NOX family NADPH oxidases in liver and in pancreatic islets: a role in the metabolic syndrome and diabetes?

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Abstract

The incidence of obesity and non-esterified (‘free’) fatty acid-associated metabolic disorders such as the metabolic syndrome and diabetes is increasing dramatically in most countries. Although the pathogenesis of these metabolic disorders is complex, there is emerging evidence that ROS (reactive oxygen species) are critically involved in the aberrant signalling and tissue damage observed in this context. Indeed, it is now widely accepted that ROS not only play an important role in physiology, but also contribute to cell and tissue dysfunction. Inappropriate ROS generation may contribute to tissue dysfunction in two ways: (i) dysregulation of redox-sensitive signalling pathways, and (ii) oxidative damage to biological structures (DNA, proteins, lipids, etc.). An important source of ROS is the NOX family of NADPH oxidases. Several NOX isoforms are expressed in the liver and pancreatic β-cells. There is now evidence that inappropriate activation of NOX enzymes may damage the liver and pancreatic β-cells. In the context of the metabolic syndrome, the emerging epidemic of non-alcoholic steatohepatitis is thought to be NOX/ROS-dependent and of particular medical relevance. NOX/ROS-dependent β-cell damage is thought to be involved in glucolipotoxicity and thereby leads to progression from the metabolic syndrome to Type 2 diabetes. Thus understanding the role of NOX enzymes in liver and β-cell damage should lead to an increased understanding of pathomechanisms in the metabolic syndrome and diabetes and may identify useful targets for novel therapeutic strategies.

Introduction

From obesity to the metabolic syndrome to Type 2 diabetes: down a slippery road

A worrying consequence of contemporary lifestyles associated with excessive caloric intake and lack of energy expenditure is an increasing accumulation of body fat. The incidence of obesity is increasing dramatically in virtually all societies of the world, and with it comes a cohort of diseases such as the metabolic syndrome and diabetes.

From obesity to the metabolic syndrome

The relationship between obesity and the metabolic syndrome is complex. Indeed, the severity of insulin resistance, a key feature of the metabolic syndrome, varies widely among obese people [1]. Nevertheless, both glucose and fatty acids may cause adaptive or toxic actions on the β-cell and non-islet tissues and contribute to the development of insulin resistance in peripheral tissues depending on their concentrations and the time during which they are elevated. After a meal in lean persons, a mild increase in glucose causes a mild increase in the release of insulin by pancreatic β-cells. Increased glycolytic flux in β-cells results in a rapid increase in the production of ATP in mitochondria, facilitating exocytosis of insulin [2]. On adipocytes and peripheral tissues such as skeletal muscles and liver, insulin activates specific signalling pathways that, in turn, cause efficient glucose uptake, which limits the increase in glucose and insulin. In obese people, however, both fat-engorged adipocytes and fat-laden myocytes are resistant to the signalling effects of insulin, a condition referred to as insulin resistance. Insufficient translocation of GLUT4 glucose transporters to the plasma membrane of adipocytes and myocytes limits glucose uptake and hence the cells require enhanced insulin secretion. This leads to ectopic fat accumulation in tissues not suited for lipid storage, such as heart, skeletal muscle, pancreas and liver. The presence of saturated fat in the liver in turn causes insulin resistance in this organ as well [3,4]. This leads to a spectrum of
NAFLDs (non-alcoholic fatty liver diseases), with manifestations ranging from pure steatosis to NASH (non-alcoholic steatohepatitis), which can evolve towards cirrhosis [5,6].

**From the metabolic syndrome to Type 2 diabetes**

Although the metabolic syndrome by itself induces a variety of pathologies, it is not synonymous with Type 2 diabetes. Thus it appears that a second hit is necessary for the progression from the metabolic syndrome to diabetes. Chronic elevation of both blood glucose and NEFAs [non-esterified (‘free’) fatty acids] are thought to exert islet toxicity and hence reduce glucose-stimulated insulin secretion, trigger the reduction of pancreatic β-cell mass, and, finally, lead to diabetes [7].

