The role of NADPH oxidases in diabetic cardiomyopathy

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Keywords

NADPH oxidases, diabetic cardiomyopathy, oxidative stress, metabolism, obesity and insulin resistance

Abbreviations

Advanced glycation end-products (AGEs), AGE receptors (RAGE), Angiotensin II (Ang II), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), diallyl trisulfide (DATS), endoplasmic reticulum (ER), endothelial and neuronal nitric oxide synthase (eNOS and nNOS), fatty acid (FA), glucose transporter (GLUT), glycated BSA (Gly-BSA), heart failure (HF), high glucose (HG), NADPH-oxidase (NOX), Na^{+}-Ca^{2+}exchanger (NCX), monoamine oxidase (MAO), perilipin 5 (Plin-5), protein kinase C (PKC), Renin-Angiotensin-System (RAS), reactive oxygen species (ROS), reactive oxygen- and nitrogen species (RONS), sarcoplasmatic reticulum (SR), Sodium-Glucose cotransporter (SGLT), Xanthine Oxidase (XO)

Abstract
Systemic changes during diabetes such as high glucose, dyslipidemia, hormonal changes and low grade inflammation, are believed to induce structural and functional changes in the cardiomyocyte associated with the development of diabetic cardiomyopathy. One of the hallmarks of the diabetic heart is increased oxidative stress. NADPH-oxidases (NOXs) are important ROS-producing enzymes in the cardiomyocyte mediating both adaptive and maladaptive changes in the heart. NOXs have been suggested as a therapeutic target for several diabetic complications, but their role in diabetic cardiomyopathy is far from elucidated. In this review we aim to provide an overview of the current knowledge regarding the understanding of how NOXs influences cardiac adaptive and maladaptive processes in a "diabetic milieu".

1. Introduction

The worldwide incidence of diabetes mellitus (DM) is increasing rapidly due to lifestyle changes. Patients with DM are two to four times more likely to develop cardiovascular disease (CVD) like high blood pressure, coronary artery disease and heart failure (HF), and have three times higher overall mortality rate compared to those without DM [1]. Diabetic cardiomyopathy is considered as left ventricular dysfunction in the absence of significant coronary or hypertensive disease [2].The development of this cardiomyopathy is multifactorial and complex and remains to be completely understood. Hallmarks of diabetes such as high blood sugar (hyperglycemia), dyslipidemia, hyperinsulinemia, activation of the Renin-Angiotensin-System (RAS) and a chronic low-grade inflammation, are believed to trigger a range of structural and functional changes at the cellular level in the diabetic heart (Figure 1). Accordingly, these hearts exhibit a range of features including oxidative stress, altered metabolism, mitochondrial dysfunction, fibrosis, apoptosis, increased ER stress, impaired autophagy, inflammation and altered calcium handling.

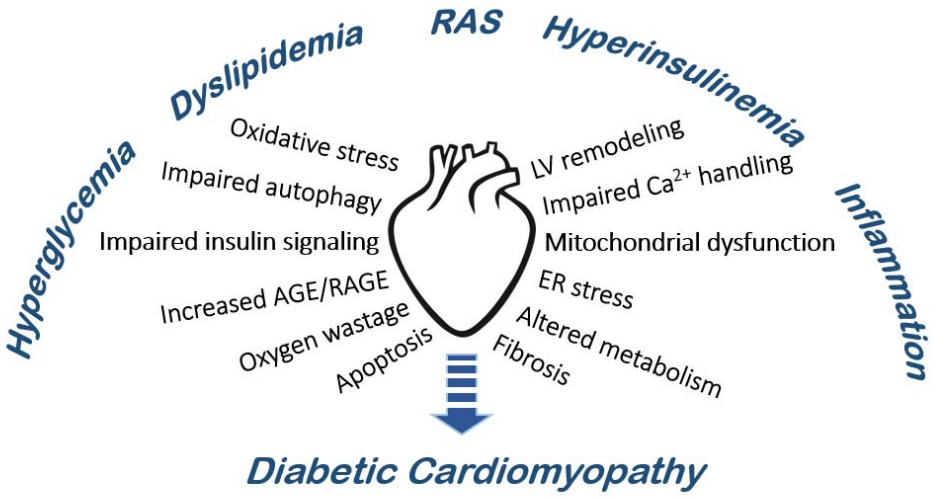

Figure 1: A range of systemic changes in diabetes such as hyperglycemia, dyslipidemia, increased activation of the Renin-Angiotensin-System (RAS), hyperinsulinemia and a chronic low grade inflammation are believed to lead to cellular changes in cardiomyocyte and consequently the development of diabetic cardiomyopathy. Advanced glycation end-products (AGE), AGE receptors (RAGE), Endoplasmic reticulum (ER).

