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Cardiac mitochondrial dysfunction during hyperglycemia—The role of oxidative stress and p66Shc signaling☆

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ABSTRACT

Diabetes mellitus is a chronic disease caused by a deficiency in the production of insulin and/or by the effects of insulin resistance. Insulin deficiency leads to hyperglycemia which is the major initiator of diabetic cardiovascular complications escalating with time and driven by many complex biochemical and molecular processes. Four hypotheses, which propose mechanisms of diabetes-associated pathophysiology, are currently considered. Cardiovascular impairment may be caused by an increase in polyol pathway flux, by intracellular advanced glycation end-products formation or increased flux through the hexosamine pathway. The latter of these mechanisms involves activation of the protein kinase C.

Cellular and mitochondrial metabolism alterations observed in the course of diabetes are partially associated with an excessive production of reactive oxygen species (ROS). Among many processes and factors involved in ROS production, the 66 kDa isoform of the growth factor adaptor shc (p66Shc protein) is of particular interest. This protein plays a key role in the control of mitochondria-dependent oxidative balance thus it involvement in diabetic complications and other oxidative stress based pathologies is recently intensively studied. In this review we summarize the current understanding of hyperglycemia induced cardiac mitochondrial dysfunction with an emphasis on the oxidative stress and p66Shc protein.

This article is part of a Directed Issue entitled: Bioenergetic dysfunction, adaptation and therapy.

1. Cardiovascular complications in diabetes – a clinical perspective

Diabetes mellitus (DM) is considered the epidemic of the XX and XXI centuries (Zimmet et al., 2001). The prevalence of this disease is higher in developed countries and in men (King et al., 1998). DM is classified in two forms. 5–10% of the patients have Type I, insulin-dependent diabetes, or juvenile onset diabetes (IDDM), caused by autoimmune destruction of pancreatic β-cells, which promotes insulin deficiency. The majority of diabetes patients have Type 2 diabetes which is non-insulin-dependent (NIDDM), an adult-onset form, which results from the combination between insulin resistance and/or β-cell secretory defect. In both types of diabetes, the decrease in the uptake of glucose by muscle and adipose tissue promotes extracellular hyperglycemia which leads to pathophysiological complications and damaged tissue (Carley and Severson, 2005; Niedowicz and Daleke, 2005). Retinopathy, nephropathy, nerve damage, heart disease and atherosclerosis are some of the complications induced by hyperglycemia that are responsible for the mortality and morbidity associated with diabetes (Table 1) (Brownlee and Cerami, 1981; Kuusisto et al., 1994; Luscher et al., 2003; Resnick and Howard, 2002).

The progression of atherosclerotic cardiovascular disease is clearly associated with the presence of insulin resistance, hyperinsulinemia, and elevated blood glucose. In this respect, coronary heart disease (CHD) is a major cause of morbidity and

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Table 1
Pathophysiology of diabetes, including organ-specific consequences.

General	
Type I	Type II
Autoimmune destruction of pancreatic β-cells	Insulin resistance
Insulin deficiency	β-Cell secretory defect
Decrease in the uptake of glucose	Decrease in the uptake of glucose
Hyperglycemia	Hyperglycemia
Organ-specific	
Retinopathy	
Nephropathy	
Nerve damage	
Atherosclerosis	
Coronary heart disease	
Coronary ischemia	
Myocardial infarction	
Silent myocardial ischemia	