**The metabolic syndrome and Type 2 diabetes: role of oxidative stress**

The molecular basis of this element of diabetogenesis has been closely linked to oxidative stress. Under conditions of elevated metabolism, many tissue-specific cells are continuously subject to insult from ROS (reactive oxygen species), such as the superoxide radical (O$_2^-$) and H$_2$O$_2$. This is probably a common feature for elements of the metabolic syndrome such as hypertension, hypertriglyceridaemia, diabetes and obesity [8–11]. Moreover, an increase in ROS production is one of the earliest events in cases of glucose intolerance, and it may be the cause of pancreatic β-cell dysfunction as well as hepatic pathologies [2,6].

Interestingly, β-cells produce ROS in response to increased glucose concentrations [2], but express relatively low levels of free-radical-detoxifying enzymes. This combination might make β-cells particularly sensitive to oxidative stress. It is also becoming more appreciated that hepatic steatosis and steatohepatitis are closely related to the generation of ROS [12].

**ROS-generating NADPH oxidases: a potential source of oxidative stress in the metabolic syndrome and diabetes**

A major source of ROS generation is the NOX family of NADPH oxidases, which includes the prototypical phagocyte NADPH oxidase NOX2. NOX-derived ROS play a physiological role in response to stimulation of various growth factors, cytokines and hormones, including insulin [13], and have pathophysiologial roles in endothelial dysfunction, inflammation, apoptosis, fibrosis, angiogenesis and important processes underlying diabetes and liver injury [14].

The prototypical NADPH oxidase NOX2, initially discovered in phagocytes, is an electron transporter [15] that catalyses the NADPH-dependent reduction of oxygen to O$_2^+$, which is the precursor of the other ROS (Figure 1). In resting cells, this multicomponent enzyme system is inactive, and its components are dispersed between the cytosol and the membranes. The flavocytochrome b$_{558}$ component, which is composed of two subunits, gp91$	ext{phox}$ (also called NOX2) and p22$	ext{phox}$, is located in the plasma membrane and in specific granules. The other components of the NADPH complex (p47$	ext{phox}$, p67$	ext{phox}$, p40$	ext{phox}$ and small G-protein Rac1/2) are cytosolic proteins. The activation of phagocytes by various stimuli, such as PMA, triggers the phosphorylation of the p47$	ext{phox}$, p67$	ext{phox}$ and p40$	ext{phox}$ cytosolic components and their translocation to the plasma membrane, where they interact with flavocytochrome b$_{558}$. Concomitantly, Rac2 dissociates from a RhoGDP dissociation inhibitor and interacts with flavocytochrome b$_{558}$ to form a binding partner for p67$	ext{phox}$. The complete assembly of NADPH oxidase components is crucial for O$_2^+$ production [16–19].

A new family of oxidases, the NOX family, has been defined on the basis of their homology with the gp91$	ext{phox}$/NOX2 catalytic subunit of phagocyte NADPH oxidase. NOX enzymes appear early in evolution, probably at the transition from unicellular to multicellular organisms [20] (Figure 1). In mammals, six NOX homologues [NOX1, NOX3, NOX4, NOX5, DUOX (dual-oxide) 1 and DUOX2] have been identified [14,17,21,22]. These homologues share many structural features with NOX2 and, similarly, are electron transporters that reduce O$_2$ to O$_2^+$. However, although the activation mechanisms of NOX1 and NOX3 includes flavocytochrome b$_{558}$ to form a binding partner for p67$	ext{phox}$, the situation is different for other NOX isoforms. NOX4 might be constitutively active, with its activity depending on the mRNA levels [23]. NOX5 contains EF-hands and is activated by Ca$_{2+}$ [24], but probably also by PKC (protein kinase C) [25]. Most mammalian cell types express at least one NOX isoform, but many cells express several of them [14,21].

Researchers in the NOX field are presently facing two important difficulties: (i) a lack of specific inhibitors (for a review, see [26]); and (ii) a lack of reliable antibodies. In terms of inhibitors, the most widely used compounds are DPI (diphenyleneiodonium) and apocynin. However, DPI is a non-selective flavoprotein inhibitor. Apocynin is an antioxidant [27], which, in inflammatory cells, is possibly converted through a myeloperoxidase-dependent reaction into a low-affinity NOX inhibitor. Thus, at this point, inhibitor data should only be used together with other arguments [e.g. knockout mice, siRNA (small interfering RNA)] to make the case for NOX involvement in a physiological or a pathological process. In terms of antibodies, very little negative data are published, but, in many instances, antibodies were not rigorously tested in knockout mice or other well-controlled systems. Thus, in general, results exclusively based on immunoreactivity with anti-NOX antibodies should be treated with caution.

