribe anti-apoptotic functions to FOXOs. Bakker *et al.* report that in fibroblasts FOXO3a activity is induced in an HIF-1 dependent manner. FOXO3a in turn represses HIF-1 mediated apoptosis via the induced expression of *CITED2*. When FOXO3a was inhibited via siRNA treatment, *CITED2* expression was reduced while the HIF-1 regulated pro-apoptotic genes *Nix* and *RTP801* were increased (Bakker, Harris [44]). Sengupta *et al.* report that in cardiomyocyte specific FOXO1/FOXO3a double knock-out mice, the pro-apoptotic gene *Bax* is up-regulated while the anti-apoptotic *Bcl2* is down-regulated (Sengupta, Molkentin [38]). Subsequently, the double knock-out mice show higher amounts of cell death during I/R injury compared to their wildtype littermates.

Last, the infiltration of neutrophils is an important mediator of I/R injury as stated previously. This immune cell population which enters the ischemic area as first during reperfusion, wreak havoc in the damaged tissue. Not much has been published on FOXO function in heart, publications on FOXO action in neutrophils is almost non-existent. Two contradicting papers exist which focus on FOXO action in neutrophils, different processes are considered however. Miyamoto *et al.* report that aged, FOXO3a deficient mice develop neutrophilia as opposed to their wildtype littermates (Miyamoto, Miyamoto [56]). This means that when FOXO3a is not present, neutrophils can freely proliferate and multiply suggesting that FOXO3a inhibits neutrophil proliferation. Over-expression of FOXO3a attenuated neutrophil over-proliferation. The authors believe that FOXO3a inhibits proliferation via Spred2 by inhibiting ERK activation.
Jonsson *et al.* on the other hand report that FOXO3a deficiency results in a less active neutrophil population. FOXO3a deficient mice are less prone to form rheumatoid arthritis, which is an inflammatory disease affecting the wrists and ankles (Jonsson, Allen [57]). The researcher prove that this phenomenon is not a result of less infiltration of neutrophils or less active neutrophils, but that neutrophils from FOXO3a deficient mice are more prone to apoptosis. Elevated levels of Fas ligand were detected in FOXO3a deficient mice as compared to wildtype littermates. Fas ligand activates Fas receptor mediated apoptosis in the FOXO3a deficient neutrophils. By using Fas ligand specific antibodies, Jonsson *et al.* show that Fas ligand is indeed the main proponent in mediating the elevated levels of apoptosis in the FOXO3a deficient cells.
Recent evidence suggests that not only the innate immune system, mainly via neutrophils, is important in I/R injury, but that the adaptive immune system (B- and T-lymphocytes) might play a role also. A rapid accumulation of T-lymphocytes is observed in myocardial I/R injury (Yang, Day [58]). RAG null mice, which are deficient in lymphocytes, are found to have smaller infarct sizes during cerebral I/R injury and myocardial I/R injury (Yilmaz, Arumugam [59], Yang, Day [58]). These findings suggest that there is an antigen-presentation independent pathway of lymphocyte activation during I/R injury. Stahl *et al.* show in their 2002 study that T-lymphocyte survival is negatively regulated by FOXO3a activity. The cytokine interleukin-2 (IL-2), which is necessary for T-lymphocyte maturation and survival, causes FOXO3a phosphorylation via Akt. Over-expressing FOXO3a results in enhanced T-lymphocyte apoptosis (Stahl, Dijkers [60]). This is confirmed by Dijkers *et al.* who also observe an apoptosis inducing function for FOXO3a in lymphocytes (Dijkers, Birkenkamp [61]).