mortality among patients with diabetes mellitus (DM) (Grundy et al., 1999). A number of important clinical studies have underscored such harmful association (Gerstein et al., 1999; Singer et al., 1992; Reaven, 1997) and have shown that individuals with DM have a higher prevalence of CHD (Fox et al., 2004), a greater extent of coronary ischemia (Granger et al., 1993), and are more likely to have a myocardial infarction (Yusuf et al., 2004; Haffner et al., 1998), and silent myocardial ischemia and infarction (Scognamiglio et al., 2006; Kannel, 1985; Margolis et al., 1973; Niakan et al., 1986), compared to individuals without diabetes. In the Framingham Heart Study, the age-adjusted risk for cardiovascular disease was doubled in men and tripled in women with DM independently of other important risk factors (Kannel and McGee, 1979). Similar data were available from the Multiple Risk Factor Intervention Trial Data where cardiovascular death rates were 9.7% among 5163 men on antidiabetic medications *vs.* 2.6% in the 342,815 men without medications for diabetes over a period of 12 years (Stamler et al., 1993). Also the Copenhagen Heart Study demonstrated type 2 DM patients, the risk of having an incident myocardial infarction or stroke is increased 2–3-fold and the risk of death is increased 2-fold, independent of other known risk factors for cardiovascular diseases (Almdal et al., 2004). Recently, the Emerging Risk Factors Collaboration study constructed a meta-analysis including 530,083 patients across 102 studies and showed that DM confers about a two-fold excess risk for a wide range of vascular diseases, independently from other conventional risk factors (Sarwar et al., 2010). Of note, both DM and impaired glucose tolerance can still remain undiagnosed in a consistent number of patients developing acute myocardial infarction (Norhammar et al., 2002). In the INTERHEART study, 15,152 cases and 14,820 controls were enrolled in a standardized case–control study of acute myocardial infarction performed in 52 countries, representing every inhabited continent (Yusuf et al., 2004). DM, among several other conditions (abnormal lipids, smoking, hypertension, abdominal obesity, psychosocial factors, low consumption of fruits and vegetables, increased alcohol intake, and absence of regular physical activity) accounted for most of the risk of myocardial infarction worldwide in both sexes and at all ages in all regions (Yusuf et al., 2004). Overall, the all-cause mortality risk associated with DM is comparable to the all-cause mortality risk associated with a prior myocardial infarction (Vaccaro et al., 2004). The importance of diabetes in the genesis of coronary heart disease has been further outlined by the National Cholesterol Education Program in 2002 which considers type 2 DM to be a CHD equivalent, placing it into the highest risk category (Haffner et al., 1998; NCEP, 2002). The relative risk for cardiovascular disease is even greater in those with type 1 DM, compared to non-diabetics of similar age (Krolewski et al., 1987). The overall impact of diabetes in cardiovascular disease is further aggravated by the frequent pre-coexistence of other atherogenic predisposing risk factors, namely obesity, hypertension, dyslipidemia, sedentary life, albuminuria, hyperhomocysteinemia, smoking, and elevated plasma fibrinogen (Grundy et al., 1999; Gerstein et al., 2001; Wachtell et al., 2003; Hoogeveen et al., 2000). Many of such factors are aggregated within the metabolic syndrome (Grundy, 2005; Petruzzelli et al., 2007; Palasciano et al., 2007).

The relative risk of coronary heart disease may also be increased in a pre-diabetic condition when higher levels of blood glucose and glycated hemoglobin are already present (Coutinho et al., 1999; Qiao et al., 2002; Ford et al., 2010). In a prospective study of 181 apparently non-diabetic patients with an acute myocardial infarction, impaired glucose tolerance was 35% while undiagnosed diabetes was 31% by glucose tolerance test but only 10% by fasting glycemia (Norhammar et al., 2002). The Emerging Risk Factors Collaboration study confirmed that in people without DM, fasting blood glucose concentration is modestly and non-linearly associated with risk of vascular disease (Sarwar et al., 2010).

Several factors are responsible for the increased cardiovascular risk and clinical presentations in diabetic and pre-diabetic patients. Apart from blood pressure and dyslipidemia, mechanisms of increased risk include increased rate of atherosclerosis (Moreno et al., 2000), structural abnormalities (*e.g.* coronary artery calcifications (Anand et al., 2006)), endothelial dysfunction, reduced myocardial flow reserve with impaired coronary vasodilator capacity (Yokoyama et al., 1997; Di Carli et al., 1999) (this latter abnormality inversely related to glycemic control (Yokoyama et al., 1997)), platelet activation (a predisposing factor to coronary thrombosis) (Davi et al., 1999), abnormalities in coagulation, hemostasis, and fibrinolysis (Kwaan, 1992). Mechanisms underlying the increased rate of silent ischemia and myocardial infarction in diabetic patients, by contrast, imply increased frequency of silent ST segment depression and coronary perfusion abnormalities during stress testing, autonomic (sympathetic) denervation of the heart associated with myocardial electrical instability (a factor potentially leading to fatal arrhythmias (Kwaan, 1992; Langer et al., 1995) and longer anginal perceptual threshold following exercise (Ranjadayalan et al., 1990). When going deeper into the cellular realm, things can be equally complex.