In the present review, we focus on two relevant aspects in the field of the metabolic syndrome and Type 2 diabetes, namely the role of oxidative stress in liver and in pancreatic β-cells. We highlight our current understanding of ROS-generating NOX enzymes in these tissues and discuss how they may contribute to the development of disease. We first describe the expression pattern of NADPH oxidase and then recall the mechanisms that associate oxidative stress with high levels of circulating glucose and fatty acids in metabolic diseases in each of the considered organs.
Figure 1 | NADPH oxidase family

The prototypical NADPH oxidase NOX2 is a multicomponent enzyme system that catalyses the NADPH-dependent reduction of oxygen to \( \text{O}_2^\cdot\). In resting cells, this multicomponent enzyme system is inactive, and its components are dispersed between the cytosol and the membranes. The flavocytochrome \( b_{558} \) component, which is composed of two subunits, gp91\textsuperscript{phox} (also called NOX2) and p22\textsuperscript{phox}, is located in the plasma membrane and in specific granules. The other components of the NADPH complex (p47\textsuperscript{phox}, p67\textsuperscript{phox}, p40\textsuperscript{phox} and small G-protein Rac1/2) are cytosol proteins. The activation of phagocytes by various stimuli triggers the phosphorylation of the p47\textsuperscript{phox}, p67\textsuperscript{phox} and p40\textsuperscript{phox} cytosolic components and their translocation to the plasma membrane, where they interact with flavocytochrome \( b_{558} \). Concomitantly, Rac2 dissociates from its RhoGDP dissociation inhibitor, which allows it to interact with flavocytochrome \( b_{558} \) to form a binding partner for p67\textsuperscript{phox}. A new family of oxidases, the NOX family, has been defined on the basis of their homology with the gp91\textsuperscript{phox}/NOX2 catalytic subunit of phagocyte NADPH oxidase. To date, six homologues (NOX1, NOX3, NOX4, NOX5, DUOX1 and DUOX2) with levels of identity with NOX2 have been identified. NOXO1 and NOXA1, which may interact with NOX1 and NOX4, are two homologues of p67\textsuperscript{phox} and p47\textsuperscript{phox} respectively. NOX family homologues have putative NADPH- and flavin-binding sites, as well as functional oxidase activity that produces \( \text{O}_2^\cdot\). NOX5, DUOX1, and DUOX2 also have a \text{Ca}^{2+}\)-binding site, whereas DUOX1 and DUOX2 have an additional transmembrane and a peroxidase-like domain.

The liver in the metabolic syndrome and diabetes: oxidative stress and NOX enzymes

Expression of NOX enzymes in the liver

Both parenchymal and non-parenchymal hepatic cells express different members of the NOX family. However, little information is currently available regarding the NOX isoforms present specifically in each hepatic cell type. The majority of the arguments for a potential role of NOX enzymes in liver cells is based on the use of non-specific inhibitors such as apocynin and DPI. Few studies have addressed the specific expression, localization and role of NOX isoforms. The presence of the phagocyte NADPH oxidase NOX2 has been suggested for a long time in the liver [28], especially in murine Kupffer cells (which are hepatic-resident macrophages) [29], in rat hepatocytes [30,31], in endothelial cells [32], and in both quiescent and culture-activated rat HSCs (hepatic stellate cells), which are responsible for vitamin A storage when quiescent and for fibrogenic mechanisms after activation [33,34].

