Nutrient regulation of β cells NADPH-oxidase activity*

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*This article is dedicated to Dr. Isabel Valverde, to homage her scientific achievements.

INTRODUCTION

Blood glucose concentration is the most important regulatory stimulus for β cell insulin secretion. The process of glucose-induced insulin secretion is fully dependent on the metabolism of the sugar in the β cell. The products of glucose metabolism, especially ATP, close the ATP potassium channel (KATP), increasing the intracellular potassium concentration that leads to a membrane depolarization (1). This phenomenon induces the opening of the voltage sensitive calcium channels (VSCC) provoking a rapid increase in calcium influx and in the intracellular calcium concentration [Ca], that triggers granule exocytosis (2, 3). On the other hand, the metabolism of glucose in the β cell also activates several enzymes, particularly phospholipase C (PLC) and adenylate cyclase (AC), which induce an increase in protein kinase C (PKC) and protein kinase A (PKA) activities, respectively (4, 5). These kinases have an important role in the mechanism of glucose-induced insulin secretion, including opening of the VSCC (6). However, PKC in particular can also stimulate other systems such as the NAD(P)H oxidase complex (7).

NADPH OXIDASE OF PHAGOCYTES

NADPH oxidase catalyses the production of the superoxide anion \( \text{O}_2^- \) by the one-electron reduction of oxygen, using NADPH as the electron donor (Fig. 1). The superoxide generated by this enzyme serves as the starting point for the production of a vast assortment of reactive oxidants, including oxidized halogens, free radicals and singlet oxygen. These oxidants are used by neutrophils to kill invading microorganisms (8), but they also cause a lot of 'damage' to nearby tissues, so their production has to be tightly regulated to ensure that they are only generated when and where required. NADPH oxidase comprises five components. In the resting cell, three of these five components, p40phox, p47phox and p67phox, exist in the cytosol...
as a complex. The other two components, p22PHOX and gp91PHOX, are located in the membranes, where they are present as a heterodimeric flavohaemoprotein known as cytochrome b558(9). Separation of these two groups of components, because of their distribution in distinct subcellular compartments, guarantees that NADPH oxidase is inactive in the resting cell(8). When the resting cell is exposed to PMA, for example, activated protein kinase C, translocated from the plasma membrane, phosphorylates the cytosolic components (Fig. 1). Subsequently, p40PHOX, which is responsible for maintaining the resting state of NADPH oxidase, is separated from other two cytosolic phox proteins following attachment of the active form of the small GTP-binding protein Rac to p67PHOX. The cytosolic duo-phox proteins (p47PHOX and p67PHOX) become conjugated with the membrane components of NADPH oxidase. This chain of events triggers the production of superoxide(10).

**NAD(P)H Oxidase in Non-Phagocytic Cells**

Recent studies have shown that several tissues and cells present NAD(P)H oxidase activity such as: endothelial cells (ECs)(11, 12), vascular smooth muscle cells (VSMCs)(13), thyroid cells(14), fibroblasts(15), lymphocytes(16), spermatozoa(17), and osteoclasts(18). In phagocytic cells, NAD(P)H oxidase produces large amounts of superoxide that leads to generation of other reactive oxygen species (ROS) devoted to kill microorganisms (bacteria, viruses, and fungi). In non-phagocytic cells, on the other hand, the role of NAD(P)H oxidase still remains to be defined. In the last years, several studies have examined the role of NAD(P)H oxidase in different tissues, such as the smooth muscle of vessels. Zalba et al.(19) demonstrated an overexpression of p22PHOX subunit of NADPH oxidase in smooth muscle of vessels from spontaneously hypertensive rats (SHR) and associate this alteration with a NAD(P)H oxidase overactivity in this tissue. There is evidence that these oxidases have a signaling role and the capacity of superoxide production is lower than in phagocytes(11). NAD(P)H oxidase found in different tissues are isoforms that usually have structural and functional differences from that present on phagocytic cells.

**NAD(P)H Oxidase Activation**

Protein kinase C (PKC) is the main regulator of NAD(P)H oxidase in leukocytes, inducing the phosphorylation-dependent activation of the cytosolic subunits(20) (Fig. 1). Inoguchi et al.(21) have also shown a PKC-dependent pathway for NAD(P)H oxidase activation in non-phagocytic cells (cultured aortic smooth cells and endothelial cells). In fact, inhibition of PKC in cultured smooth muscle cells provokes a decrease of ROS production as induced by palmitate. These authors also demonstrated that NAD(P)H oxidase of non-phagocytic cells is stimulated by high levels of glucose and free fatty acids (mainly palmitate).

**Regulation of NAD(P)H Oxidase by Metabolites**

Glucose is well known to be the main fuel for neutrophils. This metabolite is oxidized through glycolysis and pentose phosphate pathway. NADPH generated by glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase is the main substrate for NAD(P)H oxidase in phagocytes. Recent study indicates that glutamine is also actively
used by neutrophils. This amino acid metabolism provides ATP and NAD(P)H for the functioning of NAD(P)H oxidase. Also, glutamine up regulates the expression of the NAD(P)H oxidase components in neutrophils. Fatty acids also activate NAD(P)H oxidase; palmitic acid activates protein kinase C leading to superoxide production. Recent study has shown that fat-rich diets modulate the production of superoxide by neutrophils.