2. Discussion

Acute myocardial infarction (AMI) is a major clinical problem worldwide and proper treatment of the infarcted heart after an AMI is of essential importance. The primary goal should be limiting the infarct zone as much as possible, but this does not seem to be as easy as it sounds. The first thing that comes to mind when an AMI patient presents himself is restoring the blood flow as soon as possible. As with all organs, the heart especially needs constant perfusion to function properly. Since not all AMI patients can be expected to present themselves within minutes at the hospital for medical attention, the ischemic period of the infarct zone can be relatively long. The damage to the cellular components, in particular the mitochondria, during the ischemic period, forms a basis for the adverse effects during reperfusion. Ischemia/reperfusion (I/R) injury is proven to be a major factor in post treatment complications (Yellon and Hausenloy [3], Prasad, Stone [16], Eltzschig and Eckle [2]). When I/R injury was first broadly "introduced" by Yellon and Jennings in 2002, the presence and clinical relevance of reperfusion injury was hotly debated (Ferrari and Hearse [62], Piper, Garcia-Dorado [11]). Currently however it is commonly accepted that I/R injury constitutes a major part of myocardial damage after AMI.
I/R injury is a convergence of complex processes in a damaged tissue background. The reperfused infarcted area, which is already stressed and damaged during the prior ischemic period, is further damaged by the sudden shift in the extra- and intracellular conditions like pH and metabolite presence. The exact mechanisms behind I/R injury are still unclear, but possible mediators have been proposed and partially proven *in vitro* and in *in vivo* models as described in the introduction. Successes in replication and partial treatment of I/R injury in animal models caused an initial excitement about possible therapeutic treatments, but the translation to clinical trials has not been successful (Dirksen, Laarman [63]). Even though pig models represent the human situation better than mouse models, the experiments are still performed under controlled conditions. Parallel to this, in humans several drugs are administered at the same time when an AMI patient presents himself. Such drugs can obviously influence the used I/R treatment method and confound clinical outcomes of such interventions. The difficulties of such matters however are not the topic of this paper and discussion. Every researcher in the medical field is probably aware of such problems and Dirksen *et al.* summarize the difficulties with translation to clinic very nicely.

2.1 Are FOXOs up- or down-regulated during I/R injury?

The first point to consider is when and via which pathways FOXOs could be activated and regulated during I/R injury. The classical regulatory pathway for FOXO activity via insulin receptor--PI3K--pAkt was described in the introduction, and it is not hard to imagine this pathway will be inactive during the ischemic period. To reiterate, during the ischemic period FOXOs should be accumulated in the nucleus since the inhibitory action, namely phosphorylation by active Akt, is impaired. A more interesting question is what happens to FOXOs during reperfusion. As mentioned, the damage done in I/R injury is mainly due to reperfusion itself. So FOXO activation, if a cardioprotective effect from FOXOs is expected, would be desirable during reperfusion. The first thing that comes to mind while thinking about restoring blood flow after AMI is the influx of plasma compounds, among which insulin. So in essence, FOXO activity should be down-regulated by Akt during reperfusion since insulin is present. Interestingly, during chronic and acute heart failure the system is found to develop insulin resistance. Ohta *et al.* report that mice with MI develop insulin resistance in their skeletal muscle. When these mice are treated with an inhibitor against NADPH oxidase, an enzyme which converts

oxygen to superoxide radicals, the insulin resistance is attenuated (Ohta, Kinugawa [64]). Mice with an MI but without the NADPH oxidase inhibitor have lower levels of phosphorylated Akt, which leads to the conclusion that during MI, oxidative stress related insulin signaling resistance is formed via down-regulation of phosphorylated Akt. Measurements however were done four weeks after ligation of the coronary artery. The observed effects might be the result of systemic adaptation to the chronic ischemic period. The finding that inhibiting superoxide production by using an NADPH oxidase inhibitor attenuates the down-regulation of phosphorylated Akt does not correlate with a 2007 study by Chen *et al.*. Chen *et al.* report that using an NADPH oxidase inhibitor or an inactive mutant of NADPH oxidase causes lower levels of phosphorylated Akt compared to WT or un-treated mice (Chen, Zeng [65]). The major difference here is that Chen *et al.* use an I/R model as opposed to a chronic MI model by Ohta *et al.*. In conclusion, Akt seems to be positively regulated by superoxide in the short term while in the long term there is a negative regulation. The system might be protecting the organism from over-proliferation of damaged cells, since the Akt pathway is a survival and proliferation signal, during chronic stress periods.
An analysis of the insulin resistance levels of AMI patients which underwent a percutaneous coronary intervention (PCI) better resembles the situation portrayed in this paper. Patients which presented themselves with AMI were treated and so reperfusion occurred, after which the researchers determined levels of various plasma compounds with a special focus on insulin resistance (Lazzeri, Valente [66]). The researchers conclude that these patients, which basically underwent I/R injury, developed insulin resistance as judged by their insulin and glucose levels. These patients were non-diabetic according to their medical history, so the acute insulin resistance should be a direct effect of the ischemia or the I/R period.
Last, an insulin signaling blocking role for tumor necrosis factor (TNF) is plausible during I/R injury. The influx of immune cells will cause a rise in the secretion of TNF from these cells. TNF is known to be an insulin signaling inhibiting cytokine and is secreted by most, if not all, immune cells and some somatic cells (del Aguila, Claffey [67]).
The JNK pathway, which is seen to be activated by oxidative stress as described in the introduction, seems more clear-cut. JNK phosphorylates and causes FOXO translocation to the nucleus. Parallel to this AMPK also positively regulates FOXOs by phosphorylation. Taken together, it is in my opinion safe to presume that FOXOs should not be down-regulated via the insulin--Pi3K--Akt pathway due to the acute insulin resistance. Parallel to this FOXOs are activated by JNK and AMPK in a ROS dependent manner, which are abundantly present during I/R injury. This would mean that FOXOs, for good or for bad, should be able to exert their target gene activating function during I/R injury.
I/R injury animal models at the Utrecht Medical Centre are regularly performed and infarct zone tissue samples should be readily available. By doing immunohistochemistry for example, phosphorylated FOXO levels could be determined. It would be interesting to observe the difference between the ischemic and I/R period, so that a comparison in FOXO phosphorylation state can be made between the two time periods.