NOX1 is expressed in hepatic sinusoidal endothelial cells [35,36], in rat hepatocytes, and in both quiescent and culture-activated rat HSCs [34]. NOX4 is expressed in hepatocytes, Kupffer cells and culture-activated (but not quiescent) rat HSCs [34]. Finally, DUOX1 and DUOX2 are expressed in rat hepatocytes, Kupffer cells, and either activated or non-activated HSCs [34]. NOX enzymes are thus widely distributed in the whole liver. The physiological functions of NOX enzymes in the liver are only partially understood. The role of NOX2 in Kupffer cells should be similar to the role of NOX2 in other macrophages, namely killing of micro-organisms, participation in the degradation of ingested material, immunoregulation (production of the inflammatory response via the induction of mono- and poly-morphonuclear leucocyte infiltration) and possibly also cellular signalling [e.g. NF-\( \kappa \)B (nuclear factor \( \kappa \)B) activation]. Hepatic stellate cells are involved in liver development and regeneration, degradation of apoptotic bodies, production of cytokines, retinoid metabolism and secretion of lipoproteins and growth factors, but the potential physiological role of NOX enzymes has not been studied from this point of view [37]. The characteristics of liver sinusoidal endothelial cells facilitate direct contact of soluble and insoluble serum substances with hepatic parenchymal cells, resulting in enhancement of hepatic metabolic activity. In addition, sinusoidal endothelial cells have the potential to eliminate a variety of macromolecules from the blood circulation via receptor-mediated endocytosis by denaturation or modification of proteins such as AGEs (advanced glycation end-products), extracellular matrix components, including hyaluronic acid, and some lipoproteins. Sinusoidal endothelial cells have an
antigen-presenting function similar to that of dendritic cells. Taken together, it is suggested that sinusoidal endothelial cells, co-operating with Kupffer cells and hepatic dendritic cells, may partake of immunoregulatory functions in the liver, and thus may involve NOX isoforms in their physiology [38]. Finally, in hepatocytes, ROS, and thus potentially NOX enzymes, induce a number of functional adaptive changes in the capability of hepatocytes to produce bile and to secrete exogenous and endogenous compounds [39]. The potential involvement of NOX enzymes in the metabolism of carbohydrates, lipids, and proteins, and in the detoxification of xenobiotics in hepatocytes, has not been studied.

Independently of their physiological function, it is obvious that NOX enzymes may participate in the chronic production of high level ROS during hyperinsulinemia and hyperglycaemia.

**Insulin resistance, steatosis and steatohepatitis**

Different sources of fatty acids contribute to the development of fatty liver. Under conditions of insulin resistance, insulin does not adequately inhibit hormone-sensitive lipase, and lipolysis in white adipose tissue is not suppressed. Therefore peripheral fats stored in adipose tissue flow to the liver by way of plasma NEFAs. In addition, the combination of elevated plasma glucose and insulin concentrations promotes de novo fatty acid synthesis (lipogenesis) through the up-regulation of SREBP-1c (sterol-regulatory-element-binding protein 1c) and the activation of ChREBP (carbohydrate-responsive-element-binding protein), thereby contributing to the development of hepatic steatosis. After the esterification step of NEFAs into triacylglycerols, triacylglycerols can then be stored as lipid droplets within hepatocytes or secreted into the blood as VLDL (very-low-density lipoprotein) [6,40]. Because fat cannot accrue indefinitely within hepatocytes, a new steady state is reached, whereby increased input pathways are finally compensated for by increased output pathways. Two major compensatory pathways are the enhancement of mitochondrial NEFA β-oxidation and increased hepatic secretion of VLDL.

Although the hepatic accumulation of lipids is widely believed to result in insulin resistance, it remains uncertain whether a causal relationship exists [6,40].
Figure 3 | NOX family and β-cell dysfunction in Type 2 diabetes

Under normal conditions, low glucose induces a release of insulin by β-cells. Under chronic high-glucose and NEFA conditions, several modifications lead to decreased β-cell mass and Type 2 diabetes. (I) Disturbances in the ER related to development of altered glucose homeostasis are an emerging field of research. Impairment of normal protein folding in the ER during ER stress can redirect β-cells to apoptotic pathways via NOX4. (II) Hyperglycaemia is involved in cell death through the ROS inhibition of phosphatases of the JNK pathway, NF-κB activation and caspase activation. It also results in increased production of ROS by NOX1 and/or NOX2 and generation of AGEs, which in turn are associated with reduced transcription of genes involved in insulin production. (III) the endocrine pancreas is exposed not only to systemic, but also to locally produced components of the renin–angiotensin system (RAS), in Type 2 diabetes, RAS activity is inappropriately up-regulated in β-cells and in endothelial cells of the pancreatic vasculature. The physiological role of the pancreatic RAS appears to involve islet blood flow regulation, an effect capable of affecting insulin secretion that may be triggered in part by NOX1. (IV) Pancreatic fibrosis seems to involve the increased production of TGF-β1, a classic profibrotic cytokine, and PSC activation, which may involve NOX2 and/or NOX4. (V) Finally, NOX2 in macrophages and polymorphonuclear neutrophils (yellow) contribute to inflammation by producing pro-inflammatory cytokines that lead to decreased β-cell mass and Type 2 diabetes.