2. An unifying theory for DM complications?

Some hypotheses have been forwarded to explain how hyperglycemia causes diabetic-associated complications, including polyol pathway flux; increased advanced glycation end-products (AGE) formation; activation of protein kinase C (PKC) isoforms; and increased hexosamine pathway flux.

2.1. 1st hypothesis – increased polyol pathway flux

The enzyme aldose reductase is a monomeric oxidoreductase located in the cytosol that is able to catalyze NADPH-dependent reduction of several carbonyl compounds, including glucose, despite its low affinity for this compound (Brownlee, 2001; Folli et al., 2011; Giacco and Brownlee, 2010). Aldose reductase is the first enzyme in the polyol pathway and metabolism of glucose at normal concentrations by this pathway represents only a small part of the total use of glucose. Under hyperglycemic conditions, the increase in glucose increases the amount of sorbitol, which leads to the decrease in NADPH since it is consumed during the reaction. In this pathway, the enzyme sorbitol dehydrogenase is responsible for the oxidation of sorbitol into fructose, with the reduction of NAD^+ to NADH (Brownlee, 2001; Folli et al., 2011;

Giacco and Brownlee, 2010). There are several explanations for the deleterious effects of hyperglycemia through this pathway, which include sorbitol-induced osmotic stress, decreased (Na^+/K^+)ATPase activity, an increase in cytosolic NADH/NAD^+ and a decrease in cytosolic NADPH (Brownlee, 2001). The decrease of (Na^+/K^+)ATPase activity was originally thought to be mediated by a decrease in phosphatidylinositol synthesis mediated by the polyol pathway, but it has been recently shown to result instead from PKC activation. The activation of PKC promotes an increase in cytosolic phospholipase A2 activity, which increases the production of arachidonate and PGE2, inhibitors of (Na^+/K^+) ATPase (Xia et al., 1995).

The oxidation process of sorbitol increases the ratio between NADH and NAD^+, inhibiting the activity of GAPDH and increasing the concentrations of triose phosphate, which leads to increase methylglyoxal and diacylglycerol, activating PKC (Brownlee, 2001; Williamson et al., 1993). Even if hyperglycemia increases the NADH:NAD^+ ratio, this reflects a decrease in the total concentration of NAD^+ as a result of consumption by activated PARP, rather than reduction of NAD^+ to NADH (Garcia Soriano et al., 2001). The activation of PARP by hyperglycemia is mediated by increased production of reactive oxygen species (ROS) (Brownlee, 2001). Besides the explanations already mentioned, it has also been proposed that reduction of glucose to sorbitol by NADPH consumes NADPH. Since NADPH is required for the generation of reduced glutathione (GSH), this could cause or worsen intracellular oxidative stress (Brownlee, 2001). Although there are several studies regarding inhibition of the polyol pathway *in vivo*, the results are inconsistent.

2.2. 2nd hypothesis – increased intracellular formation of advanced glycation end-products

AGE are nonenzymatically glycosylated proteins and lipids which have altered biochemical and physiological properties (Ahmed, 2005). Type 2 diabetes leads to a combination of excessive protein and glucose in plasma, resulting in the formation of protein glycation end products (AGE) (Ahmed, 2005; Schleicher and Friess, 2007). These products are now recognized to exert several harmful effects (Ahmed, 2005; Schleicher and Friess, 2007). The protein glycation process starts with a nucleophilic addition reaction between free amino group of protein and carbonyl group of a reducing sugar, which is normally glucose. A Schiff's base is formed in the initial reaction, which is slow and reversible (Ahmed, 2005). In a second phase, Schiff's base reorganizes itself into ketoamines and Amadori products, which are irreversible products (Ulrich and Cerami, 2001). Besides, glycated proteins can also react with carbonyl intermediates to form the final AGE products. Increased content in AGEs have been detected in type 2 diabetes, being related with the pathophysiology and progression of diabetes. There is a second pathway for AGE production, consisting in glucose autooxidation, which also generates ROS (Wolff and Dean, 1987; Hunt et al., 1993). In the presence of transition metals, the enediols, present *in vivo*, generate anion and dicarbonyl-keto-aldehydes which react with amino groups of proteins to form ketoimines. These ketoimines are more reactive than Amadori products, contributing more to form AGE than the first pathway mentioned. Besides the above mentioned pathways, there is a third pathway that results in the formation of AGE (Ahmed, 2005). In this pathway, Amadori products are formed and converted into AGE with protein enedios and protein dicarbonyl compounds. AGE are by themselves toxic products, exerting harmful effects through ROS including the initiation of lipid peroxidation and oxidation of nucleic acids. There are several types of cell receptors for AGE and the interaction between them is believed to contribute and worsen diabetic complications (Ahmed, 2005).

