Production of Reactive Oxygen Species in the Diabetic Heart
– Roles of Mitochondria and NADPH Oxidase –

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Diabetes mellitus (DM) is an independent risk factor of heart failure. The Framingham Heart Study reported that the frequency of heart failure is 2-fold higher in male diabetics and 5-fold higher in female diabetics than in age-matched control subjects. An increase in reactive oxygen species (ROS) has been regarded as a dominant mechanism of cardiac dysfunction in patients with DM. ROS are important intracellular signaling molecules and mediate various cellular functions, including activation of transcriptional factors, protein kinases, and ion channels; however, high levels of ROS are detrimental to cardiomyocytes. Protein kinase C, angiotensin II, and advanced glycation endproducts (AGEs)/receptor for AGEs can activate NADPH oxidase. Increased intracellular calcium level mediated via the Na+-H+ exchanger and subsequent activation of Ca2+/calmodulin-dependent protein kinase II may also activate NADPH oxidase. This review presents the current understanding of the mechanisms of ROS production, focusing especially on the roles of mitochondria and NADPH oxidase. (Circ J 2014; 78: 300–306)

Key Words: Diabetes mellitus; Mitochondria; NADPH oxidase; Reactive oxygen species

Mitochondria

Mitochondrial ROS Production in DM

Mitochondria not only provide energy, but also provoke apoptosis, which is regulated by mitochondrial dynamics. Mitochondria also generate ROS as natural byproducts of oxygen metabolism in the electron transport chain. Under normal conditions, most of the electrochemical proton gradient is used to generate ATP through ATP synthase, and only 0.1% of the total oxygen consumption leaks from the respiratory chain to generate ROS. However, a high intracellular glucose concentration increases ROS production in the diabetic heart. In this review, we will summarize the mechanisms of ROS increase in the diabetic heart focusing on the roles of mitochondria and NADPH oxidase.
ROS in the Diabetic Heart

Hyperglycemia.\textsuperscript{10} ROS are more harmful close to their origin and mitochondrial DNA is a vulnerable target of ROS.\textsuperscript{12} Intracellular ROS can break the DNA strand and produce 8-hydroxydeoxyguanosine (8-OHdG).\textsuperscript{13} Mitochondrial DNA may be more sensitive to oxidative stress because 8-OHdG is 16-fold higher in mitochondrial DNA than in nuclear DNA in the rat liver.\textsuperscript{14} Therefore, 8-OHdG could be a sensitive marker for oxidative stress in mitochondria. We and another group have shown that the level of 8-OHdG is significantly increased in the streptozotocin (STZ)-induced diabetic rat heart.\textsuperscript{15,16} Mitochondria are not only a major source of ROS but also a susceptible target of ROS. In fact, sustained exposure to high glucose increases the ROS level and impairs mitochondrial morphology and function. Mitochondria were swollen and the structure of the cristae destroyed in the hearts of diabetic rats 4 weeks after STZ injection.\textsuperscript{15} Consistently, impairment of mitochondrial respiration and mitochondrial structure have been reported in animal models of type 1 and type 2 DM.\textsuperscript{17–20} Mitochondrial respiration and ATP synthesis were decreased in ob/ob mice.\textsuperscript{17} The size and the number of mitochondria were increased and respiratory function deteriorated in OVE26 mice, a chronic model of type 1 DM.\textsuperscript{20} It has also been demonstrated using electron microscopy that the density of cristae and expression of lipid droplets were reduced and mitochondrial volume increased in the hearts of type 1 diabetic Akita mice.\textsuperscript{18} Yu et al proposed that the dynamic change of mitochondrial morphology, fission and fusion contributes to ROS overproduction under high glucose conditions.\textsuperscript{21} As just described, ROS are generated by hyperpolarization of the mitochondrial inner membrane potential. Protein kinase C (PKC), angiotensin II (ATII), advanced glycation endproducts (AGEs), and calmodulin-dependent protein kinase (CaMKII) facilitate ROS production in NADPH oxidase.

Uncoupling Proteins as Regulators of Mitochondrial ROS Production

Uncoupling protein (UCP) is located in the mitochondrial inner membrane and dissipates the mitochondrial inner membrane potential. Hyperglycemia.\textsuperscript{10} ROS are generated by hyperpolarization of the mitochondrial inner membrane potential under high glucose conditions. Hence, sustained exposure to hyperpolarization-induced ROS will gradually impair mitochondrial morphology and function, leading to the generation of more ROS (Figure 2). This response may be one of the mechanisms of ROS-induced ROS release.