2.2 Would FOXOs be a positive or a negative aspect during I/R injury?

The possible positive or negative effects of FOXOs during I/R injury are more difficult to assess. Considering the complex spatiotemporal expression pattern of FOXOs and the many known target genes (Greer and Brunet [4]), it is not an easy task to pinpoint one or several FOXO target genes which could play a role in I/R injury.

The importance of ROS production during I/R injury is clear and understandable (2007 Yellon and Hausenloy [3]), the damaged and stressed cells cannot compensate for the sudden influx of metabolites and the sudden switch to fully aerobic energy production adequately. The prime FOXO target gene in this aspect seems to be manganese superoxide dismutase (*mnSOD*), an anti-oxidant enzyme which converts superoxide to the less reactive hydrogen peroxide (figure 3). Out of the three distinct types of superoxide dismutases, mnSOD is the most studied one. As early as 1998 the possible beneficial effects of mnSOD during I/R injury were observed in transgenic mice over-expressing *mnSOD*. While the earlier studies with mnSOD injection were inconclusive due to the very short half-life of injected mnSOD, Chen *et al.* showed in their 1998 study that mice over-expressing *mnSOD* had 35% smaller infarct size caused by I/R injury (Chen, Siu [68]). The protective effect of mnSOD during I/R injury was further proven in a renal I/R injury model where recombinant mnSOD, which has a longer half-life, had been injected in rats (Rahman, Mori [69]). Rats with administered rhmnSOD had attenuated tissue injury compared to non-treated rats. The inefficiency of mnSOD during I/R injury however was shown as early as 1988 by Arduini *et al.* as mentioned in the introduction. What could be the mechanism behind this inefficiency of mnSOD function during ischemia and ischemia/reperfusion injury? Peroxynitrite ($ONOO^-$) has been seen to inactivate mnSOD by nitrating internal tyrosine residues (MacMillan-Crow, Crow [70]). Peroxynitrite is a product of the O_2^- + NO reaction and is assumed to be abundantly present during I/R injury, since superoxide is excessively produced during this period. Gray *et al.* showed that animals which underwent hepatic I/R injury had a decreased mnSOD activity in the lungs, while the lungs obviously are not in close proximity to the hepatic ischemic area (Gray, MacMillan-Crow [71]). This was seen to be an effect of mnSOD nitration by peroxynitrite. So basically, the ROS scavenging system during I/R injury is stuck in a "superoxide--peroxynitrite--inactive mnSOD--more superoxide" loop, resulting in tissue damage. The question arises whether FOXO over-expression for example could overcome this inhibition of mnSOD. The findings with mice transgenic for *mnSOD* and rats injected with rhmnSOD are encouraging since the system seems to be able to overcome the proposed negative regulation by peroxynitrite. It is not hard to imagine that the overload of mnSOD decreases superoxide presence and consequently peroxynitrite production. An interesting study would be to create cardiac tissue specific *FOXO* over-expressing mice. If the hypothesis that mnSOD attenuates I/R injury is correct, over-expression of *FOXO* in the myocardium should also attenuate I/R injury via induction of *mnSOD*. Catalase, another FOXO induced anti-oxidant enzyme, is also a candidate. The regulation of this enzyme during I/R episodes however is not as extensively studied as mnSOD. It seems clear however that FOXOs can be beneficial during I/R injury due to their ability to activate transcription of different anti-oxidant enzymes, thereby exerting a ROS scavenging function.