NADPH oxidase (NOXs) are a family of enzymes whose primary function is to produce reactive oxygen species (ROS). They were first recognized as ROS-generating enzymes in professional phagocytes, playing an extremely important role in the mechanisms of host defense against infectious agents. NOXs are also believed to be a major source of ROS in different organs. Many of the systemic changes in diabetes are known activators of NOXs [3], and NOXs have therefore been suggested as a therapeutic target for diabetic complications (as reviewed by Gorin and Block [4]). However, studies undertaking the role of NOXs report both detrimental and protective effects of different NOX isoforms in the cardiovascular system [5, 6], and the role of NOXs in diabetic cardiomyopathy is far from elucidated. The aim of the present review, is to provide an overview of the current knowledge regarding the understanding of how NOXs influences cardiac adaptive and maladaptive processes in a "diabetic milieu".

1.1. Cardiac redox-signaling and oxidative stress

In response to specific stimuli (acute, transient or sustained), reactive oxygen- and nitrogen species (RONS) are produced through various enzymes in cardiomyocytes (Figure 2). Under physiological conditions, RONS are known to play key roles in different signaling pathways through their oxidation of specific targets, so-called redox signaling [7, 8]. However,

following increased activation of RONS-producing enzymes and/or impairment of endogenous antioxidant capacity, oxidative stress may occur [9]. Hence, redox signaling comes in “different flavors” where reversible modification may transiently change protein activity involved in physiological adaptations, while irreversible oxidations may lead to pathophysiological processes such as in HF [10] (Figure 2). A vast amount of clinical and experimental studies support increased oxidative damage in diabetic hearts [11-13].

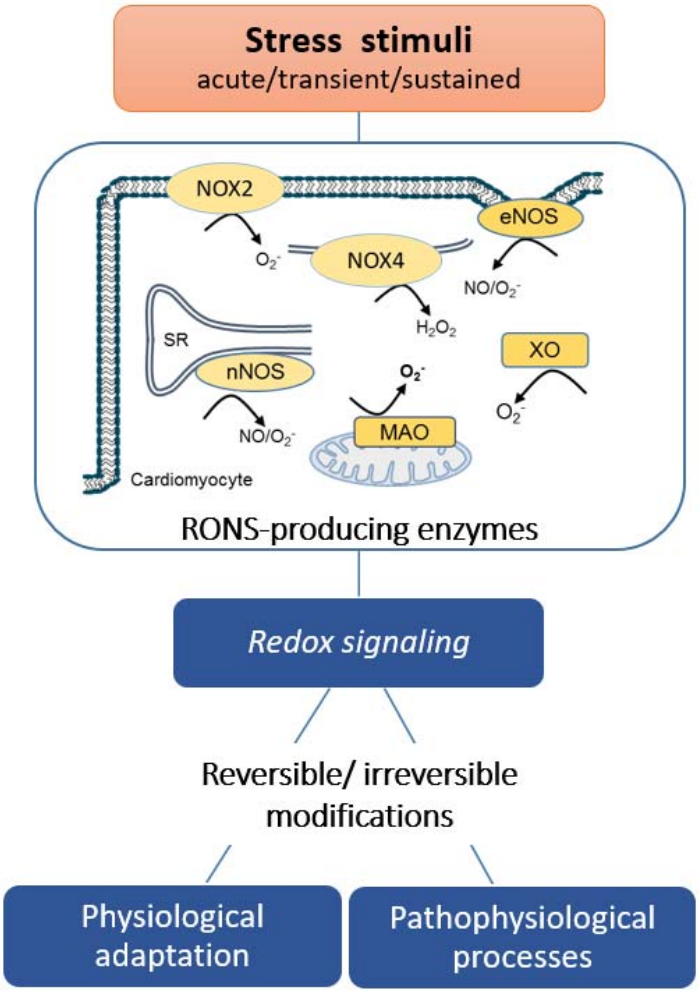

Figure 2: In response to various stimuli, several enzyme systems in the cardiomyocyte produce reactive oxygen- and nitrogen species (RONS). These enzymes (NADPH oxidase (NOX), endothelial and neuronal nitric oxide synthase (eNOS and nNOS), monoamine oxidase (MAO), xanthine oxidase (XO)) can through redox signaling mediate reversible or irreversible modification leading to physiological adaptive or pathophysiological processes. Sarcoplasmatic reticulum (SR).