**Figure 1.** Oxidative stress-induced cardiac dysfunction in diabetes mellitus. Oxidative stress facilitates inflammation, apoptosis, and fibrosis in the heart and induces cardiac dysfunction. ROS, reactive oxygen species.

**Figure 2.** Interaction of multiple factors to increase production of reactive oxygen species (ROS) in diabetes mellitus. NADPH oxidase and mitochondria play a pivotal role and mutually stimulate to enhance ROS production. UCPs regulate ROS production in mitochondria by dissipating the mitochondrial inner membrane potential. Protein kinase C (PKC), angiotensin II (ATII), advanced glycation endproducts (AGEs), and calmodulin-dependent protein kinase (CaMKII) facilitate ROS production in NADPH oxidase. UCP, uncoupling protein.
membrane and partially dissipates the proton electrochemical gradient. UCP2 and UCP3 are homologs identified as the UCP family and are expressed in the heart. Because the mitochondrial inner membrane potential controls ROS production, UCP may reduce ROS production in mitochondria by dissipating the electrochemical gradient. Indeed, overexpression of UCP2 increased oxygen consumption and attenuated ROS production in cultured cardiomyocytes stimulated by H$_2$O$_2$ or doxorubicin. Knockout of UCP2 resulted in a concomitant increase of ROS in islets and macrophages. Consistently, isolated mitochondria from UCP3-knockout mice generated more ROS than wild-type mice. Overexpression of UCP1 also prevented an increase in ROS associated with hyperglycemia. However, the effect of UCP1 on ROS production is still controversial, because there is no effect on superoxide production in adipose tissue from UCP1-knockout mice. These results indicate that the principal role of UCP2 and 3 may be regulation of ROS production in mitochondria. Mitochondrial uncoupling increased in accordance with upregulation of UCP2 and 3 in the heart of db/db mice, Zucker diabetic rats, and STZ-induced diabetic rats. UCPs can be directly activated by superoxide to induce mitochondrial inner membrane depolarization and thereby decrease ROS production. Therefore, upregulation of UCP expression in DM is regarded as an adaptive response to an increase in the ROS level. Interestingly, UCP2 may contribute to the pathogenesis of type 2 DM. Because UCP2 is expressed ubiquitously and is present in pancreatic islets and β cells, an increase in its expression in islet tissue and β cells impairs glucose-stimulated insulin secretion by suppressing ATP levels. Conversely, UCP2-deficient mice show high islet ATP levels and increased glucose-stimulated insulin secretion.

Although UCPs can reduce ROS production in cardiomyocytes, prolonged UCP activation may have detrimental effects in the heart. Upregulation of UCP2 and UCP3 expression in the rat heart increased mitochondrial uncoupling and decreased myocardial energy efficiency. Further, overexpression of UCP2 inhibited mitochondrial Ca$^{2+}$ uptake, and prolonged the decay phase of cytosolic Ca$^{2+}$ transient and increased intracellular Ca$^{2+}$ spark activity. These effects may increase arrhythmogenic potential.

**NADPH Oxidase**

**ROS Production in NADPH Oxidase**

NADPH oxidase produces superoxide, which is highly reactive, short-lived, and rapidly dismutates to the more stable and diffusible H$_2$O$_2$. Superoxide is produced through the 1-electron reduction of O$_2$ catalyzed by NADPH oxidase. Among the NADPH oxidase isoforms, gp91phox (Nox2) and Nox4 are abundantly expressed in cardiomyocytes. Nox2 is located on the plasma membrane of cardiomyocytes and has a heterodimeric structure comprising Nox2 and p22phox subunits. Nox2 activation requires combination with p47phox, p67phox, p40phox, and Rac1, whereas, Nox4 is constitutively active and it does not require cytosolic subunits to increase its activity. NADPH oxidase is activated by a wide range of agonists and stimulation and is implicated in the ROS increase in DM. However, the most of the information has been derived from analysis of endothelial cells and vascular smooth muscle cells. Recently, several studies revealed that NADPH oxidase plays a role as a primary source of ROS production in the heart. The increase in ROS production by NADPH oxidase is well documented in cultured cardiomyocytes exposed to high glucose and in the hearts of diabetic animal models; although which of Nox2 and Nox4 mainly contributes to ROS production is still obscure. Exposure to high glucose concentrations upregulated the expressions of p47phox and p67phox, and increased ROS levels in cultured cardiomyocytes in an apocynin-inhibitable manner. Another group showed that the activity of NADPH oxidase and the expression of Nox4 increased in cardiomyocytes exposed to high glucose. That group also demonstrated that inhibition of Nox4 by antisense oligonucleotides decreased NADPH oxidase activity in the STZ-induced diabetic rat heart. These results indicate that NADPH oxidase contributes to DM-induced ROS increase. Furthermore, inhibition of NADPH oxidase by apocynin alleviated myocardial contractile dysfunction and endothelial dysfunction in DM. Cardiomyocyte-specific Rac1 deficiency attenuated ROS production and prevented cardiac hypertrophy and fibrosis in STZ-induced diabetic mice. The same group also demonstrated that Rac1 is necessary for induction of apoptosis in hyperglycemia through Nox2 activation and ROS production. Heart-specific Rac1 overexpression increased the atrial collagen content and NADPH oxidase activity, resulting in a high incidence of atrial fibrillation. Taken together, NADPH-derived ROS contribute to various types of cardiac complications in DM.

