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# The regulation of cardiac intermediary metabolism by NADPH oxidases

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## Abstract

NADPH oxidases (NOXs), enzymes whose primary function is to generate reactive oxygen species, are important regulators of the heart's physiological function and response to pathological insults. The role of NOX-driven redox signalling in pathophysiological myocardial remodelling, including processes such as interstitial fibrosis, contractile dysfunction, cellular hypertrophy, and cell survival, is well recognized. While the NOX2 isoform promotes many detrimental effects, the NOX4 isoform has attracted considerable attention as a driver of adaptive stress responses both during pathology and under physiological states such as exercise. Recent studies have begun to define some of the NOX4-modulated mechanisms that may underlie these adaptive responses. In particular, novel functions of NOX4 in driving cellular metabolic changes have emerged. Alterations in cellular metabolism are a recognized hallmark of the heart's response to physiological and pathological stresses. In this review, we highlight the emerging roles of NOX enzymes as important modulators of cellular intermediary metabolism in the heart, linking stress responses not only to myocardial energetics but also other functions. The novel interplay of NOX-modulated redox signalling pathways and intermediary metabolism in the heart is unravelling a new aspect of the fascinating biology of these enzymes which will inform a better understanding of how they drive adaptive responses. We also discuss the implications of these new findings for therapeutic approaches that target metabolism in cardiac disease.

## Keywords

NADPH oxidases • Cardiac metabolism • Intermediary metabolism • Redox signalling

## 1. Introduction

The heart must contend with two interconnected functions: (i) to provide continuous, yet rapidly responsive contractile activity to pump blood to the tissues to meet varying demand and (ii) to undergo chronic remodelling of structure and function to maintain homeostasis and adapt to longer-term physiological stressors. For instance, during acute physiological fluctuations (e.g. fight or flight responses), high contractile activity and energetic efficiency are needed, whereas more chronic physiological stressors such as pregnancy, exercise, and growth from childhood to adulthood also require adaptive myocardial hypertrophy.<sup>1</sup> To fulfil both functions, the heart has evolved elaborate metabolic adaptations to alter its substrate utilization, in the form mainly of carbohydrates and fatty acids, both to deliver high aerobic energy production for contractile function and to maintain cellular structures and integrity and any requirements for remodelling.<sup>2,3</sup> Alterations to intermediary metabolism, which describes how substrates and their downstream metabolite intermediates integrate in various metabolic pathways to

maintain physiological function, may be key to understanding pathological states such as hypertensive heart disease, ischaemia, and diabetes or obesity-related cardiomyopathies that lead to heart failure.<sup>2,4</sup>

The failing heart is characterized by several changes in metabolism. These include a reduction in glycolysis and altered glucose oxidation and fatty acid oxidation leading to the energetic deficit and pump failure,<sup>5</sup> as well as interrelated changes affecting key cellular functions such as excitation–contraction coupling, cell hypertrophy, cell viability, fibrosis, and other processes.<sup>2,4</sup> Emerging data indicate that enzymes of the NADPH oxidase (NOX) family are key regulators of intermediary metabolic processes in the heart. Here, we review the main NOX isoforms that mediate the redox regulation of pathways involved in cardiac intermediary metabolism. We highlight the emerging links to heart function including effects on the cellular integrated stress response (ISR), mitochondrial function, calcium homeostasis, cellular differentiation, and hypertrophy. A better understanding of this complex regulation by NOX enzymes will provide new mechanistic insights into cardiac metabolic reprogramming and adaption to pathological stress and

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may aid the development of novel therapeutic strategies for heart disease.

## 2. NOX isoforms and the heart

NOXs use NADPH to reduce molecular oxygen ( $O_2$ ) and form the reactive oxygen species (ROS), superoxide anions ( $O_2^-$ ), and hydrogen peroxide ( $H_2O_2$ ). The prototype of this family of enzymes is the NOX2 isoform (originally known as gp91phox oxidase). In the last 20 years, seven distinct isoforms (NOX1-5 and DUOX1-2) have been identified in diverse cells and tissues.<sup>6</sup> The NOX1-4 isoforms associate with p22phox protein to form a membrane-bound heterodimer critical to their catalytic activity while NOX5 and the DUOXs retain the catalytic core but without the requirement for the p22phox subunit. The membrane-spanning NOX-p22phox heterodimer allows for the electron transfer from NADPH to bound FAD, two haem moieties, and then to molecular  $O_2$  on the other side of the membrane to generate superoxide or hydrogen peroxide. Whereas the activation of NOX1 and NOX2 requires association of the membrane-bound heterodimer with regulatory subunits (*described below for NOX2*), NOX3, and NOX4 have no such requirement. NOX5 is different from the other NOXs in being calcium-activated via its EF-hand motifs at the N-terminus and not requiring binding to p22phox. The reader is directed to several comprehensive reviews describing in detail the structure and biology of the different NOX isoforms.<sup>7,8</sup> The effects of NOXs in cardiac hypertrophy and failure have also been reviewed.<sup>9,10</sup> Here, we provide a brief overview of NOX2 and NOX4, which are the main isoforms found in the heart (*Figure 1*), and then focus on their roles in the regulation of intermediary metabolism.

NOX2 is normally quiescent but is activated by stimuli such as G-protein coupled receptor agonists (e.g. angiotensin II, endothelin-1), growth factors, cytokines, and mechanical forces in a process that involves the binding of four regulatory subunits (p67phox, p47phox, p40phox, and Rac1) to the NOX2-p22phox complex at the sarcolemma (including T-tubules) of the cardiomyocyte.<sup>11</sup> NOX4 is reportedly found in many intracellular locations, notably the endoplasmic reticulum (ER),<sup>12-14</sup> nucleus,<sup>15</sup> and mitochondria.<sup>16</sup> In cardiomyocytes, NOX4 is mainly localized to the ER<sup>13,17</sup> and possibly mitochondria.<sup>18</sup> More recently, we have identified NOX4 to be present at ER-mitochondrial contacts known as the mitochondrial-associated membrane (MAM),<sup>14</sup> which may have important implications for its functional roles in regulating metabolism as well as accounting for prior reports that it is located in mitochondria.

In contrast to NOX2, NOX4 does not require additional regulatory subunits and is constitutively active. Its activity depends upon its abundance which is increased by a wide variety of agonists (such as angiotensin II, platelet-derived growth factor, tumour necrosis factor alpha, and mechanical forces)<sup>19</sup> and cellular stresses (hypoxia, ER and metabolic stress, serum, and glucose withdrawal) both through transcriptional and post-translational mechanisms.<sup>13</sup> For instance, NOX4 is a transcriptional target of hypoxia-inducible factor 1-alpha (HIF1 $\alpha$ ) during hypoxia<sup>20</sup> and of activating transcription factor 4 (ATF4) during diverse stress situations (*discussed later*). E2F transcription factors also regulate NOX4 expression, at least in vascular smooth muscle cells.<sup>21</sup> There is also a suggestion that epigenetic mechanisms (enhanced histone acetylation) may increase NOX4 expression.<sup>22</sup> Post-translational regulation of NOX4 may occur at the level of its protein stability. For example, the binding of the protein Poldip2 to the NOX4-p22phox heterodimer is

reported to increase its stability and overall ROS-generating activity.<sup>23</sup> How the intracellular localization of NOX4 is achieved still requires more investigation. However, interaction with cellular compartment-specific proteins may be at least part of the mechanism. For example, calnexin has been shown to be a specific NOX4-interacting protein and this may explain its localization to the ER<sup>24</sup> and the MAM.<sup>25</sup> As will be explored in later sections, such specific targeting of NOX4 may be key to determining its role in metabolism.