1.2. NADPH oxidases in the heart

Of the seven mammalian NOX isoforms (Nox1-5 and Duox1-2), NOX2 and NOX4 are expressed in the heart [5]. Both isoforms exist as a heterodimeric flavocytochrome with a p22phox subunit, but they differ in their structure, activation, subcellular localization, type of ROS produced as well as in the specific signaling pathways they induce [14]. NOX2 activation requires the recruitment of several cytosolic subunits (p47phox, p67phox, p40phox and Rac1) which bind to the flavocytochrome to induce production of mainly superoxide. NOX4 on the other hand, is situated at internal membranes such as the endoplasmic reticulum (ER) and the mitochondria, is constitutively active, produces hydrogen peroxide and is mainly transcriptionally regulated [5]. Interestingly, these two enzymes have been shown to have distinct physiological and pathophysiological roles in the heart. In response to physiological stressors, NOX2 have been reported to be involved in stretch-induced calcium release, EC-coupling, and preconditioning [15-17]. NOX4 have been shown to play important roles in endogenous detoxifying responses [18], angiogenesis [19], ER-stress and protein unfolding stress response [20], substrate utilization [21] and in mediating metabolic stress responses [22]. Following different types of sustained stress, NOX2-dependent signaling promotes several detrimental processes in cardiac pathology, including cardiomyocyte hypertrophy, contractile dysfunction, arrhythmia, interstitial fibrosis, cell death, and cardiac rupture after myocardial infarction as reviewed by Zhang *et al* [3]. In contrast, in the setting of chronic hemodynamic stress, NOX4 have been shown to mediate protective effects such as adaptive remodeling with better preserved function and reduced hypertrophy [19, 23].

2. Hyperglycemia and NOX activity in the diabetic heart

Elevated glucose (hyperglycemia) is an important risk factor for developing cardiovascular disease. In addition to generating pyruvate for oxidation, elevated plasma levels of glucose may also affect non-oxidative pathways including the polyolhexosamine biosynthetic pathway, protein kinase C (PKC) activation and production of advanced glycation end-products (AGEs). There are growing evidence that hyperglycemia can induce NOX activity through various pathways in the heart (Figure 3).

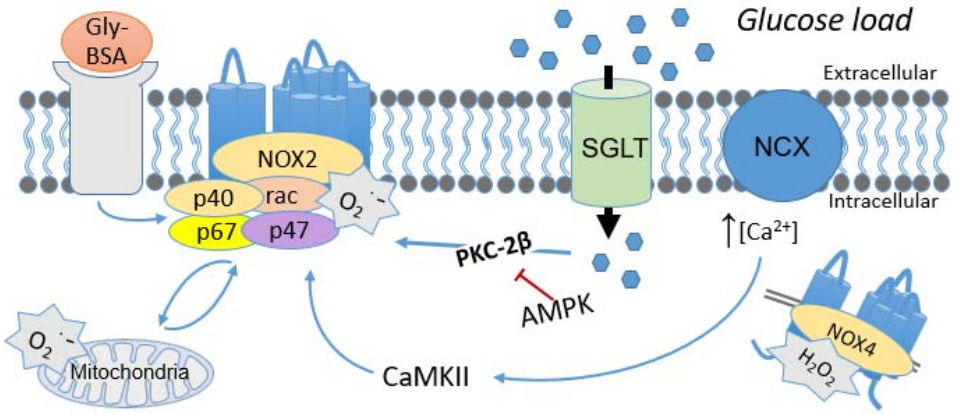

Figure 3: Schematic diagram of proposed mechanisms for NADPH oxidase (NOX) activation in cardiomyocytes exposed to high glucose (HG) load. HG load through the Sodium-glucose cotransporter (SGLT) leads to activation of protein kinase C-2 β (PKC), recruitment of catalytic subunits and consequently increased production of NOX2-derived superoxide. HG-induced elevation in intracellular Ca^{2+} ($[Ca^{2+}]$) activates Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and consequently increases NOX2 activity. Activation results in NOX2 superoxide ($O_2^{\cdot -}$) production, which promotes mitochondrial ROS production in a positive feedback loop. NOX4 has also been shown to be activated by HG through unknown mechanisms. NOX2, but not NOX4 activity is increased following stimulation of glycated BSA (Gly-BSA). AMP-activated protein kinase (AMPK), Na^{+}-Ca^{2+}exchanger (NCX).