Insulin resistance

Under physiological conditions, the involvement of transient small bursts of ROS in insulin action has been suggested for decades (Figure 2). NOX4 has been shown to oxidize and inhibit the reduced cysteine residues containing PTP1B (protein tyrosine phosphatase 1B), facilitating the tyrosine phosphorylation of IRS1 (insulin receptor substrate 1) and thus glucose uptake (reviewed in [13]). Nevertheless, recent studies have identified chronic high production of ROS as a common pathway causing steatosis. Indeed, a high-fat diet coupled to antioxidant administration decreased steatosis in C57BL/6 mice [41].

Chronic elevation of ROS leads to impaired insulin signalling by way of a complex mechanism. This involves increased JNK (c-Jun N-terminal kinase) 1-mediated-serine/threonine phosphorylation of IRS1, increased proteasomal degradation of IRS1, impaired insulin-stimulated redistribution of IRS1 and PI3K (phosphoinositide 3-kinase) activity causing reduced Akt/PKB (protein kinase B) phosphorylation [2]. Enhanced JunD/JNK1-dependent liver injury correlated with the acute induction of NOX-dependent O2•− production following hepatic ischaemia/reperfusion. JunD and JNK1 may act in concert to control NOX2 and NOX4 gene expression in the liver, which influences ROS-dependent
AP-1 (activator protein 1) response [42]. Furthermore, Akt activity and cell growth are stimulated significantly by treating hepatoma cells with low concentration of ROS. Finally, the PI3K inhibitor wortmannin inhibited Akt/PKB phosphorylation induced by ROS in these cells [43].

Thus NADPH oxidases, especially NOX2 and NOX4, seem to be involved in pathways leading to insulin resistance and steatosis in the liver, constituting the first line of the ‘two risk factors’ theory for NASH pathogenesis: steatosis (insulin resistance) and inflammation/fibrosis. Oxidative stress, cytokine expression and lipid peroxidation may play a role in the development of inflammation and fibrosis. However, the mechanism leading from steatosis to steatohepatitis is still unknown.

**Hyperglycaemia and fibrosis**

Hyperglycaemia has been singled out as a harmful prognostic factor in the evolution of NASH towards fibrosis via activation of HSCs [44]. High glucose stimulates HSCs to proliferate and to produce collagen through free radical production [45,46]. Interestingly, DPI and apocynin, specific NOX inhibitors, inhibit the PDGF (platelet-derived growth factor)-induced-proliferation of the human HSC line Li-90 and murine primary-cultured HSCs [47]. Furthermore, TGF-β (transforming growth factor β) induces NOX4 expression upon the transdifferentiation of activated HSCs to myofibroblastoid cells, demonstrating the important role of NOX in HSC activation and the onset of fibrosis [48]. Moreover, knockout mice for p47<sup>phox</sup>, which have a defect in activation of both NOX1 and NOX2, show attenuated liver injury and fibrosis after bile duct ligation. A deficiency of angiotensin II signalling may be responsible for this protection [49].

NOX4, the expression of which increases during activation of HSCs, and NOX1, which is involved in angiotensin II signalling, are the most promising fibrogenic NOXs. Nevertheless the involvement of each NOX may be dependent on the hepatic pathology model used (Figure 2).

**NEFAs and fibrosis**

Increased plasma concentration of NEFAs and fibrosis are also associated with liver insulin-resistant states. It has been shown that a high-fat diet in fa/fa obese rats increases NOX-mediated ROS production. Interestingly, in the same study, hyperlipidaemia decreased xanthine oxidase and cytochrome P450 2E1 activity, excluding a role of these other sources of ROS [51].

Plasma levels of adiponecin, an adipocyte-derived anti-diabetic, anti-atherogenic adipocytokine, are inversely correlated with insulin resistance, fibrosis and diabetes in obese individuals. High-molecular-mass adiponecin inhibited PDGF-induced proliferation of HSCs via suppression of ROS production and subsequent inhibition of the Akt/PKB pathway. ROS production by PDGF-stimulated HSCs was inhibited by DPI, implicating NADPH oxidase as the source [52].