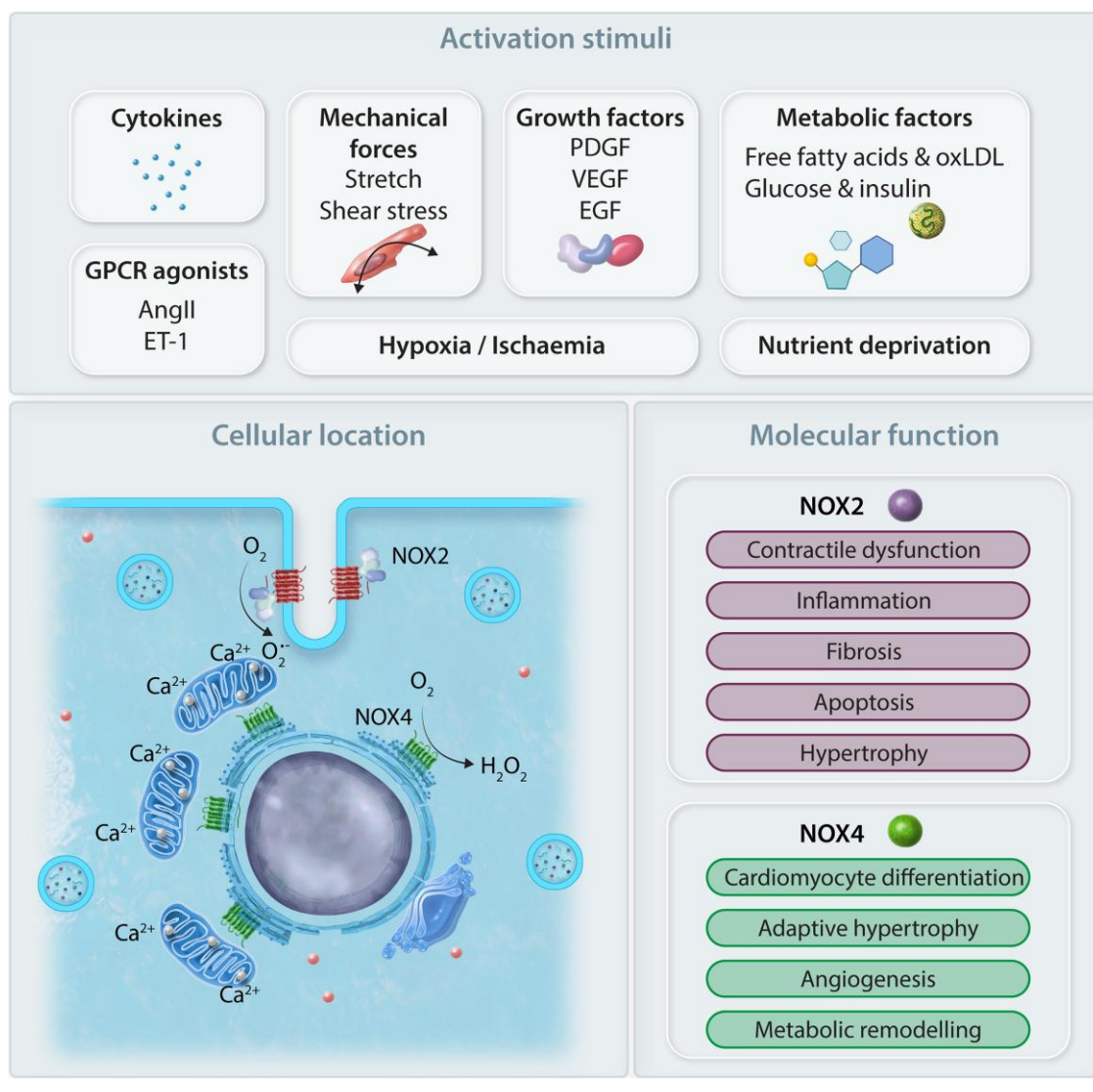
Another important difference between NOX4 and NOX2 is that NOX4 produces predominantly  $H_2O_2$  (a property that appears to have a specific structural basis), whereas NOX2 generates which can then be dismutated to  $H_2O_2$ .<sup>26,27</sup> An especially important implication of this difference is in relation to crosstalk with vascular nitric oxide synthase (NOS) signalling: superoxide reacts rapidly with vasodilator nitric oxide (NO) and inactivates it, whereas  $H_2O_2$  can increase endothelial NOS expression and/or have direct vasodilator actions (*further highlighted in the section on endothelial cells later*). NOX4 produces localized low levels (nanomolar range) of  $H_2O_2$ <sup>14,17,28</sup> that are implicated in the regulation of redox-sensitive cellular processes including cell differentiation, proliferation, and migration.<sup>19</sup> In the heart, changes in the expression level of NOX4 were described as enhancing the differentiation of mouse embryonic stem cells into beating cardiomyocytes<sup>29</sup> although NOX4 knockout mice are born with normal hearts.<sup>17,18</sup> It should be mentioned that NOX4 can exist as splice variants,<sup>30</sup> in particular, NOX4D which is expressed in many cell types and localizes to the nucleus, which distinguishes it from NOX4.<sup>31</sup> Of interest, an observational study of human hearts from ischaemic cardiomyopathy patients indicated a down-regulation of a splice variant consistent with NOX4D.<sup>32</sup> However, the relevance of NOX4D splice variants to cardiac pathology is still largely unknown.<sup>33</sup> It was shown in vascular smooth muscle cells that NOX4D increases the phosphorylation of extracellular-signal-regulated kinase1/2 and the nuclear transcription factor Elk-1 and may play a role in nuclear signalling and DNA damage, effects distinct from NOX4.<sup>31</sup> NOX4D-mediated nuclear ROS production was also reported in cancer cells and COS cells where it may modulate the cell cycle.<sup>34</sup>

NOX2 and NOX4 are both up-regulated during the response to hypertrophic stimuli but appear to have distinct effects in the heart.<sup>35</sup> NOX2 activation broadly speaking drives a detrimental outcome by amplifying pro-fibrotic, pro-hypertrophic, contractile dysfunction, and cell death pathways, whereas NOX4 is associated with activating a complex protective signalling response (*Figure 1*). Among the features of the adaptive cardiac stress response triggered by NOX4 are the activation of a network of pro-survival transcription factors including HIF1 $\alpha$ ,<sup>17,36,37</sup> ATF4,<sup>28</sup> and nuclear factor erythroid 2-related factor 2 (NRF2),<sup>37-41</sup> that may all modulate processes of intermediary metabolism—either individually or collectively.<sup>42</sup>

While the major focus of this review is on the effects of NOXs on cardiac intermediary metabolism, it should be noted that NOXs have important roles in other cardiovascular conditions such as hypertension and atherosclerosis that may also impact the heart. The interested reader is directed to comprehensive reviews of these areas.<sup>7,19,43,44</sup>

## 3. Influence of NOXs on the regulation of cardiac glucose and fatty acid oxidation

Recently, we identified that NOX4 can drive a substantial rewiring of substrate metabolism in the heart which appears, at least in part, to

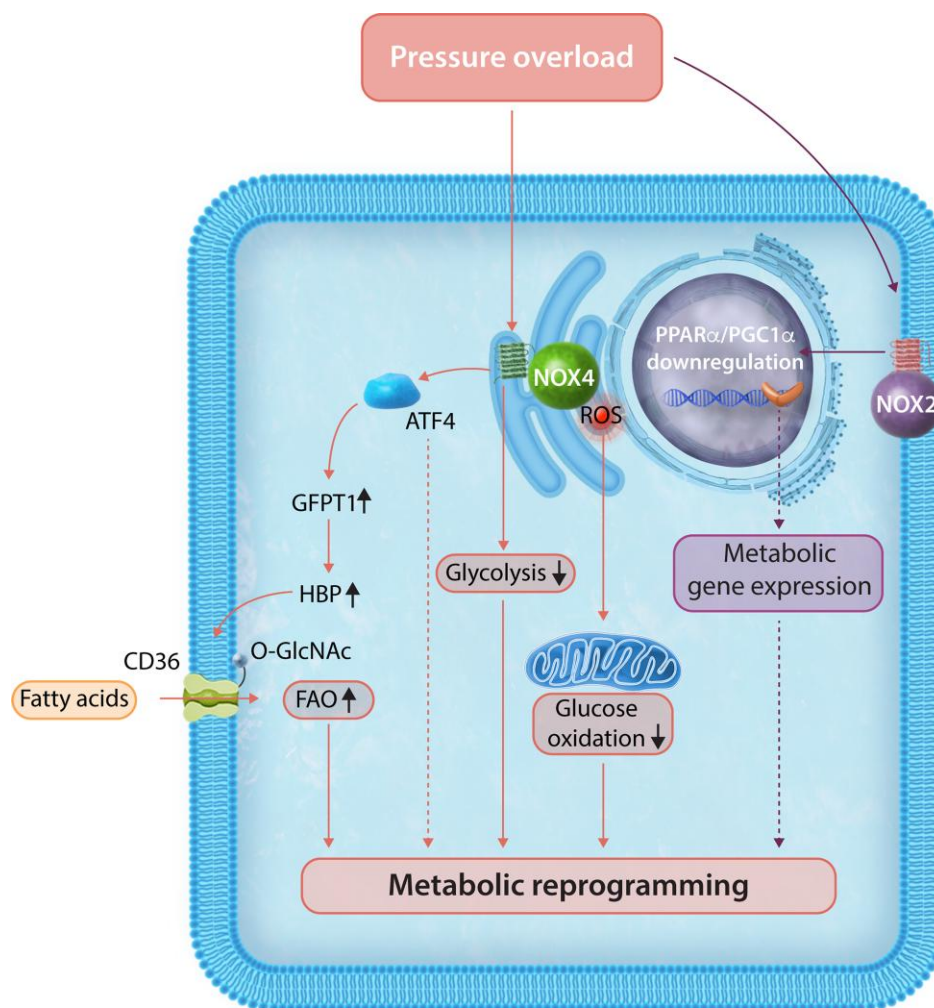


**Figure 1** NOX2 and NOX4 are the major isoforms present in the cardiomyocyte. They are activated by a variety of stimuli (e.g. cytokines, mechanical stretch, growth factors, nutrient deprivation, metabolites) and G-protein coupled receptor agonists such as angiotensin II (Ang II) and endothelin-1 (ET-1). Their intracellular locations are also distinct, with NOX2 being located at the sarcolemma and NOX4 located in intracellular membrane compartments (ER/mitochondria/MAM). Another distinction between isoforms is the ROS produced. NOX2 generates superoxide ( $O_2^-$ ) but NOX4 generates predominantly hydrogen peroxide ( $H_2O_2$ ). It is likely that the differing intracellular locations and ROS-generating properties underlie their functional differences. PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; oxLDL, oxidized LDL.