Since the heart is essentially a big muscle and muscle cell proliferation after differentiation is almost non-existent, cell death during I/R injury is detrimental. Adult stem cells, or rather cardiac progenitor cells in this case, have been identified and are seen to differentiate *in vitro*. This however could not as of yet be repeated *in vivo* nor could endogenous regeneration of any kind be observed in infarcted myocardium. FOXO influence on cell death however seems to be complex. While a regulated form of necrosis, named necroptosis, has been proposed (Vandenabeele, Galluzzi [72]), it is still controversial and the regulatory mechanisms behind this form of necrosis are not fully understood. Regulation of apoptosis during I/R injury however is a better candidate to consider. FOXOs are classically considered as pro-apoptotic factors, as described in the introduction (figure 6). When the relation between apoptosis and I/R injury is considered simplistically, the conclusion would be that FOXO

activation during I/R injury is detrimental since this will trigger cell death. Since cellular recovery in the myocardium is almost non-existent, this would mean irreversible damage to the heart. Parallel to cell loss, early apoptosis is thought to be an inducer of inflammation during I/R injury (Daemen, van 't Veer [73]). Inflammation is a strong mediator of I/R injury as described in the introduction. Sengupta *et al.* however showed a positive effect for FOXO1 and FOXO3a expression in the infarcted heart. As described in the introduction, gain-of-function and loss-of-function assays *in vitro* and *in vivo* in mouse hearts show that the presence of these two FOXOs attenuates ROS presence and cell death (Sengupta, Molkentin [38]). The down-regulation of *CITED2* shown in FOXO1/FOXO3a double knock-out mice also correlates with the observations made by Bakker *et al.,* this group shows that FOXO3a represses HIF-1 induced apoptosis via CITED2 (Bakker, Harris [44]). The question arises whether FOXOs are pro-apoptotic in the heart and whether FOXOs are a positive effect. Why not both? The pro-apoptotic function of FOXOs have been unequivocally proven, but the ROS scavenging function described previously might prevail in the heart. Most of the research into the apoptotic role of FOXOs have been performed in tumor environments. While the ischemic core of a tumor might represent the situation of an ischemic heart to a degree, the reperfusion period is drastically different. The excess of ROS during the reperfusion period might have the upper hand in apoptosis induction. The research done by Sengupta *et al.* is encouraging in this context since it is clearly shown that FOXO1 and FOXO3a function is necessary to limit the infarct size in mice *in vivo* and cardiomyocyte damage *in vitro*. This could mean that during I/R injury, apoptosis induced by FOXO expression might be negated by the ROS scavenging function of FOXOs. The study by Juhasz *et al.*, in which is shown that infarct size is limited by Bromelain treatment via activation of Akt and subsequently inhibition of FOXO3a, does not coincide with this hypothesis (Juhasz, Thirunavukkarasu [55]). The results given by this group are however incomplete in my opinion. First, the limited infarct size might be directly related to increased Akt activity. FOXO3a inactivation might just be a 'side-effect' of the Bromelain treatment, while the activated Akt, which is considered a propagator of a pro-survival pathway, might exert the infarct size attenuating effects via other mediators. Second, Bromelain itself might influence other cellular pathways involved in cell survival. Juhasz *et al.* do not take these points into consideration and therefore present a very narrow conclusion. Using a constituvely active form of FOXO3a in tandem with Bromelain treatment might have made things more clear.
From the above arguments it can be concluded that FOXO activation during I/R injury will be beneficial for cell survival. FOXOs are widely considered as pro-apoptotic, but the ROS scavenging function of FOXOs will dominate during I/R injury in my opinion. The massive amount of ROS produced during I/R injury will be much more detrimental to cell viability than apoptosis caused by FOXO activation. An easy experiment would be to compare FOXO null cardiomyocytes and FOXO over-expressing cardiomyocytes viability during H2O2 treatment, FOXO over-expressing cardiomyocytes should be taken as controls. If the hypothesis that ROS scavenging function of FOXOs overrules the pro-apoptotic function is correct, FOXO null cells treated with H2O2 should show more apoptosis compared to the FOXO over-expressing H2O2 treated cells.