2.3. 3rd hypothesis – activation of protein kinase C (PKC)

The PKC family includes at least eleven isoforms, representing major targets for lipid second messengers or phorbol esters (Nishizuka, 1992, 1995; Liscovitch and Cantley, 1994). PKC isoforms are divided into conventional and new PKCs. The conventional type is dependent on Ca^{2+} and binding site of DAG. The so-called new PKCs are sensitive to DAG but are independent of Ca^{2+} (Koya and King, 1998). The source of DAG that is responsible for the activation of PKC can derive from the hydrolysis of phosphatidylinositides or from the metabolism of phosphatidylcholine (Koya and King, 1998). It has been demonstrated that intracellular hyperglycemia increases the amount of DAG in a variety of tissues such as retina, heart and renal glomeruli, which interestingly are affected during diabetic complications (Shiba et al., 1993; Inoguchi et al., 1992; Craven et al., 1990; Ishii et al., 1996). These effects seem to be caused by the increase in *de novo* synthesis of DAG which consequently activates PKC (Brownlee, 2001). Isoforms β and δ appear to be activated first, although others are also activated (Brownlee, 2001). Besides the direct ways, hyperglycemia can activate PKC isoforms through ligation of AGE receptors and polyol pathway by increased ROS generation (Portilla et al., 2000; Keogh et al., 1997). The abnormal activation of PKC has been implicated in decreased production of nitric oxide in smooth muscle cells and glumeruli (Brownlee, 2001). Besides all the already mentioned effects, the activation of PKC by hiperglycemia is also involved in the overexpression of the fibrinolytic inhibitor PAI-1, activation of NF-κB and the regulation and activation (Brownlee, 2001; Yerneni et al., 1999; Pieper and Riaz ul, 1997) of several membrane-bound NAD(P)H-dependent oxidases (Feener et al., 1996). Several studies demonstrate that the inhibition of PKC activation is able to ameliorate the diabetic patients condition (Brownlee, 2001).

2.4. 4th hypothesis – increased flux through the hexosamine pathway

Another pathway of glucose metabolism that can mediate some deleterious effects is the hexosamine pathway (Brownlee, 2001; Du et al., 2000). Around 5% of the total glucose that enters cells is incorporated in this pathway which begins with the conversion of fructose 6-phosphate to glucosamine 6-phosphate by glutamine:fructose-6-phosphate amidotransferase (James et al., 2002). Under hyperglycemic conditions, in which a significant increase in the availability of nutrients exists, glucose is directed to the hexosamine pathway, in which UDP-N-acetylglucosamine is produced as an end-product (McClain and Crook, 1996). This compound is a substrate for glycosylation of intracellular factors, affecting the expression of several genes, including plasminogen activator inhibitor-1 (PAI-1) leading to the development of the microvascular complications that characterize diabetes (Gabriely et al., 2002; Goldberg et al., 2002).

Although there is no hypothesis capable of unifying all four mechanisms, there is strong evidence that the overproduction of superoxide anion by the mitochondrial electron transport chain may be one possible unifying factor, cause and consequence (Rolo and Palmeira, 2006). Superoxide anion is the initial oxygen free radical formed by mitochondria, which is then converted in other reactive species that cause mitochondrial perturbations in the heart, contributing to or synergizing with other deleterious effects of each one of the four above described pathways (Brownlee, 2001; Du et al., 2000; Nishikawa et al., 2000).