be transcriptionally mediated and contributes to the adaptive response to chronic haemodynamic overload. We observed that hearts overexpressing NOX4 had reduced glycolysis and glucose oxidation but still had maintained glucose uptake, whereas fatty acid oxidation was significantly increased.<sup>45</sup> Using targeted metabolomics, we found that there was a build-up of proximal glycolytic intermediates along with evidence of an increased flux through the hexosamine biosynthetic pathway (HBP), resulting in an increase in the production of O-GlcNAcylated proteins. This post-translational modification at serine and threonine residues involves the conjugation of a monosaccharide (N-acetylglucosamine) derived from the glycolytic intermediate fructose-6-phosphate and may result in specific modifications of protein function or stability.<sup>46,47</sup> We found that the enhanced HBP activity involves NOX4-mediated activation of ATF4 (discussed further in a later

section), which increases the level of the rate-limiting HBP enzyme, glutamine fructose-6-phosphate aminotransferase 1 (GFPT1).<sup>45</sup> Notably, the NOX4-ATF4-dependent increase in protein O-GlcNAcylation was linked to the increase in fatty acid oxidation observed in these hearts. We found that the cardiomyocyte fatty acid membrane transporter, CD36, was O-GlcNAcylated under these conditions and that this was accompanied by higher fatty acid uptake and oxidation.<sup>45</sup> Therefore, the shift in cardiac energy substrate utilization from glucose to fatty acids, that is, sustained by NOX4 is related, at least in part, to an ATF4-dependent increase in HBP activity (Figure 2).

One of the factors suggested to be involved in the progression from cardiac hypertrophy to heart failure is a decline in fatty acid oxidation that may cause a deficit in ATP production and consequent contractile dysfunction.<sup>48,49</sup> Augmentation of fatty acid oxidation has been



**Figure 2** Pressure-overload activates both NOX2 and NOX4 in the heart. NOX2 down-regulates the transcription factor PPAR $\alpha$  and PGC1 $\alpha$ . This may have subsequent downstream effects on metabolic gene programmes including glucose oxidation and FAO and potential for lipotoxicity. NOX4 augments FAO in cardiomyocytes via an ATF4-dependent mechanism leading to preserved myocardial energetics. Increasing flux through a branch pathway of glycolysis, the HBP, via up-regulation of glutamine-fructose-6-phosphate transaminase 1 (GFPT1) expression leads to the formation of UDP-N-acetylglucosamine (UDP-GlcNAc). UDP-GlcNAc is used by O-GlcNAc transferase (Ogt) to post-translationally modify specific serine and threonine residues of proteins by O-linked-N-acetylglucosaminylation (O-GlcNAcylation). Such O-GlcNAc modification of Cd36/fatty acid translocase results in an increased uptake and metabolism of fatty acids. It should be noted that broad suppression of NOX-derived ROS causes an up-regulation of PPAR $\alpha$  leading to lipotoxicity in the setting of ischaemia–reperfusion injury.

associated with preserving ATP production and ameliorating the effects of pressure-overload-induced failure.<sup>50–52</sup> Our studies of pressure-overload hypertrophy in NOX4 gene-modified mice support this notion. We found that NOX4-overexpressing hearts not only have a higher fatty acid oxidation both at baseline and after chronic pressure overload but also demonstrate preserved cardiac energetics and have better contractile function and less remodelling than their wild-type counterparts.<sup>53</sup> A role of NOX4 in augmenting fatty acid oxidation has subsequently also been observed in macrophages,<sup>54</sup> endothelial cells (ECs),<sup>55</sup> and hepatocytes,<sup>56</sup> suggesting that this may be a more general effect of NOX4.

The transcription factor peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ) regulates genes involved in fatty acid oxidation and a down-regulation of PPAR $\alpha$  is associated with reduced fatty acid

oxidation and a transition to heart failure in pressure-overload-induced cardiac hypertrophy. In the study above,<sup>45</sup> although PPAR $\alpha$  levels were not directly assessed, several genes of fatty acid oxidation (FAO) and their protein expression were found to be down-regulated by NOX4 overexpression despite an increase in FAO rates. Therefore, the NOX4-induced changes in FAO appear unrelated to PPAR $\alpha$ . However, in a study in which ROS production from both NOX2 and NOX4 was genetically suppressed, an increase in PPAR $\alpha$  expression was observed and taken to suggest that basal levels of NOX-derived ROS are required for PPAR $\alpha$  regulation.<sup>57</sup> In this study, overactivity of PPAR $\alpha$  was linked to lipotoxicity after ischaemia–reperfusion injury. Another study showed that NOX2 activation in the heart drives PPAR $\alpha$  down-regulation which in turn leads to maladaptive pressure-overload hypertrophy.<sup>58</sup> Indeed, NOX2-deficient hearts did

not demonstrate a down-regulation of PPAR $\alpha$ , implying that NOX2 is required for down-regulation of PPAR $\alpha$  and subsequent cardiac dysfunction. Intriguingly, the authors noted an interesting crosstalk between NOX2 and PPAR $\alpha$ , finding that PPAR $\alpha$ -deficient hearts failed to show an up-regulation of NOX2 when subjected to pressure-overload hypertrophy, suggesting PPAR $\alpha$  regulates NOX2 *in vivo*. The effect of NOX2/PPAR $\alpha$  crosstalk on downstream intermediary metabolism was not addressed in this study but does suggest that NOX2 may exert inhibitory effects on fatty acid metabolism through modulation of PPAR $\alpha$ .

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1 $\alpha$ ) alongside PPAR $\alpha$  is another major regulator of glucose and fatty acid oxidation with its down-regulation being associated with adverse cardiac remodelling.<sup>59</sup> As described in the next section, NOX2 activation has been associated with the impaired cardiac contractility and mitochondrial dysfunction seen in high-fat diet. A direct assessment of NOX2-dependent changes in substrate handling in hearts has yet to be performed. However, a recent study demonstrated that NOX2 drives activation of Calpain-1 degradation of cardiac Erk5, causing a down-regulation of PGC1 $\alpha$ -mediated increases in both glucose and fatty acid oxidation and leading to lipotoxicity.<sup>60</sup>

Interestingly, in skeletal muscle, bouts of moderate exercise can induce cytosolic ROS production from NOX2, resulting in exercise-induced glucose uptake via GLUT4 translocation.<sup>61</sup> However, it is unknown if NOX2 can regulate cardiac glucose uptake in a similar manner and how this affects downstream intermediary metabolism. In contrast to skeletal muscle NOX2, NOX4 in skeletal muscle ECs was found to be required for metabolic adaptation to chronic exercise, specifically by promoting fatty acid oxidation,<sup>55</sup> supporting findings that NOX4 regulates FAO in multiple cell types.

An increasingly studied area in heart metabolism is the use of ketone bodies for fuel in addition to the well-described metabolism of glucose and fatty acids. It has been observed in the failing heart that ketone bodies are preferentially oxidized for energy. Indeed, a switch to increasing ketone fuels may ameliorate adverse remodelling in the failing heart and has led to the idea of therapeutic ketosis as a treatment for heart failure.<sup>62,63</sup> To date, no studies have specifically looked at the role of NOXs in cardiac ketone body metabolism or how ketone bodies affect NOX activity in the heart. Indirectly, global NOX4 deficiency has been associated with ketonaemia, likely to be through its effects on hepatic metabolism,<sup>56</sup> but the effects on heart biology are unknown.

Taken together, these studies suggest that NOX2 and NOX4 may have opposite effects on FAO in the setting of pressure-overload hypertrophy. Increasing evidence suggests that NOX4 can enhance FAO in a variety of cell types. In contrast, less is known about the role of NOX2 but it seems clear that it can down-regulate cardiac PPAR $\alpha$ /PGC1 $\alpha$  transcriptional activity and affect subsequent utilization of glucose and fatty acid substrates.