Oxidative Stress in Pancreatic Islets

Oxidative stress is a rupture in the balance between oxidant and antioxidant agents resulting in an increase in cellular free radicals, which can react with biological molecules causing cell injuries.

There are several indications for the occurrence of oxidative stress in pancreatic ß cells. Therefore, pancreatic islets isolated from rats and incubated in the presence of high glucose concentration (16.7 mM) for a short period of time (60 min) have shown increased activity of superoxide dismutase (SOD) and glutathione peroxidase (GSHPx). This activation may result from ROS stimulation, which is generated during glucose metabolism. Chronic high glucose level increases the production of advanced glycosylation end-products (AGEs) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) in ß cells. These metabolites are important markers of ROS generation and oxidative stress that leads to stimulation of antioxidant enzyme expression (heme oxidase-1 and GSHPx). The decreased transcription of the insulin gene induced by chronic elevation of glucose is prevented by antioxidants such as aminoguanidine and N-acetyl-L-cysteine (NAC). These findings support the proposition that hyperglycemia is associated with ROS generation by ß cells.

Islets are very susceptible to free radicals due to the low activity and expression of antioxidant enzymes. During the last years, we developed a series of experiments attempting to better understand the glucose effect on the oxidative stress. Our experiments utilizing the NBT (nitroblue tetrazolium) reduction have shown that both glucose and palmitate induce an acute increase in ROS production by one hour incubated islets (unpublished). Considering that mitochondria is the main site of ROS production in other cells, the action of α-ketoisocaprate (α-KIC), which is exclusively metabolized in the mitochondria, on the production of these species was tested. After one hour incubation, α-KIC fail to induce the production of ROS by rat pancreatic isolated islets, showing that the production of these species does not occur in the mitochondria during glucose and palmitate metabolism. We also demonstrate that during the glucose metabolism there is an increase of CuZnSOD activity which is an strong indicative that ROS production occurs mainly in cytosol.

Evidence for the Presence of NAD(P)H Oxidase in Pancreatic Islets

Recently we have developed an extensive investigation on the presence of NAD(P)H oxidase in rat pancreatic islets. The indications for the presence of this enzyme in ß cells are listed below.

1. Pancreatic islets present a consistent reduction of NBT up to 2 hours incubation under physiological glucose concentration (5.6 mM).
2. PMA (an activator of PKC) increased the ROS generation in the presence and absence of glucose (5.6 mM) or palmitate (0.1 mM).
3. DPI (diphenylene iodonium), an inhibitor of NAD(P)H oxidase, markedly reduced glucose and palmitate-induced ROS production.
4. The expression of the p47PHOX and p67PHOX subunits was demonstrated by Western blotting.
5. The expression of three NAD(P)H oxidase components was demonstrated by RT-PCR analysis: gp91PHOX and p22PHOX, both membrane-binding subunits, and p47PHOX, a cytosolic subunit.
6. The expression of p47PHOX, and insulin, was evaluated in consecutive pancreas sections by immunohistochemistry. Similarly to insulin, p47PHOX was mainly found in the central area of the islet, suggesting the expression of this NAD(P)H oxidase subunit by insulin-producing cells.

These findings indicate that pancreatic ß cells express NAD(P)H oxidase and this enzyme is activated by glucose and palmitate through, at least in part, of a dependent-PKC pathway.

Significance of NAD(P)H Oxidase in Non-Phagocytic Cells

The complete mechanism involved in the activation of NAD(P)H oxidase
and its physiological role in pancreatic β cells still remain to be fully established. In several cell types, ROS produced through NAD(P)H oxidase may have a signaling role. In endothelial cells, for example, NAD(P)H oxidase-derived H₂O₂ increases the release of intracellular Ca²⁺. In pancreatic islets, this effect on Ca²⁺ mobilization would be of great importance for regulation of insulin secretion. In general, ROS act as second messengers on cell signaling cascades and on regulation of several biological processes, such as cell growth, gene expression, kinase activation and modulation of ionic channels. On the other hand, ROS-mediated oxidative stress also produces damage in several tissues. In endothelial cells, ROS generated by NAD(P)H oxidase are implicated in impairment of cell function and pathophysiology of disorders such as hypertension, hypercholesterolemia and atherosclerosis. ROS generation is also related to cell death either by apoptosis or necrosis. Activation of NAD(P)H oxidase and subsequent generation of ROS is the main mechanism to trigger apoptosis in some cell types. This oxidase, for example, contributes directly to neuronal apoptosis. Islet cells possess low expression and activity of ROS-scavenging enzymes, being then susceptible to the toxicity of these oxidants. Therefore, a chronic activation of NAD(P)H oxidase may be related to the process of glucotoxicity and lipotoxicity, leading to a consequent failure of β cell function and/or death (Fig. 2).

CONCLUDING REMARKS

In summary, we found that pancreatic β cells express a phagocyte-like NAD(P)H oxidase. The cytosolic subunit p47^phox is mainly expressed in β cells, as shown by immunostaining of consecutive pancreas sections with an anti-insulin antibody. Glucose stimulates ROS production in islets through a PKC-dependent activation of NAD(P)H oxidase and this effect is potentiated by palmitate. Since ROS are recognizably toxic, hyperglycemia and high plasma FFA levels may lead to β cell death through NAD(P)H oxidase activation.

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