2.1. NOX2 activation by glycated proteins

Both intracellular and extracellular lipids and protein exposed to high levels of sugars may undergo glycosylation. Zhang and co-workers [24] found glycated BSA (Gly-BSA) to induce ROS production and increase NOX2 activity in cardiomyocytes. They also reported that the activation of NOX2 was PKC-dependent and associated with translocation of the nuclear factor κB (NF-κB) to the nucleus. Interestingly neither NOX4, xanthine oxidase (XO), nitric oxide synthase (NOS) nor mitochondrial ROS, seemed to a play role in this process. Although AGEs may be important in the pathogenesis of diabetic cardiomyopathy [25], there are no direct evidence of AGE-induced NOX activation in diabetic hearts. Circumstantial evidence, however, suggests that increased AGE accumulation and AGE receptor (RAGE) expression in diabetic hearts are coupled with increased expression of NOX2 and its catalytic subunits [26].

2.2. NOX-activation by acute high glucose exposure

A vast amount of cell studies have demonstrated glucose-toxicity to be mediated through NOX2 activation. Exposing cardiomyocytes to a high glucose (HG) media enhance protein expression of NOX2 and its catalytic subunits [27-29], induce translocation of catalytic

subunits to the cell membrane [28, 30, 31], and increase overall NOX2 activity [32-34]. Multiple interventions to inhibit NOX2 activity have clearly demonstrated abrogation of HG-induced elevation of ROS in cardiomyocytes [30, 33-35]. NOX2 inhibition also ameliorates the detrimental cellular effects of HG, as indicted by improved insulin signaling [30, 33], increased endogenous antioxidant capacity [33], reduced apoptosis/cell death [27, 28, 32, 34, 36] and increased cardiomyocyte contractility [29]. Although less studied, HG-induced increase in NOX4 expression has also been reported in cardiomyocytes [37, 38]. Transfecting cultured cardiomyocytes with dominant negative NOX4 was able to reduce HG-increased expression of fetal gene program [37], suggesting NOX4 to play a role in HG-induced detrimental effects in cardiomyocytes.

2.3. NOX-activation by chronic high glucose exposure

Animal studies also report increased NOX activity in hearts following chronic hyperglycemia. Increased cardiac NOX2 activity has been found in hearts from both type I [26, 27, 34-36, 38-43] and type II [42, 44, 45]diabetic models. Furthermore strategies to directly reduce NOX2 activity in diabetic hearts have been shown to abolish many of the detrimental changes associated with diabetes. Reduced NOX2 activity in streptozytocin-induced diabetic hearts following cardiac specific knock down of the catalytic subunit Rac1 was associated with reduced cardiac oxidative stress [34, 39], ameliorated diabetes-induced collagen deposition, decreased inflammation [39], reduced markers of apoptosis [34] and reduced ER-stress [39]. These beneficial cellular effects were accompanied by reduced myocardial remodeling and improved cardiac function [34, 39]. Using a therapeutic approach, long term treatment with the NOX2 inhibitor apocynin has also been able to ameliorate many of the diabetes-induced adverse cellular effects and improve systolic and diastolic ventricular function [34, 39, 43, 46]. Increased expression of NOX4 has also been reported in hearts from diabetic models [37, 38, 47, 48], and anti-diabetic treatments and exercise have been shown to normalize this expression [38, 48]. Maalouf *et al.* demonstrated direct cardiac effect of NOX4 inhibition as administration of antisense NOX4 oligonucleotides (NOX4-AS) decreased diabetes-induced cardiac ROS production associated with improved mechanical function [37]. Surprisingly, diabetes was not associated with a change in NOX2 activity or expression in this study, a finding that is commonly reported [27, 34-36, 38-45].