## 4. NOXs in metabolic cardiac disease

NOX2 may indirectly affect metabolism, secondary to increases in oxidative stress and imbalance in redox signalling which leads to mitochondrial dysfunction. Recent studies implicate NOX2 in cardiac dysfunction associated with obesity. A feature of obesity-induced cardiac dysfunction is an increase in myocardial O<sub>2</sub> consumption and cardiac inefficiency, and it was found that ROS generation from myocardial NOX2 is an important contributor.<sup>64</sup> The main effect was through increased

non-mechanical work and work associated with excitation–contraction coupling. A postulated mechanism may be the induction of mitochondrial uncoupling, leading to O<sub>2</sub> wasting. The effects of high saturated fat diets and lipotoxicity may in part involve ROS-induced mitochondrial dysfunction, with the consequent development of heart failure. Ablating NOX2 has been shown to reduce high saturated fat and ROS-induced mitochondrial dysfunction in cardiomyocytes.<sup>65</sup> In addition, another study identified that high-fat diet-induced cardiac hypertrophy was driven by NOX2, with deficiency of NOX2 protecting hearts against LVH.<sup>66</sup> Another study in an obesogenic model of cardiac dysfunction and fatty acid overload in cardiomyocytes found that NOX2-ROS impaired autophagy by reducing autophagosome clearance, which then led to cardiac dysfunction.<sup>67</sup> During ischaemia–reperfusion, diabetes and elevated glucose aggravate myocardial injury and cell death. This was found to be improved by inhibiting NOX2 expression. Indeed, NOX2 regulation may be AMPK-dependent as it was shown that increasing AMPK activity could reduce NOX2-dependent oxidative stress and myocardial injury.<sup>68</sup> Recently hyperglycaemia-associated increases in cardiomyocyte ROS were shown to involve the activation of CaMKII by O-GlcNAcylation and a subsequent generation of ROS by NOX2.<sup>69</sup>

There are some reports that NOX4 may aggravate myocardial injury in models of Type 1 diabetes or in cultured cells treated with high glucose although the mechanism was not fully elucidated.<sup>70</sup> On the other hand, endothelial NOX4 expression might also protect against adverse cardiac remodelling in experimental diabetes.<sup>71</sup> Indeed, the diabetic state results in glycated proteins which have been found to induce ROS production from NOX2 rather than NOX4.<sup>72</sup> Interestingly, in a model of diabetes-induced diastolic dysfunction, both NOX2 and NOX4 expression could be attenuated by AAV6-targeted up-regulation of a constitutively active PI3K, leading to a decrease in ROS and protection against diastolic dysfunction.<sup>73</sup> However, it was not clear which isoform was pathological in this setting. In another model of diastolic dysfunction induced by diet-induced hypercholesterolaemia, NOX4 was found to be up-regulated relative to NOX2 in the hearts of cholesterol-fed rats.<sup>74</sup> Furthermore, myocardial microRNA-25 was found to be a direct target for NOX4 and was decreased in cholesterol-fed rat hearts, with *in vitro* studies demonstrating a direct link between reduced microRNA-25, increased NOX4 expression, and oxidative stress. Although microRNAs are also reported to affect NOX2 expression in the heart<sup>75</sup> and could be exploited to protect against NOX2-driven adverse effects after myocardial infarction,<sup>76</sup> the effect on metabolic cardiac disease is not known. MicroRNA-488, a known regulator of NOX2 in the heart, is significantly reduced in obesity and would be of interest to investigate with respect to its effects on NOX2 in metabolic cardiac disease.<sup>77</sup> Overall, the effects of NOX4 are less clear than those of NOX2.

In summary, these studies implicate NOX2 and to a lesser extent NOX4 in metabolic causes of heart failure including obesity, high-fat diet, and diabetes. In general, this appears to involve ROS production induced by the condition (e.g. obesity) and subsequent ROS-induced myocardial injury rather than a direct redox modulation of metabolic pathways.

## 5. NOX, the ISR, and cardiac metabolism

There are numerous published reports that NOX4 is induced during the unfolded protein response (UPR, also known as the ER stress

response).<sup>78,79</sup> This was initially reported in vascular smooth muscle cells during the induction of ER stress and cell death by the oxysterol, 7-ketocholesterol, an oxidized form of LDL, that is, detected at high levels in human atherosclerotic plaques.<sup>80</sup> Other reports include increased NOX4 expression in response to ER stress agonists such as tunicamycin (an inhibitor of *N*-glycosylation) and thapsigargin (which disrupts ER calcium homeostasis) in cardiac myocytes,<sup>28</sup> vascular ECs,<sup>12</sup> and embryonic fibroblasts<sup>28</sup>—suggesting that this may be a universal response across multiple cell types.

The UPR is a highly conserved signalling pathway that senses ER stress—which results in protein misfolding in the organelle—and communicates with the nucleus to promote an adaptive response that rescues folding capacity and restores protein homeostasis.<sup>81–83</sup> Its importance in the heart is underlined by several studies that show perturbing its homeostatic function leads to cardiovascular disease including heart failure.<sup>84</sup> The ultimate effects of UPR activation depend upon multiple factors including the duration and intensity of stress, and may result in cell survival, dysfunction, or death. UPR activation comprises three main limbs: the inositol-requiring enzyme 1 alpha—X-box binding protein 1 (IRE1 $\alpha$ -XBP1), activating transcription factor 6 (ATF6), and protein kinase R (PKR)-like ER kinase/eukaryotic translation initiation factor 2 alpha (PERK/eIF2 $\alpha$ ) pathways. IRE1 kinase activation leads to the splicing of XBP1 mRNA and the formation of XBP1s which has potent transcriptional activity. Activation of the transcription factor, ATF6, during the UPR involves its translocation and proteolysis in the Golgi. In the third limb, PERK activation via autophosphorylation leads to the phosphorylation of the  $\alpha$  subunit of eIF2 $\alpha$ . This, in turn, results in attenuation of global protein synthesis but enhanced translation of a subset of proteins among which the most important is the transcription factor ATF4.<sup>85</sup> Phosphorylation of eIF2 $\alpha$  and increase in ATF4 levels can also be mediated by three other kinases that are activated by diverse stresses unrelated to ER stress, including hypoxia, starvation, viral DNA, and free haem. This overall eIF2 $\alpha$ /ATF4 response is termed the integrated stress response (ISR) and drives an extensive pro-survival transcriptional programme that includes a broad range of metabolic pathways such as phospholipid synthesis and increased amino acid biosynthesis and transport.<sup>86</sup>

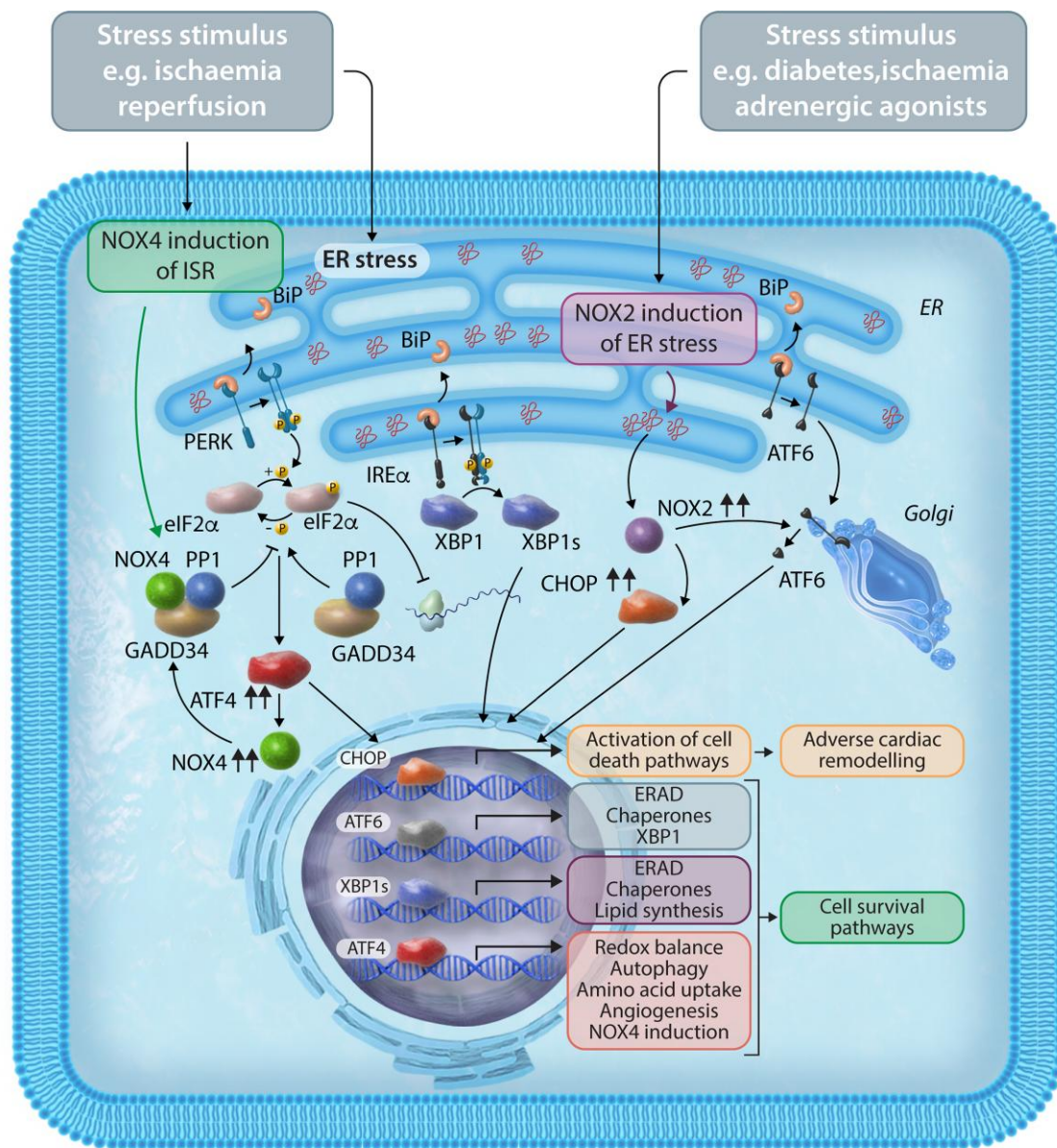
Recently, we identified an obligatory role for NOX4 in enhancing eIF2 $\alpha$ -ATF4 activation through an intricate localized redox signalling pathway at the ER.<sup>28</sup> While UPR signalling was evoked by ER stress agonists in cardiac cells (as well as embryonic fibroblasts), the effects of NOX4 were interestingly restricted predominantly to the eIF2 $\alpha$ -ATF4 limb of the UPR (Figure 3). We found using super-resolution microscopy and cellular fractionation that NOX4 is located predominantly at the ER and that its mRNA levels are increased, in part, via the transcriptional effects of ATF4. The mechanism by which NOX4 augments eIF2 $\alpha$  phosphorylation involves the redox inhibition of protein phosphatase-1 (PP1), which normally dephosphorylates eIF2 $\alpha$ . We found that NOX4 interacts with the protein, GADD34, which binds to and targets PP1 specifically to the ER. Through the proximity to ER-related PP1 via its binding to growth arrest and DNA damage-inducible 34 (GADD34), NOX4 mediates oxidation of the PP1 metal centre to inactivate the enzyme—a mechanism that contrasts to the cysteine oxidation, that is, the well-established mechanism of redox inhibition of protein tyrosine phosphatases. Notably, this localized inhibition of ER-located PP1 means that other PP1 targets in the cell are unaffected. The consequence of PP1 inhibition at the ER is to sustain eIF2 $\alpha$  phosphorylation and ATF4 translation. Thus, NOX4 acts both upstream and downstream of ATF4 to mediate a positive feedback loop to eIF2 $\alpha$ -ATF4 signalling.<sup>28</sup>