3. Mitochondrial abnormalities in the diabetic heart

As described in a previous section, diabetic patients are at increased risk of hypertension, coronary heart disease and

myocardial infarction, being cardiovascular disease the leading cause of mortality (Stamler et al., 1993; Abbott et al., 1988; Cohen-Solal et al., 2008). Diabetic cardiomiopathy is now the term used to describe ventricular dysfunction in diabetic patients (Margolis et al., 1973; Bell, 2003). Diabetes is characterized by high levels of circulating fatty acids (FA) resulting in increased cardiac fatty acid uptake, storage and metabolism (Stanley et al., 1997; Rodrigues et al., 1995). Cardiomyocytes accumulate fatty acids that are transported to mitochondria and oxidized to generate ATP (Lopaschuk et al., 2010). Similarly to what occurs in mitochondria, fatty acids can also be beta-oxidized in peroxisomes (Reszko et al., 2004; Marin-Garcia and Goldenthal, 2002) or instead incorporated into triglycerides (TAG) (Duncan, 2011). Normally, the heart does not accumulate significant amounts of lipids but when fatty acid supply is high, triglycerides can accumulate in cardiomyocytes. Increased intra-cellular triglyceride content was already demonstrated in human and animal models of Type 2 diabetes (Sharma et al., 2004; Murthy and Shipp, 1977; Szczepaniak et al., 2007). Substrate preference in the heart varies between glucose and fatty acids in a dynamic way in order to achieve the needs of this very active tissue (Duncan, 2011). The diabetic heart uses mainly fatty acid β-oxidation (FAO) to obtain energy since glucose transport through the sarcolemma is diminished because of insulin resistance (Duncan, 2011). In response to the increase in FA concentration, cardiomyocytes upregulate the expression of the enzymes necessary for β-oxidation (Marin-Garcia and Goldenthal, 2002). Because of this metabolic shift and insufficient mitochondrial FAO β-oxidation, inhibition of pyruvate dehydrogenase activity occurs, which impairs myocardial energy production and leads to accumulation of glycolytic and lipid intermediates, promoting lipo-glucotoxicity. Despite acting as an adaptative mechanism, extensive FAO can result in an impairment of the mitochondrial respiratory chain (Duncan, 2011).

Being the fulcrum of glucose and fatty acid metabolism, mitochondrial physiology can be altered by the impaired metabolism which is associated with diabetes (Duncan, 2011). Abnormalities in mitochondria are present in the skeletal muscle of humans with diabetes and insulin resistance (Mootha et al., 2003; Patti et al., 2003). Besides, the expression of genes associated with mitochondrial oxidative phosphorylation is also decreased (Mootha et al., 2003; Patti et al., 2003). There was a notable decrease in peroxisome proliferator-activated receptors (PPAR), co-activator of PGC-α, which coordinates gene expression of pathways that are involved in the biogenesis of mitochondria (Finck and Kelly, 2002). Furthermore, type 2 diabetes promotes a reduction in ATP synthesis and mitochondrial content (Morino et al., 2005). In skeletal muscle mitochondria from diabetic patients, morphological alterations and decreased activity of the oxidative phosphorylation was observed (Kelley et al., 2002; Ritov et al., 2005). Although fewer data are available for cardiac mitochondria, still several studies showed results of altered mitochondrial function in diabetic individuals (Diamant et al., 2003). As opposed to humans, cardiac mitochondrial function has been well explored in animal models for diabetes.

Using Type 1 diabetes animal models, increased mitochondrial biogenesis associated with a decrease in mitochondrial function and mitochondrial structural abnormalities were already demonstrated (Shen et al., 2004). Oliveira et al. demonstrated that heart mitochondria from streptozotocin (STZ)-treated diabetic Wistar rats not only have decreased mitochondrial respiration but also increased induction of the calcium-mediated mitochondrial permeability transition (Oliveira et al., 2003). The majority of hyperglycemia-induced cardiac mitochondrial alterations occur in the interfibrillar sub-population, which is critical for the process of energy generation for muscle contractility (Lumini-Oliveira et al., 2011). Interestingly, the deleterious effects of STZ-induced mitochondrial dysfunction in the heart were attenuated by endurance exercise (Lumini-Oliveira et al., 2011). A relationship between mitochondrial dysfunction and diastolic dysfunction of STZ-treated rats was already demonstrated by Flarsheim et al. (1996), suggesting that loss of cardiac calcium control by mitochondria in diabetic animals may also contribute to the deleterious effects of hyperglycemia on hemodynamics. A specific work indicated that hyperglycemia per se is not the culprit for mitochondrial alterations; instead, the authors pointed the finger to a multi-factorial mechanism including the overall metabolic remodeling (Lashin and Romani, 2004).