**Ca$^{2+}$/Calmodulin-Dependent Protein Kinase II**

The Na$^+$/H$^+$ exchanger (NHE) is a major mechanism of proton removal from the cytosol under conditions of intracellular acidosis. Exposure to high glucose concentrations increases NHE expression and activity in various types of cells. Under physiological conditions, the Na$^+$/Ca$^{2+}$ exchanger (NCX) extrudes Ca$^{2+}$ from the cells in exchange for Na$^+$. Under conditions where the intracellular Na$^+$ levels increase, the NCX works in reverse mode: Na$^+$ excretion in exchange for Ca$^{2+}$. Therefore, the NHE can increase intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]I) mediated via NCX in reverse mode. The [Ca$^{2+}$]I level is increased in cardiomyocytes isolated from STZ-induced diabetic rats and Goto-Kakizaki diabetic rats mediated via NHE activation. Increased [Ca$^{2+}$]I levels can activate Ca$^{2+}$/calmodulin-dependent protein kinase (CaMK) II, a multifunctional serine/threonine protein kinase, by the formation of a Ca$^{2+}$/calmodulin complex. CaMKII is implicated in the pathogenesis of a variety of cardiac diseases such as arrhythmias, cardiac hypertrophy, heart failure, and ischemia/reperfusion injury. NHE-mediated [Ca$^{2+}$]I loading increased the phosphorylation of CaMKII and NADPH oxidase expression in cultured cardiomyocytes exposed to high glucose concentrations and also in the hearts of STZ-induced diabetic rats. These results demonstrate the possible contribution of NHE and CaMKII to NADPH oxidase-mediated ROS production in the diabetic heart (Figure 3). Activation of CaMKII may also increase ROS production in mitochondria. CaMKII facilitates mitochondrial permeability transition pore opening and mitochondrial ROS production by increasing the cytosolic Ca$^{2+}$ concentration. In addition to the widely-accepted mechanism of Ca$^{2+}$/calmodulin-mediated activation, CaMKII can also be activated by ROS-induced oxidation in a Ca$^{2+}$-independent manner. For example, hydrogen peroxide activates CaMKII in Jurkat T cells. Thus, overproduction of ROS induced by Ca$^{2+}$-dependent CaMKII activation may further activate CaMKII in a Ca$^{2+}$-independent manner to promote more ROS production. Furthermore, CaMKII can directly enhance the activity of the NHE, which may activate another vicious cycle leading to excessive ROS generation by mutual enhancement between CaMKII and the NHE.
ROS in the Diabetic Heart

**Protein Kinase C**
A number of studies have shown that hyperglycemia activates protein kinase C (PKC) in various types of cells and tissue. In addition, hyperglycemia also promotes the synthesis of diacylglycerol, an activator of PKC. PKC regulates NADPH oxidase activity by phosphorylating the p47phox subunit. The phosphorylation of p47phox induces their translocation from the cytosolic component to the cellular membrane, where they can combine with other NADPH oxidase subunits to become a fully activated NADPH oxidase complex.

PKC can also activate the NHE. Therefore, exposure to high glucose concentrations may activate the NHE via PKC-dependent mechanisms, and the consequent [Ca^{2+}] increase will activate CaMKII and NADPH oxidase to generate more ROS (Figure 3). The importance of PKC activation in ROS production in DM was also demonstrated in several reports. The ROS increase induced by high glucose exposure was partially suppressed by a PKC inhibitor. Inoguchi et al showed that high glucose exposure stimulated ROS production through PKC-dependent NADPH oxidase activation in cultured vascular smooth muscle cells and endothelial cells.