**Inflammation**

Importantly, activity and expression of the phagocyte NADPH oxidase NOX2 are increased in subjects with the metabolic syndrome [53], suggesting a possible role for NOX2 in hepatic inflammation. Nevertheless, the involvement of NOX2 may be more discrete as Nox2<sup>−/−</sup> mice fed a steatohepatitis-inducing, methionine- and choline-deficient diet are not protected [54] (Figure 2).

**Apoptosis**

Enhanced hepatocellular apoptosis is a feature of most chronic liver disorders. A subset of patients with NAFLD develop progressive NASH in which disease activity correlates with CD95 and TNFR1 [TNFα (tumour necrosis factor α) receptor 1]-dependent hepatocyte apoptosis [55,56]. CD95 and TNFR1 also increase in experimental models of NASH, and CD95L and TNFα promote hepatocyte apoptosis and inflammation in these fatty liver models [55]. Importantly, CD95L induces oxidative stress in rat hepatocytes by activation of NOX1 or NOX2 via signalling through sphingomyelinase, ceramide and PKCζ [34]. For the vascular part, VEGF (vascular endothelial growth factor)-resistant apoptosis of sinusoidal endothelial cells may be driven by NOX1 [35].

Hepatocellular apoptosis and phagocytosis of apoptotic bodies by Kupffer cells and HSCs may cause inflammatory reactions such as macrophage TGF-β secretion and p47<sup>phox</sup>-dependent O2•− production [57].

Finally, hepatic metabolic disorders can induce ER (endoplasmic reticulum) stress [58]. Liver cells cope with ER stress by an adaptive protective response termed the UPR (unfolded protein response), which includes enhancing protein folding and degradation in the ER and down-regulating overall protein synthesis. When the UPR adaptation to ER stress is insufficient, the ER stress response unleashes pathological consequences including hepatic fat accumulation, inflammation and cell death, which can lead to liver disease or worsen underlying causes of liver injury, such as NASH [59]. It has also been shown that oxysterol induces oxidative stress and/or apoptotic events in human aortic smooth muscle cells. This specific effect is mediated by a robust up-regulation of NOX4, an activation of ER stress (transient intracellular Ca2+ oscillations, induction of the cell death effector CHOP [C/EBP (CCAAT/enhancer-binding protein)-homologous protein] and GRP78 (glucose-regulated protein 78 kDa)/BiP (immunoglobulin heavy-chain-binding protein)-chaperone via the activation of IRE-1 (insulin-response element 1), all hallmarks of the unfolded protein response) linking UPR-mediated apoptosis to NOX enzymes [60,61] (Figure 2).

In conclusion, liver fat is a critical determinant of metabolic fluxes and inflammatory processes. Altogether, these data place NOX enzymes in the middle of NASH pathogenesis, thereby representing an important therapeutic target in insulin resistance and hepatic complications such as fibrosis, inflammation and apoptosis.
**β-Cells in the metabolic syndrome and diabetes: oxidative stress and NOX enzymes**

**Expression of NOX enzymes in pancreatic β-cells**
O$_2^•−$ generation was first demonstrated by the cytochrome c reduction method in insulin-producing cell lines [62]. Then, the phagocyte-like NADPH oxidase NOX2 (gp91phox and p22phox) and its related subunits (p47phox and p67phox) followed by NOX1, NOX4, NOXO1 and NOXA1, have all been shown to be expressed in pancreatic islets [63–67]. Recently, mRNA for NADPH oxidase components NOX1, NOX2 and NOX4, and protein for NADPH oxidase subunits NOXA1, p22phox, p47phox and p67phox, have been detected in human and rat PSCs (pancreatic stellate cells) [68,69]. The physiological role of NOX enzymes in pancreatic islets is poorly understood. On the basis of studies using the non-selective flavoprotein inhibitor DPI and the antioxidant/NOX inhibitor apocynin [27], a role for NOX enzymes in insulin secretion [67,70–72] has been suggested. However, so far, no deficiency in insulin secretion has been reported in various NOX-knockout mice.

**β-Cell dysfunction**
The mechanisms by which hyperglycaemia and hyperlipidaemia exert their deleterious effects on the β-cell include among others ROS generation. In contrast with most other mammalian cell types, β-cells have relatively low levels of free-radical-detoxifying and redox-regulating enzymes [73,74]. The consequence of limited scavenging systems is that, upon normal or excessive Ca$^{2+}$ stimulation, ROS concentrations in β-cells may increase rapidly and reach damaging levels easily.