Several signaling pathways may mediate the HG-induced activation of NOX2 (Figure 3). Phosphorylation of p47phox and consequent translocation to the plasma membrane is known to be catalyzed by several types of PKCs in neutrophils [49]. HG can activate PKC- β2 in cardiac caveolae [50], and in line with this, a PKC- β2-inhibitor was shown to reduce the HG-induced p47phox translocation [31]. Baltau and coworkers [30] reported that inhibition of glucose uptake through glucose transporter 1 and 4 (GLUT1 and GLUT 4) did not affect HG-induced ROS production, and that the HG-induced NOX2 activation and consequent ROS production could be mimicked by using non-metabolizable glucose-analogs. They therefore suggested that glucose transport through the sodium-glucose cotransporter (SGLT) is

responsible for the activation of NOX2, and not glucose utilization per se. In a follow-up study [31] they found that AMP-activated protein kinase (AMPK) activity could inhibited HG-induced NOX2 activity by blocking the PKC-β2 pathway and the subsequent translocation of p47phox to the membrane. In the same study, p47phox was shown to translocate to caveoline-3 and that disruption of the caveolar structure prevented HG-induced ROS. Together, these data strengthen the notion of a HG-induced signalosome located in cardiac caveolae.

A cross-talk between other ROS-producing enzymes and NOX activity has been suggested following acute HG-exposure in cardiomyocytes. Both inhibition of either mitochondrial superoxide or NOX2 was found to prevent HG-induced ROS [36]. Chronic antioxidant supplementation using diallyl trisulfide (DATS) [27], coenzyme Q10 [40, 41], mito-TEMPO [36] or N-acetyl-L-cysteine (NAC) [42] has also been shown to reduce myocardial NOX2 expression and activation in diabetes, resulting in ameliorated morphological remodeling and improved ventricular function. Cardiac specific knock-down of cardiac Rac1 also reduced mitochondrial superoxide production [34, 39], suggesting that NOX2 could contribute to mitochondrial ROS in hyperglycemic hearts. Furthermore, apocynin has also been suggested to limit diabetes-induced eNOS uncoupling in cardiomyocytes [43]. Together, these studies clearly suggest an interaction between different ROS sources in the cardiomyocyte where NOX2 activity in the diabetic heart may both modify and be modified by other ROS-producing enzymes.

3. Dyslipidemia and NOX activation in the diabetic heart

In addition to elevated glucose, diabetes is associated with dyslipidemia where both elevated fatty acid (FA) uptake and oxidation is believed to induce cardiac lipotoxicity. Growing evidence suggest that exposure to a high lipid load result in activation of NOX2 [42, 44, 45, 51, 52] (Figure 4).

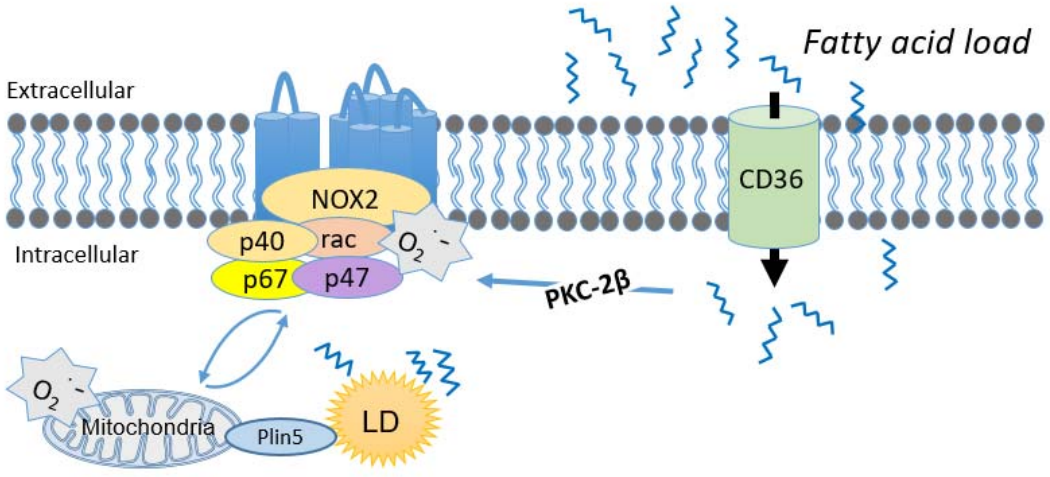

Figure 4: Schematic diagram of proposed mechanisms for NADPH oxide (NOX) activation in cardiomyocytes exposed to saturated fatty acids (FA). Transport of FA through CD36 leads to activation of protein kinase C-2 β (PKC-2β), which promotes recruitment of NOX2 catalytic subunits and activation of NOX2. This activation results in superoxide ($O_2^{\cdot -}$) production, which consequently promotes mitochondrial ROS production in a positive feedback loop. Lipid droplets (LD) release FA in to the cytosol and are transported into the mitochondria with assistance from perilipin 5 (Plin5). FA released from the LDs promotes NOX2 activation.