**Angiotensin II**
Hyperglycemia stimulates the production of angiotensin II and develops oxidative stress in cardiomyocytes. Stimulation of the angiotensin type 1 (AT1) receptor by angiotensin II activates NADPH oxidase and facilitates ROS production in cardiomyocytes and endothelial cells. A contractile defect in cardiomyocytes induced by angiotensin II was prevented by apocynin, an inhibitor of NADPH oxidase. Apocynin also abolished angiotensin II-induced apoptosis in embryonic rat cardiomyocytes. Angiotsin II-induced apoptosis is mediated via NADPH oxidase-dependent peroxinitrite formation and consequent DNA damage.

These results indicate that angiotensin II-induced cardiac dysfunction is mediated via, at least in part, ROS production in NADPH oxidase. Conversely, ROS may also work as an upstream signaling molecule of angiotensin II.

**AGEs/RAGE Interaction**
Advanced glycation endproducts (AGEs) are generated through nonenzymatic glycation, in which reducing sugars such as glucose react nonenzymatically with proteins, lipids, and nucleic acids. This leads to the formation of Schiff bases, Amadori products, and finally AGEs. AGEs are regarded as key molecules in the progression of diabetic complications. AGEs bind with their receptor (RAGE) and activate several signaling pathways including an activation of NADPH oxidase.

In human endothelial cells, exposure to high glucose concentrations may activate the NHE via PKC-dependent mechanisms, and the consequent [Ca^{2+}] increase will activate CaMKII and NADPH oxidase to generate more ROS (Figure 3). The importance of PKC activation in ROS production in DM was also demonstrated in several reports. The ROS increase induced by high glucose exposure was partially suppressed by a PKC inhibitor. Inoguchi et al showed that high glucose exposure stimulated ROS production through PKC-dependent NADPH oxidase activation in cultured vascular smooth muscle cells and endothelial cells.

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mitochondrial ROS production by impairing their function.

Besides ROS production, a number of studies have shown other detrimental effects of AGES/RAGE interaction. AGES/RAGE have been shown to change the expressions of saccosomal reticulum calcium ATPase, phospholamban, and NCX, and induce cardiac dysfunction by impairing the [Ca^{2+}]_i transient. AGES/RAGE may also stimulate release of pro-inflammatory cytokines and transcription factor such as nuclear factor-kappa B, which facilitates apoptosis. Atria from diabetic rats exhibited structural remodeling characterized by diffuse interstitial fibrosis mediated via AGES/RAGE interaction. AGES temporarily activated ERK1/2, P38 MAPK, and nulear O-GlcNAcylation, and increased apoptosis. We should note the possibility that some of these detrimental effects of AGES/RAGE interaction may be caused by an increase in ROS.

**ROS-Induced ROS Release**

There is a functional connectivity between intracellular sites of ROS production, which is termed ROS-induced ROS release. This crosstalk is a common mechanism of ROS amplification and regional ROS production. The morphology of mitochondria is remarkably altered in STZ-induced diabetic hearts. The mitochondria are significantly larger and luminosity, which indicates collapse of the cristae, was significantly higher in STZ-induced diabetic hearts than in controls. However, these changes were prevented by administration of KN-93 and apocynin, inhibitors of CaMKII and NADPH oxidase, respectively. Thus, CaMKII may promote ROS production in NADPH oxidase, and induce morphological changes in the mitochondria leading to mitochondrial malfunction and more ROS production (Figure 2). There is a crosstalk between mitochondria and NADPH oxidase, which represents a feed-forward vicious cycle of ROS production. In human 293T cells, ROS generated from mitochondria stimulated activated Nox1.

Conversely, NADPH oxidase may induce ROS production in mitochondria. Superoxide generated by NADPH oxidase triggers rapid Ca^{2+} mobilization followed by mitochondrial ROS production in endothelial cells. There are other systems of ROS-induced ROS release. NADPH oxidase-derived ROS induced conversion of xanthine dehydrogenase to xanthine oxidase, which is a well-known ROS-generating enzyme. ROS accumulation in cardiomyocytes triggers a depolarization of the mitochondrial transmembrane potential by mitochondrial permeability transition, and induces a burst of mitochondrial ROS generation. This system may also facilitate ROS production in DM.

**Conclusions**

An increase in the ROS level in the diabetic heart is induced by multiple mechanisms. Among these, NADPH oxidase and mitochondria play a pivotal role and mutually stimulate to enhance ROS production. UCPs regulate ROS production in mitochondria by dissipating the mitochondrial inner membrane potential. PKC, angiotensin II, AGES/RAGE, and CaMKII facilitate ROS production in NADPH oxidase. The mechanisms of ROS increase in DM are complex because the multiple factors interact and enhance each other.

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**Disclosures**

Conflict of interest: None.

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