**Hyperglycaemia**
In β-cells, hyperglycaemia stimulates a rapid and proportional increase in glycolytic flux and reducing equivalents due to channelling of glucose carbon into the tricarboxylic acid cycle. An excessive increase in intracellular Ca$^{2+}$, via PKC activation, may enhance NOX-dependent generation of ROS and thus induce oxidative stress and/or apoptosis [63,67,75–77]. In early Type 1 diabetes, systemic markers of oxidative stress correlate with insulin requirements. It has been suggested that this reflects oxidative stress in the pancreatic islets damaging insulin-secreting β-cells [78]. In Type 2 diabetes, exposure of isolated islets to high glucose induces increases in intracellular peroxide levels, and treatment with antioxidant partially protects animal models of Type 2 diabetes against the development of hyperglycaemia [79]. Furthermore, an increased expression of NOX2 occurs in islets and may exacerbate disease aggravation over time by damaging insulin-producing cells [64]. Chronic hyperglycaemia inhibits insulin secretion, activates angiotensin II type 1 receptor, and increases O$_2^•−$ production and p47phox and p22phox expression in a rat insulin-producing cell line and in isolated human pancreatic islets [65,80,81] (Figure 3).

**Hyperlipidaemia**
NOX enzymes appear to be especially important in redox signalling in that they are specifically activated by diverse agonists and regulate the activation of downstream protein kinases, transcription factors and other biological molecules. In vascular tissues, NEFAs in conjunction with high glucose stimulate the PKC pathway and subsequently stimulate NOX-dependent O$_2^•−$ production [82]. In addition, NEFAs induce activation of NF-κB, p38 MAPK (mitogen-activated protein kinase) and JNKs/SAPKs (stress-activated protein kinases), along with the activation of the AGES/receptor for AGEs, PKC and sorbitol stress pathways. Studies with antioxidants such as vitamin E, α-lipoic acid and N-acetylcysteine suggest that new strategies may become available to treat these conditions [83]. In the long run, inhibitors of NOX isoforms might provide a more powerful and more specific approach to limit oxidative damage in pancreatic β-cells [26].

**Fibrosis**
Activated PSCs play an important role in pancreatic fibrosis, and inflammation and oxidative stress are also implicated in the pathogenesis. NOX might be a source of ROS in the injured pancreas. In a model of pancreatitis, NOX enzymes were implicated in the inflammatory response that resulted in tissue inflammation and destruction [71,84]. DPI inhibits the transdifferentiation of quiescent PSCs to activated PSCs, which produce collagen and pro-inflammatory cytokines. Pancreatic fibrosis was also inhibited by DPI in vivo, suggesting a role for NOX isoforms in the activation of PSCs [68]. Inversely, angiotensin II type 1 receptor blockers, such as candesartan and losartan, improved glucose tolerance, increased β-cell mass, reduced fibrosis, decreased oxidative stress and decreased p22phox and NOX2 expression in db/db obese mice [66] (Figure 3).

**Apoptosis and ER stress**
Recent genetic and biochemical evidence in both humans and mice supports a requirement for the UPR to preserve ER homeostasis and prevent the β-cell failure that may be fundamental in the aetiology of diabetes. Chronic or overwhelming ER stress stimuli associated with the metabolic syndrome can disrupt protein folding in the ER, reduce insulin secretion, invoke oxidative stress and activate cell death pathways [85]. Since the UPR is related to oxidative stress, NOX enzymes might be implicated in β-cell apoptosis [60,61] (Figure 3).

**Conclusion and outlook**
Traditionally, mitochondria were thought to be the most important, if not only, source of ROS in metabolism [86]. It is now becoming clear that ROS generation by NADPH oxidases of the NOX family may also be an important player. However, the conclusions of many studies are based on inhibitors, and, unfortunately, there are no specific NOX inhibitors available as yet. Thus studies using mice deficient in specific NOX isoforms will be crucial. The development
of more specific NOX inhibitors will also be an important task. Indeed, unlike mitochondria, NOX enzymes have a major potential as drug targets [26]. Thus, if the concepts discussed above can be proved, NOX inhibitors might become useful drugs in the medical management of the metabolic syndrome and diabetes.

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