This NOX4-eIF2 $\alpha$ -ATF4 pathway not only enhanced cardiac cell survival in response to ER stress agonists but also mediated cardioprotection and cell survival during heart ischaemia–reperfusion, indicating that NOX4 mediates a broader regulation of the ISR. Overall, this is one of the first detailed mechanisms to be elucidated through which NOX4-dependent compartmentalized redox regulation mediates stress adaptation.<sup>28</sup>

NOX4-dependent regulation of the ISR is likely to have multiple effects on intermediary metabolism. In the study discussed above, among the genes regulated via NOX4-eIF2 $\alpha$ -ATF4 signalling were several involved in the transmembrane transport and synthesis of amino acids such as asparagine and glycine.<sup>28</sup> In ECs, we have shown that an important ATF4 target gene is cystathionine gamma-lyase which acts in the trans-sulfuration pathway that converts cystathionine derived from methionine into cysteine.<sup>87</sup> The downstream consequences of the modulation of this pathway may include altered cellular glutathione synthesis (which requires cysteine) and therefore redox state, as well as altered production of the signalling molecule hydrogen sulphide which is produced via the action of cystathionine gamma-lyase.<sup>87</sup> NOX4 was also found to regulate the flux of homocysteine to cysteine in the liver (which will then lead to increased glutathione synthesis) although it is not clear whether this is ATF4 regulated.<sup>88</sup> In a study investigating cardioprotective effects evoked by aldehyde stress in an *in vivo* mouse model subjected to ischaemia–reperfusion,<sup>89</sup> it was reported that ATF4 mediated reprogramming of glucose catabolism to increase pentose phosphate pathway (PPP) sugar intermediates and glutathione synthesis—thereby conferring resistance against acute oxidative stress. However, it remains to be established whether NOX4 is involved in this response.

Another important response may be the triggering of autophagy which itself is intricately linked with alterations in cardiac metabolism.<sup>90</sup> In cardiac myocytes subjected to glucose deprivation, it was shown that NOX4 levels increase in the ER and enhance eIF2 $\alpha$ /ATF4 signalling to induce autophagy.<sup>13</sup> This is in line with prior data that key genes involved in autophagy, such as Unc-51 like autophagy activating kinase 1 (ULK1) and Beclin-1, are transcriptional targets of ATF4 and stimulate autophagy in other cells and tissues such as fibroblasts<sup>91</sup> and cancer cells.<sup>92</sup> NOX4-ATF4-dependent enhancement of autophagy appears to be an important protective pathway in the heart as it was found to mediate cardioprotection in response to ischaemia–reperfusion in mice.<sup>13,28</sup> As mentioned in the previous section, NOX2-ROS can impair autophagy leading to cardiac dysfunction in the setting of obesity<sup>67</sup>—illustrating another instance where the two NOX isoforms have contrasting effects.

In contrast to NOX4, NOX2 may impair myocardial remodelling due to activation of ER stress (Figure 3). In the context of diabetes, adverse cardiac remodelling was abrogated by Rac1 deletion in cardiomyocytes leading to less NOX2 activation and PERK/ATF-6 induced ER stress.<sup>93</sup> In a rabbit model of myocardial infarction, remote myocardial NOX2 expression was found to be increased, in association with increased oxidative stress, elevated C/EBP homologous protein (CHOP), and cleaved ATF6. All the changes were attenuated with apocynin treatment, a non-specific inhibitor of NOX activity, suggesting an association between NOX2, ER stress, and impaired remote myocardial remodelling post-MI.<sup>94</sup> In cultured cardiomyocytes treated with phenylephrine, either the silencing of NOX2 or its pharmacological inhibition resulted in attenuation of the ER stress response—indicating that phenylephrine-induced ER stress was NOX2 dependent.<sup>95</sup> Previous studies in macrophages and kidney tissue also demonstrate that ER stress itself can lead to ROS production via NOX2, which in turn can



**Figure 3** ER stressors activate/induce both NOX2 and NOX4 but lead to different effects on cell fate. ER stress activates the UPR through the interaction of molecular chaperones like BiP with specific pathways that can lead to either cell death or cell survival. NOX2 induces ER stress leading to apoptosis in response to a variety of ER-stress stimuli including diabetes, ischaemia, and adrenergic agonist stimulation. NOX2 can increase levels of CHOP which signals to activation of cell death pathways and adverse cardiac remodelling. NOX2 can also induce ATF6 at least in chronic hyperglycaemic conditions. During ischaemia–reperfusion, ER-located NOX4 can specifically activate the ISR leading to the activation of cell survival pathways. Specifically, NOX4 mediates oxidation of the PP1 metal centre to inactivate the enzyme and sustain eIF2 $\alpha$  phosphorylation and ATF4 activation (eIF2 $\alpha$ -ATF4 limb). This is independent of the other arms of the UPR, namely the IRE1 $\alpha$ -XBP1 pathway (shown for illustration) or the ATF6 pathway. ERAD, ER-associated protein degradation; GADD34, growth arrest and DNA damage-inducible 34; BiP, binding immunoglobulin protein.

further potentiate CHOP activity leading to apoptosis.<sup>96</sup> The authors found this was specific for a non-PERK-activated phospho-eIF2 $\alpha$  pathway rather than global ER stress. Such a NOX2-driven activation of the UPR/apoptosis pathways may be relevant in the heart, but further studies are required.

In summary, the relationship between NOXs and the ISR is complex. There is an emerging picture for isoform-specific effects, with NOX4-ATF4 pathways driving a cytoprotective ISR through intricate redox signalling and with widespread effects on intermediary

metabolism and autophagy. In contrast, NOX2 may enhance ER stress and CHOP-mediated adverse cardiac remodelling.

## 6. NOX enzymes and metabolic regulation during hypoxia

It is well established that HIF1 $\alpha$  increases the expression of glycolytic enzymes such as phosphoglycerate kinase 1, glyceraldehyde 3-phosphate



dehydrogenase, and lactate dehydrogenase (LDH) during hypoxia<sup>97</sup> or ischaemia of the heart.<sup>98,99</sup> HIF1 $\alpha$  is also a key regulator of intermediary metabolism in the heart during pathological hypertrophy, directly regulating glycolysis and other aspects of lipid intermediary metabolism.<sup>100</sup> Numerous studies indicate that NOX4 can regulate HIF activity. Similar to the regulation of ATF4, NOX4 may be both downstream and upstream of HIF. HIF1 $\alpha$  transcriptionally activates NOX4 in response to hypoxia in vascular cells,<sup>20</sup> whereas in renal cell carcinoma NOX4 may help maintain HIF2 $\alpha$  expression.<sup>101</sup> However, NOX4 can also regulate HIF protein levels through the inhibition of prolyl hydroxylase-2 (PHD2, which regulates HIF stability). We found that NOX4-PHD2-dependent up-regulation of cardiomyocyte HIF1 $\alpha$  induces vascular endothelial growth factor-mediated adaptive proangiogenic signalling during cardiac hypertrophy.<sup>17</sup> Similar NOX4-mediated up-regulation of HIF signalling is also reported in microvascular ECs after ischaemia/reperfusion,<sup>102</sup> in pulmonary artery smooth muscle cells,<sup>103</sup> and in cerebellar progenitor cells.<sup>104</sup> In ECs subjected to disturbed flow, it was recently shown that HIF activation downstream of NOX4 drives metabolic reprogramming involving an increase in glycolysis and a decrease in mitochondrial respiratory capacity due to increased expression of pyruvate dehydrogenase kinase-1.<sup>105</sup> It will be of interest in future studies to also examine the paracrine crosstalk between cardiomyocytes and cardiac ECs during adaptive responses to stress since NOX4-HIF1 $\alpha$  signalling occurs in both cell types.<sup>106</sup>