3.1. NOX activation by acute lipid load

Cardiomyocytes exposed to the saturated FA, palmitate, exhibit increased levels of p47phox in the membrane and elevated ROS production [51, 52]. Oleate, an unsaturated FA did not increase superoxide production, suggesting that this effect is not a general effect of a FA load, but rather a result of high levels of saturated FA [51]. In a NOX2 KO model, high levels of palmitate did not induce higher levels of ROS, mitochondrial dysfunction [51, 52] or sarcoplasmic reticulum calcium-leak [52]. In agreement with this, NOX2 inhibitors (apocynin and gp91 ds-tat) and SiRNA-mediated depletion of p47phox prevented palmitate-induced ROS formation [52]. Interestingly, NOX2 inhibition also restored lysosome acidification and enzyme activity as well as reduced autophagosome accumulation in palmitate-treated cardiomyocytes [51]. In contrast, inhibiting NOS, another known source of ROS, had minimal effect on palmitate-induced ROS formation [52].

Both PKC activation and ROS-induced ROS-release are proposed to mediate the palmitate-induced NOX2 activation in cardiomyocytes, as inhibition of PKC prevented palmitate-induced NOX2-derived ROS production [51, 52]. Furthermore, mitochondrial ROS seems to be an important contribution to the total ROS levels induced by elevated palmitate levels, as mito-TEMPO eliminated the palmitate-induced ROS formation in cardiomyocytes. Interestingly the palmitate induced ROS production from NOX2 seemed to precede palmitate induced mitochondrial ROS production [52].

3.2. NOX activation by chronic dyslipidemia

The palmitate-induced activity of NOX2 is supported by studies on animal models of obesity and diabetes. First, lipid lowering treatment has been shown to lower the NOX-dependent ROS production in obese and diabetic animals [44, 45]. Treating Diabetic *db/db* mice with a cholesterol-lowering drug, resulted in reduced NOX2 activity in the heart, which could have contributed to the attenuation of oxidative stress [45]. In addition silencing of the FA-transporter CD36 decreased NOX2-dependent ROS production in hearts from *ob/ob* mice. This was accompanied by prevention of cardiac steatosis, as well as increased insulin sensitivity and glucose utilization in the heart [44]. Perilipin 5 (Plin5) is essential to protect lipid droplets in the cardiomyocyte. It is abundantly expressed in the heart and is thought to stabilize lipid droplets by preventing accumulation of lipotoxic intermediates. Interestingly, Kuramoto and colleagues found that the suppression of myocardial lipid droplet accumulation in diabetic Plin5-KO mice was associated with attenuation of diabetes-induced

cardiac dysfunction. These hearts, which were protected against functional remodeling, and also exhibited decreased assembly of NOX2, reduced membrane translocation of PKC-2β and lower levels of ROS [42].

4. Activation of NOX by the Renin-Angiotensin System (RAS) in the diabetic heart

Angiotensin II (Ang II) is a well-known activator of NOXs, that has been shown mediate a range of pathological cardiac changes such as fibrosis, apoptosis and hypertrophy [3, 8]. Huynh and colleagues [40] reported that the ACE-inhibitor ramipril was effective in preventing diabetes-induced upregulation of p47phox, p22phox and NOX2 expression together with reduced NOX2 driven myocardial superoxide production. This was accompanied by reduced apoptosis, fibrosis and hypertrophic gene expression. Also, blocking AT1 with candesartan in *db/db* mice ameliorated NOX2 and p22phox expression, superoxide content and macrophage infiltration in the heart [53]. In cardiomyocytes, the use of an Ang II type 1 (AT_1) antagonist could ameliorate HG-induced increase in p47phox expression and prevent HG-induced abnormalities [29]. Thus, the increased RAS activity in diabetes most likely support the interplay between Ang II and NOX activity in the diabetic heart.