Data regarding type 2 diabetes models also indicate that mitochondrial physiology is altered (reviewed in Rolo and Palmeira, 2006). One piece of data shows that calcium tolerance is actually increased in the hearts of 26-week-old Goto-Kakizaki (GK) rats, a model for type 2 diabetes (Oliveira et al., 2001), which suggests possible adaptations at the mitochondrial level. The experiment was not repeated with older animals, in order to question whether advancing age would lead to progressive mitochondrial alterations. Decreased cardiac mitochondrial antioxidant defenses were present in the hearts of 12-week-old GK rats, which explained increased susceptibility to lipid peroxidation (Santos et al., 2003). In order to maintain ATP production, the heart is continuously maintaining high oxidative phosphorylation fluxes, which explains why about 40% of the total volume of cardiomyocytes is occupied by mitochondria, a high number when comparing to other tissues (Duncan, 2011). Although FAO oxidation is the main generator of ATP in the heart, a further increase in this type of metabolism is observed in the diabetic heart, which may contribute to increased damage to the contractile apparatus (Duncan, 2011; Buchanan et al., 2005; Mazumder et al., 2004). In tissues with high rates of respiration, such as cardiomyocytes, mitochondria are the major source of ROS production (Song et al., 2007). In the respiratory chain, the electron transport produces superoxide anion radicals at complexes I and III (Raha and Robinson, 2000; Turrens, 2003). Besides mitochondria, other mechanisms contribute to the increase in ROS in diabetes including accumulation of AGE (mentioned above) and NADPH oxidase activity (Coughlan et al., 2009; Li and Renier, 2006). Intra-mitochondrial-generated ROS are involved in mitochondrial DNA (mtDNA) damage, oxidative protein alteration and lipids peroxidation products, which can further contribute to induce protein and phospholipid damage (Duncan, 2011; Wallace, 1992). Moreover, superoxide anion can generate peroxynitrite after reacting with nitric oxide (NO) which causes intracellular nitrosylation and damage to mitochondrial structures (Turko et al., 2003). Oxidative stress has been implicated in the pathophysiology of diabetes and its complications (Fox et al., 2004; Folli et al., 2011), but this issue will be discussed in further sections.

4. p66Shc and intracellular oxidative stress

The p66Shc protein (ShcA family member), a growth factor adapter protein, is important in oxidative stress-induced deleterious events and evidence exists that putative therapeutic opportunities using p66Shc as a suitable target for the prevention of ROS-related tissue damage may be achievable (Arany et al., 2010; Zaccagnini et al., 2004).

The p66Shc protein differs from other ShcA family members (p56Shc and p46Shc) by the presence of an additional N-terminal domain (CH2) with a critical serine in position 36 (Ser36). It has been repeatedly demonstrated that its phosphorylation is important for the "pro-oxidative" and "pro-apoptotic" properties of p66Shc protein (Migliaccio et al., 1999; Pellegrini et al., 2005). Under physiological conditions, p66Shc takes part in the signal transduction from epidermal growth factor (EGF) receptor to the nucleus as a dominant negative regulator of Ras signaling pathway,