In cardiomyocytes, hypoxia-induced generation was found to be NOX2 mediated, with its inhibition preventing hypoxia-induced apoptosis.<sup>107</sup> In this study, NOX2 deficiency achieved by adenoviral antisense NOX2 delivery into cardiomyocytes also attenuated hypoxia-induced HIF1 $\alpha$  expression. NOX2 can also potentiate HIF signalling in several other cell types. For example, NOX2 activates HIF1 $\alpha$  and enhances glycolysis in M1 macrophages.<sup>108</sup> Part of the mechanism is thought to involve protein tyrosine oxidation and AKT activation causing an increase in glucose uptake. Of note, PHD2 regulation of HIF activity is important in determining macrophage metabolism and function.<sup>109</sup> In human ECs, NOX2 was found to promote angiogenesis via activation of HIF1 $\alpha$ .<sup>110</sup> In this study, urotensin-II, an angiogenic factor, was found to generate ROS, which could be ablated by inhibiting NOX2 expression. Interestingly, depletion of NOX4 levels unlike NOX2 was less effective in reducing ROS levels in response to urotensin-II. Indeed, vessels from NOX2-deficient mice had a diminished capacity to form new vessels upon urotensin-II stimulation. Interestingly, urotensin-II treatment increased HIF1 $\alpha$  expression which when inhibited reduced NOX2 levels, whereas augmenting HIF1 $\alpha$  increased NOX2 levels and the associated ROS production. Another interesting finding was that NOX2 itself can regulate HIF1 $\alpha$  induced by urotensin-II. The authors conclude that NOX2 is both a HIF1 $\alpha$  target gene and is required for increasing HIF1 $\alpha$  levels. So as with NOX4, NOX2 can also be upstream and downstream of HIF activation. Studies are required to specifically examine the effect of such activation on intermediary metabolism.

## 7. NOX4 and the activation of NRF2

The transcription factor NRF2 may be activated by oxidative or electrophilic stress as well as several endogenous mediators (e.g. NO, nitro-fatty acids).<sup>111,112</sup> NRF2 can induce an extensive array of genes involved in cytoprotection, among which are many that are involved in glutathione biosynthesis and several other intermediary metabolic pathways. In other organs, NRF2 regulates intermediary metabolism through

glycolytic branch pathways such as the PPP.<sup>113</sup> In the liver, NRF2 can alter lipogenic as well as gluconeogenic and glycolytic pathways leading to fatty liver disease.<sup>114,115</sup> In cancer cells, NRF2 activation is linked to increased expression of PPP enzymes and tumour progression.<sup>116,117</sup>

Although NRF2 may in principle be activated by any source of ROS, intriguingly, we have found that endogenous NOX4 is an obligatory activator of NRF2 in the heart both during pathological cardiac hypertrophy<sup>38</sup> and acute physiological exercise.<sup>39</sup> As such, NOX4 knockout mice subjected to chronic pressure overload failed to up-regulate the NRF2 pathway and diverse antioxidant and glutathione biosynthetic genes. This was accompanied by evidence of increased oxidative stress and increased mitochondrial DNA damage, indicating that NOX4 regulates redox state in this setting via the up-regulation of NRF2.<sup>38</sup> The importance of NOX4 in NRF2 activation is not limited to the heart but also evident in mouse embryonic fibroblasts and renal proximal tubular cells.<sup>118</sup> Intriguingly, there may be crosstalk between NOX2 and NOX4 with down-regulation of NOX4 and NRF2 pathways and up-regulation of NOX2 and NF- $\kappa$ B pathways evident in the hearts and kidneys of animal models with diabetes-induced oxidative stress.<sup>40</sup> In contrast to these reports, a study in human lung fibroblasts suggested that NOX4 may inhibit NRF2 activation but the mechanism underlying this surprising finding was not defined.<sup>119</sup>

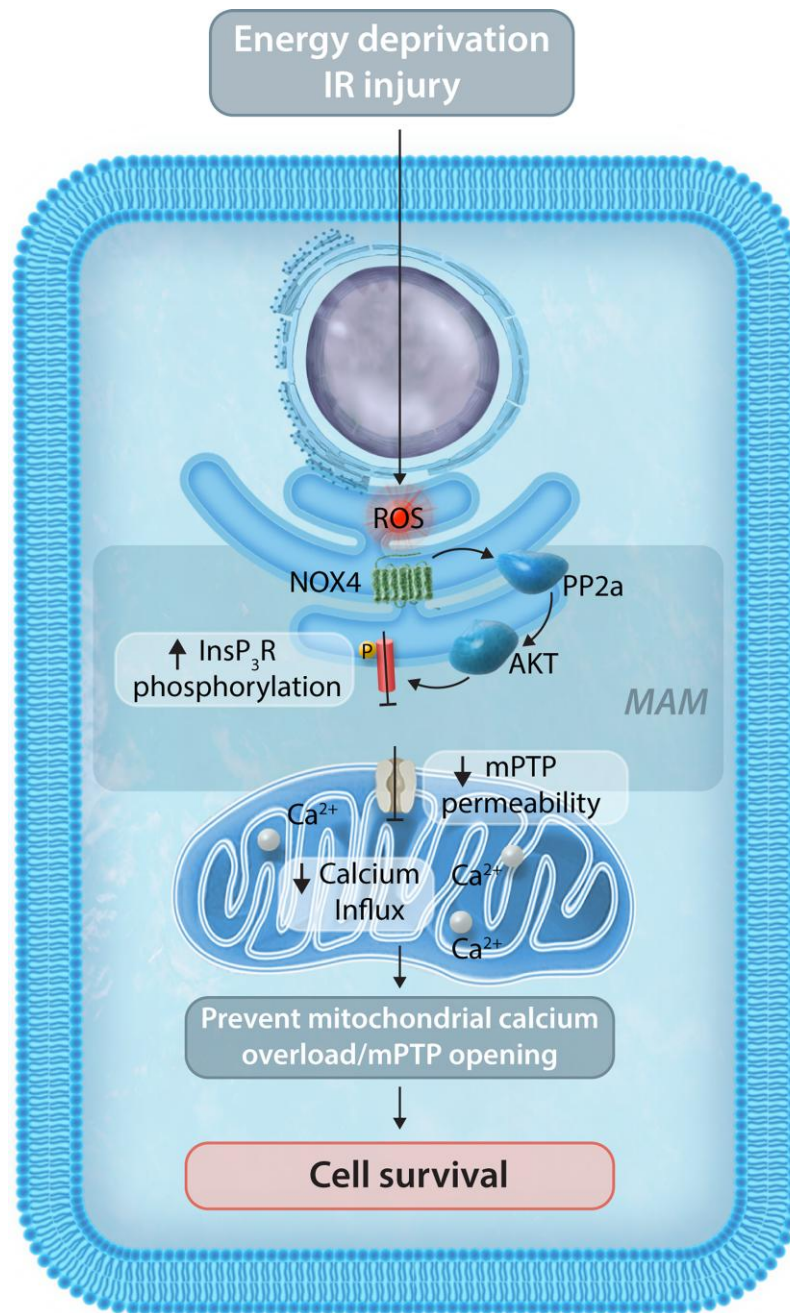
NOX4 is also crucial in maintaining a normal mitochondrial redox state during repeated acute physiological exercise. We found that mice with either a global or cardiac-specific deficiency of NOX4 had a reduced maximal exercise capacity and impaired cardiac contractile function during treadmill running.<sup>39</sup> The deficiency of NOX4 in the heart led to impaired activation of NRF2, which in turn resulted in an impaired mitochondrial redox state and respiration during acute exercise. A similar phenotype was observed in mice with a cardiac-specific deficiency of NRF2.<sup>39</sup> Either enhancing NRF2 activation with sulforaphane or augmenting mitochondrial antioxidant capacity by administration of the mitochondrial-targeted antioxidant, Mito-Q, was able to rescue the impaired exercise phenotype in these animals. Therefore, maintaining mitochondrial redox balance via the NOX4-NRF2 axis is a key component of ensuring that the heart can maintain cellular energetics for contractile function during peak exercise. Beyond the maintenance of redox state, whether NOX4 modulates intermediary metabolism or other NRF2-dependent effects in the heart is an interesting question for future work.

Much less has been published regarding the interplay of NOX2 and NRF2. A high fructose diet has been linked to the development of cardiometabolic disease and in a murine model of high fructose diet, myocardial NOX2 in contrast to NOX4 expression was found to be elevated alongside increased hypertrophy and reduced NRF2/KEAP1 expression.<sup>120</sup> Exercise attenuated NOX2 expression, increased NRF2/KEAP1 ratio, and reduced fructose-mediated hypertrophy.