5. Impaired calcium handling and NOX activation in the diabetic heart

Alteration in calcium handling and the excitation-contraction coupling machinery is profound in the diabetic heart [54]. Although not clearly demonstrated, impaired cardiac calcium handling has been suggested to modulate NOX activity. Exposure to HG was shown to increase intracellular calcium ($[Ca^{2+}]_i$) through the sodium-calcium exhanger (NCX) in cardiomyocytes. This consequently increased Ca^{2+}/calmodulin-dependent protein kinase II CaMKII activation which was associated with increased NOX2 activation [35]. Inhibition of CaMKII activity reduced NOX2 activity and ROS production in diabetic hearts, indicating a link between activated CaMKII and the activation of NOX2 [35]. Conversely, CaMKII and other calcium handling proteins are redox sensitive and their activity may consequently be altered by NOXs [55]. It is tempting to speculate that some of the observed beneficial effects on ventricular function following NOX2 inhibition could be mediated through improved calcium handling in diabetic hearts. Apocynin treatment did however fail to alter diabetes-induced effects on protein expression of sarcoplasmic reticulum ATPase as well as phospholamban phosphorylation in type 1 diabetic hearts, despite improved contractile properties [43]. However, restoring the optimal redox state for intracellular Ca^{2+}-handling proteins, may very well not be reflected in the overall protein expression levels.

6. Gaps in current knowledge and future perspectives

The current knowledge regarding the role of NOXs in diabetic cardiomyopathy is mostly from animal- and cell studies, as there are few clinical studies. Increased NOX activity accompanied by translocation of p47phox to the cardiomyocyte sarcolemma has however

been reported in failing human myocardium [56]. NOX-derived ROS has also been suggested to be involved in the development of vascular disease in diabetic patients [57] . All though ROS seem to play a major role in the pathology of cardiovascular diseases, clinical trials with general exogenous antioxidant treatments have been largely unsuccessful in terms of preventing or treating such diseases [58, 59]. Specific-ROS-producing enzymes like the NOXs have therefore emerged as potential therapeutic targets, as recently reviewed by several groups [4, 60, 61]. However, a major challenge with the development of NOX inhibitors is that they are often un-specific and not isoform selective, they also may exhibit general ROS-scavenging properties [61]. In addition, NOXs also have important validated physiological functions which need to be sustained. Mutations in humans leaving a dysfunctional NOX2 protein leads to chronic granulomatous disease and NOX2 KO mice display impaired immune defense against pathogens [62]. Therefore, complete abrogation of NOX2 activity does not seem to be an acceptable therapeutic approach. In contrast, genetic deletion of NOX4 have revealed no spontaneous pathologies and a dual NOX1/4 inhibitor have been tested in the clinic with good tolerability [60]. However, the role of NOX4 in pathology is controversial as indicated by studies both reporting beneficial [19] and detrimental [63] effects of NOX4 in experimental HF. One factor that can explain these discrepancies is the severity of the HF applied in the different studies, where NOX4 may mediate beneficial effects through increased angiogenesis in the progression of a less severe HF. This topic is not studied in different animal models of diabetes where the progression to diabetic cardiomyopathy may vary greatly. Therefore there is a still a need for more understanding of the individual roles of NOX homologues in molecular mechanisms and signaling cascades in pursuing potential therapeutic interventions.

7. Conclusion

Significant progress has been made to elucidating the role of NOXs in diabetic cardiomyopathy. Anti-diabetic treatments and correction of dyslipidemia are associated with both reduced NOX2 and NOX4 activity in the heart, suggesting diabetes-induced systemic activators of NOXs. Cell studies clearly suggest a detrimental role for increased NOX2 activity following exposure to high glucose, elevated glycated proteins, dyslipidemia and increased activity of RAS. Also, reducing NOX2 activity in chronic models of diabetes, through NOX2 inhibition/deletion consistently reports to be associated with amelioration of adverse cardiac effects. The role of NOX4 in diabetic cardiomyopathy is however less elucidated, but studies so far suggest NOX4 to mediate adverse effects. Several studies indicate a marked complexity in the activation of NOXs which present a challenge when studying the role of these enzymes. Certain activators of NOXs also seems to be targets of NOX-induced redox modulation, creating feedback-loops and potential amplifying signaling cycles. Although cell and animal studies clearly suggest a role for NOXs in some of the pathological processes, there is still the need for more understanding of the individual role of NOX homologues in the progression of diabetic cardiomyopathy.

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