Last, the immune reaction during I/R injury should be taken into consideration. Drawing clear conclusions about FOXO action in neutrophils, which are proposed to be a major propagator of I/R injury, is near impossible. Publications focusing on FOXO regulation and action in neutrophils are limited as shown in the introduction. The two publications described contradict each other, but it must be mentioned that different processes are considered. It must be commented however that the

observations done by Jonson *et al*, in which FOXO3a is proposed to inhibit neutrophil apoptosis (Jonsson, Allen [57]), contradicts with previous research done focusing on the apoptotic effect of FOXOs. FOXO3a is found to suppress Fas ligand in neutrophils by Jonsson *et al.*, which leads to neutrophil survival. Fas ligand is commonly considered as a positively regulated target gene of FOXOs (figure 6). It is possible of course that the function of FOXOs in neutrophils differs from somatic cells. Such a radical change in function however, an inhibitory effect on Fas ligand instead of a commonly accepted inducing effect, is improbable.
More promising are the findings concerning FOXO action in lymphocytes. These cells of the adaptive immunity were found to be present during I/R injury as described in the introduction. FOXO activation was seen to induce apoptosis in these cells, thereby attenuating infarct size since inflammation was reduced.

To conclude, It is plausible that FOXO activation during I/R injury could be beneficial for cell survival and for limiting infarct size. The pro-apoptotic function of FOXOs should be overruled by their beneficial, ROS scavenging, effects in cardiac cells. Parallel to this, the apoptotic function of FOXOs in the immune cells should cause less accumulation of these cells during I/R injury. ROS scavenging function of FOXOs should not play a critical role in this case, since the immune cells obviously did not undergo an ischemic period leading to an excess in ROS production. So FOXO activation during I/R injury should save cardiac cells by scavenging the excessively produced ROS, and FOXO activation should induce apoptosis in the immune cells and so reduce infarct size by limiting inflammation.

2.3 Can FOXOs, or a specific FOXO, in the heart be therapeutically influenced?

As with all drug administration, specifically targeting a drug to a single organ is as of yet impossible. Especially with a transcription factor such as FOXO which seems to be ubiquitously expressed throughout the whole body, finding a method to induce FOXO expression in the infarcted heart will be difficult. FOXO3a repeatedly comes forward as a potential candidate for specific cardiac FOXO targeting, since most of the research done in myocardial FOXO effects are focused on FOXO3a. This however is most likely the result of research subject bias since a big portion of FOXO related research is focused on FOXO3a in general. FOXO3a's expression pattern is not unequivocally related to the myocardium, as such systemic inhibition of FOXO3a would not be desirable in AMI patients. Systemic inhibition of any of the FOXOs would be unwise since all family members play many and critical roles in various tissues. Local administration of a drug, or of FOXOs themselves, during a percutaneous coronary intervention might be a possibility. When the catheter is introduced to open the blocked artery, drugs can be locally administered via this catheter to the injured tissue. The problem remains however that the drug administered via this method will be dispersed into the blood flow since the catheter used during PCI is introduced via the femoral artery. Advances have been made in injecting stem cells locally into the infarcted myocardium (Williams, Trachtenberg [74]), which can possibly be used to administer FOXOs or a drug activating FOXO transcription. Several strategies have been proposed over the years for local drug delivery, among which ultrasound triggered drug release (Li, Zheng [75]) and guidance of magnetic particles by MRI (Pouponneau, Savadogo [76]). These methods however are as of yet inefficient and have technical problems, so local injection via a catheter is the most plausible method of administration.
The biggest hurdle however is the timing of FOXO target gene activation. Since FOXO in this case serves as an intermediary, administering FOXO does not instantly lead to an effect. The transcriptional and translational machinery first needs to start in order for FOXOs to exert their I/R

injury attenuating function. Ischemia/reperfusion injury however is an acute process and thus requires an acute intervention. Preventive medication is a possibility, but the localization problem remains since oral administration would be the only possible solution in such a case. Preventive medication for AMI patients however would be impossible since the acute onset of this pathological condition is not predictable.

In my opinion a combination of recombinant mnSOD and FOXO injection is more desirable to achieve a positive effect. While recombinant mnSOD will clear the blunt of the excessively processed ROS immediately after administration, thereby giving the tissue more time to resist cell death, the injected FOXO will activate target genes and cause a delayed reaction. So in essence recombinant mnSOD will act as the frontier drug until degradation, while FOXO takes over after the initial mnSOD action. This of course hinges on the hypothesis that ROS are the most damaging components in I/R injury as proposed, and thus this should be further studied.

While in my opinion FOXOs do have positive therapeutical potential in I/R injury, it is obvious that administration and upkeep of FOXOs or FOXO activating drugs will be a major hurdle to overcome. Advances with local administration of drugs are being made, so local administration or activation of FOXOs should be possible in the near future. How much effect this administration or activation will have however remains the question, since FOXOs need time to achieve their effects via their target genes and resulting proteins, while I/R injury is a relatively acute process. While FOXO might not be ideal on its own, it might be a good candidate as a support therapy.

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