## 8. NOX4-dependent regulation of mitochondrial calcium homeostasis and cell viability

Recent work indicates an important role for NOX4 in regulating ER to mitochondrial calcium transfer and, through this mechanism, modulating a key cellular pro-survival mechanism.

We demonstrated the enrichment of NOX4 at the mitochondria-associated membranes (MAM) in several tissues including the heart and human-induced pluripotent stem cell-derived cardiomyocytes.<sup>14</sup>



**Figure 4** The MAM represents a unique compartment enabling NOX4-localized ROS signalling and ER-mitochondrial calcium communication. Serum starvation or ischaemia–reperfusion (IR) injury can lead to mitochondrial calcium overload and cell death but an increase in NOX4 levels at the MAM prevents excessive calcium transfer between the ER and mitochondria, thereby inhibiting opening of the mitochondrial permeability transition pore and cell death. Mechanistically, NOX4 results in the enhancement of MAM-located Akt activity via a redox inhibition of the phosphatase PP2a. Akt in turn is able to phosphorylate ER InsP<sub>3</sub>R channels which decrease the release of calcium from the ER to the mitochondria.

These sites of contact between the ER and mitochondria are a critical crosstalk platform between the organelles and thus are pivotal in regulating diverse cellular functions, including mitochondrial calcium homeostasis, metabolism, and cell survival.<sup>121</sup> During severe cell stresses, the MAM may mediate substantial calcium transfer into the mitochondria, resulting in calcium overload and the triggering of mitochondrial permeability transition pore-dependent regulated cell death. The control of

this pathway is therefore critical in determining cell survival during many stresses, for example, myocardial ischaemia–reperfusion. We found that NOX4 levels at the MAM increase in response to serum starvation of cardiomyocytes and abrogate calcium transfer from the ER to mitochondria by inhibiting calcium release through the ER inositol triphosphate receptor channels (InsP<sub>3</sub>R).<sup>14</sup> This in turn prevents mitochondrial calcium overload and the mPTP and enhances cell survival.

The mechanism underlying NOX4-mediated inhibition of  $\text{InsP}_3\text{R}$  involves an enhancement of MAM-located Akt activity via a redox inhibition of the phosphatase PP2a (which normally dephosphorylates Akt). Akt is known to inhibit  $\text{InsP}_3\text{R}$  via phosphorylation and the increase in Akt activity, therefore, abrogates calcium release (see Figure 4). This is another example of NOX4-mediated specific compartmentalized redox signalling. NOX4-mediated inhibition of calcium overload-induced cell death was found to be important in driving protective effects against ischaemia–reperfusion injury in hearts, suggesting a key role for MAM-localized NOX4 signalling in mediating cytoprotective functions. Mitochondrial calcium levels are also a key determinant of mitochondrial metabolism but whether NOX4 modulates this aspect under physiological conditions has not yet been studied.

It should be noted that studies in which NOX4 was overexpressed in cardiac cells have reported that the consequent increase in ROS production may lead to mitochondrial dysfunction and cell death, i.e. effects opposite to those described above.<sup>122</sup> These apparently discrepant findings may be reconciled by considering that specific compartmentalized signalling (e.g. at the MAM) is dependent upon local low-level ROS production, whereas the overexpression of unphysiologically high levels of NOX4 and ROS will have non-specific damaging effects. Consistent with this idea, it was shown that complete abrogation of NOX-derived ROS production was detrimental during myocardial ischaemia–reperfusion.<sup>57</sup>

The role of NOX2 in mitochondrial calcium homeostasis and cell viability has been studied in sepsis-induced cardiomyopathy.<sup>123</sup> LPS-induced ROS production requires NOX2 activation. The associated reduction in myocardial contractility could be reversed by NOX2 inhibition which was found to impair sarcoplasmic calcium release. Furthermore, the authors found that *in vivo* LPS-induced myocardial expression of mitochondrial metabolic genes including *Ppara*, *Pgc1a*, and *Cpt1b* was down-regulated, which could be normalized by treatment with apocynin—suggesting NOX2 directly impairs not just calcium handling but also mitochondrial function and metabolism. How NOX2 effects are communicated between ER calcium and mitochondrial function remains unclear in this study.

## 9. NOX and EC metabolism

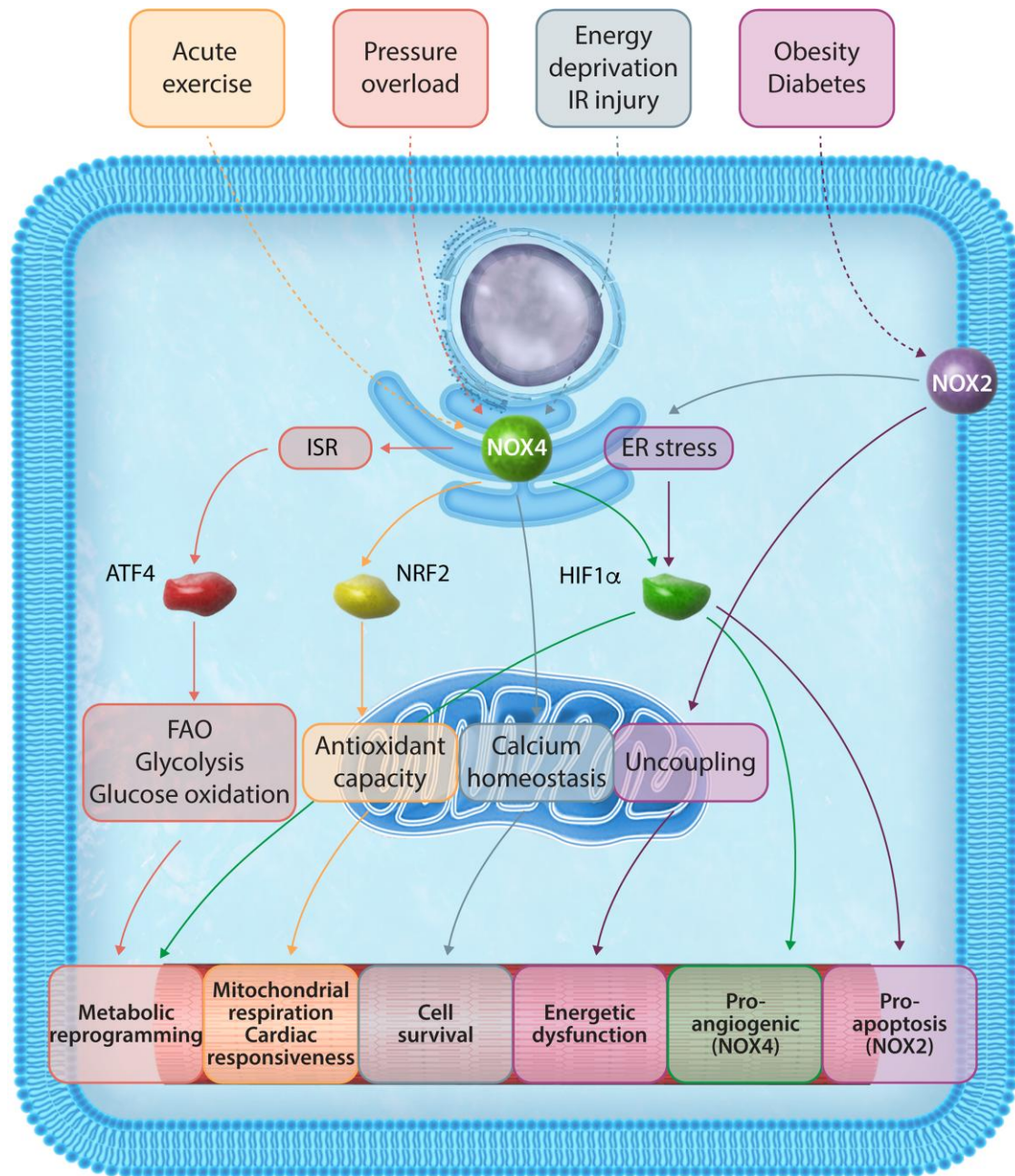
EC function is intricately linked with cardiac function both by affecting myocardial perfusion and through paracrine crosstalk with cardiomyocytes. EC dysfunction contributes to the pathophysiology of heart failure,<sup>124</sup> myocardial ischaemia,<sup>125</sup> and diabetic cardiomyopathy.<sup>126</sup> NOX enzymes are considered to be the main sources of ROS in ECs. NOX4 is the major isoform expressed in ECs but these cells also express NOX2 and NOX1 (and NOX5 in humans).<sup>127,128</sup> It is known that ECs rely on glycolysis rather than oxidative metabolism for the majority of their ATP (in contrast to cardiomyocytes).<sup>129</sup> Indeed, during proliferation, ECs are able to rewire their intermediary metabolism to support growth, including increasing glycolytic flux toward branch pathways such as the PPP and HBP, and harnessing FAO to provide TCA intermediate for the generation of nucleotides.<sup>130</sup> Both NOX2 and NOX4 have been associated with ROS generation and EC proliferation.<sup>131</sup> As described in the section on hypoxia, NOX enzymes are also important in regulating EC HIF activity. It is tempting to speculate that NOX activity may be required for the metabolic flexibility of these cells during proliferation, but direct data on this question are lacking. In diabetes, both NOX1 and NOX2 are considered important mediators of vascular dysfunction.<sup>132</sup> This is in part thought to be due to increased

oxidative stress arising from hyperglycaemia induced activation of NOX2. Oxidative stress has subsequent downstream effects on inhibiting glycolysis, increasing proximal metabolites, and feeding into branch pathways and causing defects in EC behaviour.<sup>133</sup> In contrast to NOX2, NOX4 has a vasoprotective role by maintaining EC function.<sup>134</sup> This difference is likely to be related to the generation of  $\text{H}_2\text{O}_2$  by NOX4 (whereas NOX2 generates superoxide) and subsequent localized  $\text{H}_2\text{O}_2$ -specific signalling which may mediate downstream vasoprotective signalling.<sup>132</sup> NOX2-derived superoxide reacts with and inactivates NO (also generating peroxynitrite) and may uncouple endothelial eNOS, resulting in amplification of production. In contrast, NOX4-derived  $\text{H}_2\text{O}_2$  does not induce these detrimental effects, especially if  $\text{H}_2\text{O}_2$  levels are not supra-pharmacological.<sup>37</sup> Moreover,  $\text{H}_2\text{O}_2$  can have direct vasodilator activity, can activate protein kinase G to stimulate vasodilation,<sup>135</sup> can increase eNOS expression, and can stimulate eNOS activity—all actions that may contribute to vasoprotective activity.<sup>37,136</sup> Such effects are evident in NOX4-deficient mice where the development of atherosclerosis is enhanced in high-fat diet<sup>137</sup> and diabetic models.<sup>138</sup> NOX4 has also been shown to mediate anti-inflammatory properties in the vasculature and the heart, which are most likely underpinned by  $\text{H}_2\text{O}_2$ -specific signalling, and contrast with the pro-inflammatory effects of enhanced NOX2 signalling.<sup>137–139</sup> These effects of NOX4- $\text{H}_2\text{O}_2$  may be exerted not only at the level of ECs but may also involve the direct modulation of macrophage polarization and phenotype.<sup>140,141</sup>

Given the intricate links between ROS biology and intermediary metabolism, the role of NOX-ROS in regulating intermediary metabolism in ECs is ripe for further exploration. Furthermore, the crosstalk between ECs and cardiomyocytes may be an important mechanism through which cardiac metabolism is also affected, for example, in metabolic cardiac disease.<sup>142,143</sup>

## 10. Summary of NOX effects on cardiac intermediary metabolism

The roles of NOX enzymes in redox signalling pathways in the heart are quite well characterized. However, only recently have the effects of NOXs in regulating cardiac metabolism in the heart emerged (Figure 5). NOX4 appears to be a crucial component of cellular stress responses and its effects are typically mediated by highly specific and compartmentalized redox signalling dependent both on its subcellular location and the repertoire of proteins that it interacts with under different stress situations. It is notable that such signalling involves the modulation of levels of several transcription factors, including NRF2, ATF4, and HIF1 $\alpha$ . Each of these stress-activated transcription factors have direct effects on intermediary metabolism pathways, at least some of which have been found to be regulated by NOX4. In addition, NOX4 may exert metabolic effects through other pathways such as the regulation of ER-to-mitochondrial calcium transfer during severe cell stress. Interestingly, some NOX4 effects are important in physiological settings. For instance, NOX4 is crucial for the hormetic activation of NRF2 in response to acute exercise, with an important impact on mitochondrial redox balance and function and thus exercise capacity. This may comprise a novel physiological mechanism underpinning the benefits of repetitive exercise on mitochondrial 'fitness' via NOX4 activation. On the other hand, during chronic stresses such as pressure-overload or ischaemia–reperfusion, NOX4-dependent ATF4 activation drives a broader array of adaptive changes. These include augmentation of fatty acid oxidation with the preservation of energetics, and cytoprotective



**Figure 5** Both NOX2 and NOX4, the main NOX isoforms in cardiomyocytes, can regulate intermediary metabolism in response to a variety of stresses. Several mechanisms are involved, including activation of transcription factors or in the case of NOX4 location at the MAM, targeted ROS signalling to influence mitochondrial function and cell viability. ATF4, activating transcription factor 4; NRF2, nuclear factor erythroid factor 2-related factor 2; HIF1 $\alpha$ , hypoxia-inducible factor 1-alpha; ISR, integrated stress response.

responses such as the ISR and autophagy. NOX2-mediated effects on metabolism may be especially relevant in obesity, associated with a pro-inflammatory/pro-oxidative response that impairs mitochondrial function. NOX2-ROS generation may be important in driving detrimental ER stress during metabolic cardiac disease. Emerging work also indicates that NOX2 can influence (down-regulate) PPAR $\alpha$  and PGC1 $\alpha$ , with consequent effects on fatty acid metabolism. However further work is needed to establish how NOX2 regulates these factors, including characterization of the downstream effects on cardiac metabolic pathways.

## 11. Implications and avenues for further research

Intermediary metabolism directly influences several vital functions in tissues. The regulation of NADP/NADPH balance impacts not only on redox state through the regeneration of glutathione but also affects numerous metabolic pathways (e.g. lipid synthesis). Many intermediary metabolic pathways are important in generating cellular building blocks for biosynthesis, such as amino acids, nucleotides, and lipid precursors.

They also generate metabolites that may act as signalling molecules.<sup>144</sup> While the intricacies of these functions have been increasingly established in cancer and other tissues, they remain relatively underexplored in the heart. Alterations in redox state are well established as being an important determinant of chronic changes in cardiac function, for example, during chronic haemodynamic overload or in response to ischaemia–reperfusion. However, less is known about the other aspects of intermediary metabolism. Conceptualization of the heart as a balance between catabolic and anabolic states may provide a useful framework for considering changes such as cardiac remodelling in response to chronic physiological or pathological stress.<sup>1</sup> It is evident that for the developing or remodelling heart, there will be a need to maintain both growth and contractile function to maintain cardiac output. The regulation of intermediary metabolic pathways may be particularly important in achieving this state.<sup>145</sup>

It is intriguing that NOX enzymes, and NOX4 in particular, appear to impact intermediary metabolic pathways during cardiac stress responses. While the studies to date have begun to outline the upstream components of NOX4-dependent signalling, fully dissecting the links to chronic changes in cardiac structure and function requires further work. NOX4-dependent modulation of protein O-GlcNAcylation is especially interesting in relation to intracellular signalling, with prior data in the literature that it may contribute to hypertrophic signalling.<sup>146</sup> The broader effects of NOX4-dependent ATF4 activation in the heart are also important to elicit, given the evolutionary significance of the ATF4-regulated ISR. Likewise, it will be important to establish how NOX4-mediated regulation of NRF2 fits within the wider scheme of NRF2 regulation and functions in the heart. It is of interest that NOX4 has been associated with driving metabolic alterations in other cell types including cancer cells<sup>16,147</sup> and macrophages,<sup>54</sup> suggesting that it could have a fundamental role in modulating cellular intermediary metabolism. Integrative approaches that combine, for example, transcriptomic and proteomic data with metabolic fluxomics are likely to be especially informative in providing a more global picture of the effects in the heart.

It should be recognized that the heart is made up of multiple cell types including cardiomyocytes, fibroblasts, ECs, and immune cells. While we have taken a predominantly cardiomyocyte-centric view in this review, metabolic crosstalk between different cell types may be important in the heart—analogueous to emerging data in cancer.<sup>148</sup> It will be important to develop further research in this area to understand cell-specific effects and the overall impact on cardiac remodelling.

It is probably premature to speculate on the global therapeutic implications of the research findings to date. However, some aspects such as modulation of fatty acid oxidation in the stressed heart or the prevention of mitochondrial calcium overload have obvious relevance to heart failure and ischaemia–reperfusion, respectively. Understanding the role of NOXs in other aspects of substrate metabolism such as ketone body utilization would also be of importance since this is an area of significant therapeutic interest. Given the distinct signalling networks that appear to be activated by NOX2 vs. NOX4, targeting specific isoforms or part of the downstream networks is a potentially attractive proposition to pursue. In this regard, it should be noted that developing specific pharmacological inhibitors of individual NOX isoforms has been challenging to date. Furthermore, the desired aim in many cases might be to enhance rather than inhibit NOX4 signalling. We believe a more comprehensive elucidation of the complex networks regulated by the NOX isoforms and how this affects intermediary metabolism will clarify what are the optimal therapeutic targets to focus on.

## 12. Conclusions

Recent insights into the regulation of key transcription factors (such as NRF2, ATF4, and HIF1 $\alpha$ ) as well as mitochondrial processes by NOX enzymes suggest that these enzymes have an important role in regulating metabolism, and especially intermediary metabolism, in the heart. The effects of NOX4 are notable in involving highly specific and compartmentalized redox signalling within cells, even extending to physiological settings. These data not only highlight a further facet of NOX biology but also suggest new avenues for research to understand the complexity of chronic changes in cardiac structure and function in an effort to define new therapeutic opportunities for heart disease.

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## Data availability

Data sharing not applicable – no new data generated.

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