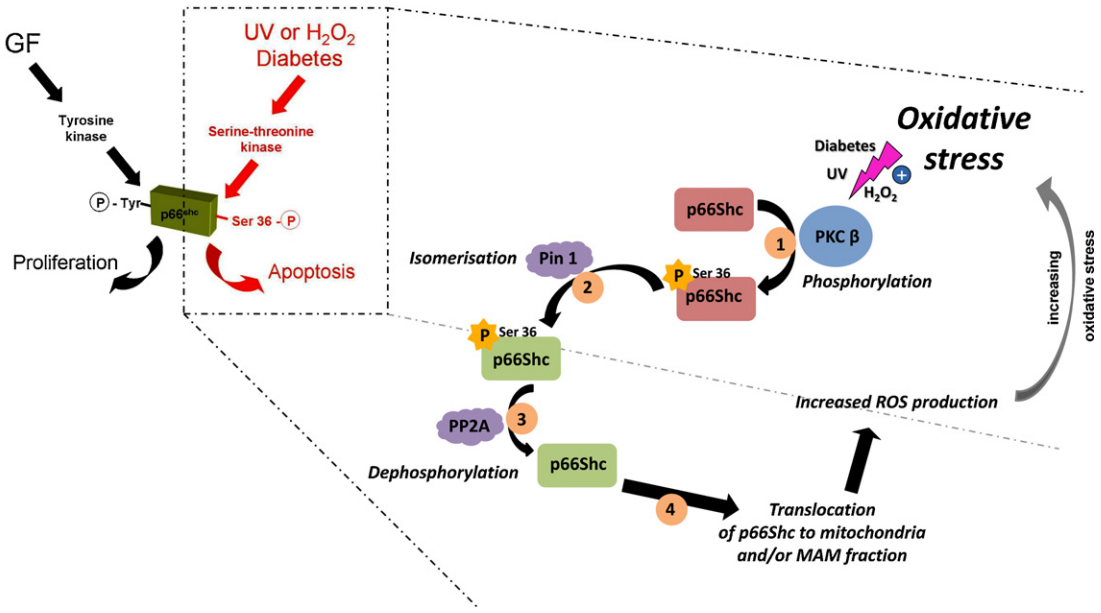

Fig. 1. Scheme presenting the steps of the oxidative stress-related activation of p66Shc pathway, leading to further increasing mitochondrial ROS production. All the intermediate steps are described in more detail in Section 4.

while under cellular oxidative stress, p66Shc is phosphorylated at Ser36 by serine-threonine kinases, including protein kinase Cβ (PKCβ). This signaling pathway has been described by us previously and four important steps have been identified (see Fig. 1). Step 1 – phosphorylation of p66Shc at Ser36 by PKCβ in response to the oxidative. Step 2 – isomerization of Ser36-phosphorylated p66Shc by a prolyl isomerase Pin1. Step 3 – dephosphorylation of isomerized Ser36-P-p66Shc by phosphatase A2 (PPA2). Step 4 – translocation of modified p66Shc to the mitochondria. Apoptosis signal-regulating kinase 1 (ASK1) (Li et al., 2007) and p38 MAP kinase (p38 MAPK) may also be involved in the phosphorylation of p66Shc (Yannoni et al., 2004). Additionally, p38 MAPK is sensitive to variety of factors increasing ROS generation, including osmotic and thermic shock, inflammatory cytokines, lipopolysaccharides, UV, interleukin 1 and H_2O_2 (Yannoni et al., 2004). Increased p66Shc content in mitochondria was correlated with alteration of mitochondrial structure, decrease of mitochondrial calcium uptake and enhanced mitochondrial ROS production triggering the mitochondrial route of apoptosis (Pinton et al., 2007). Recently, the presence of the p66Shc in plasma membrane-associated membrane (PAM) and in mitochondria-associated membrane (MAM) fractions has been confirmed, where it plays a role in signal transduction and in cellular response to the oxidative stress, respectively (Lebiedzinska et al., 2009). Nemoto et al. (2006) demonstrated decreased NADH metabolism in p66Shc$^{-/-}$ cells. Moreover, induction of glycolytic pathway has been observed in those cells (Nemoto et al., 2006).

4.1. p66Shc and ROS-related pathologies

The p66Shc Ser36 phosphorylation pathway is known to be activated in many various pathologies associated with oxidative stress, including mitochondrial defects/disorders, ischemia/reperfusion, diabetes, in tumors, during the aging process, as well as in neurodegenerative diseases, such as Alzheimer disease. p66Shc also participates in the obesity process, which may contribute to accelerate the aging process. Intracellular oxidative stress with a mitochondrial origin activates p66Shc phosphorylation on Ser36 which results in additionally increased ROS production. This in consequence initiates a vicious circle of p66Shc phosphorylation and p66Shc-dependent ROS production. Interestingly, the PKCβ inhibitor hispidin reduces phosphorylated p66Shc and significantly ameliorates mitochondrial bioenergetics parameters (Lebiedzinska et al., 2010).

In a classical example, tissue ischemia and reperfusion (I/R), the deprivation of oxygen due to blood flow cessation, followed by a re-oxygenation are events well known to induce increased ROS generation. An *in vitro* model of I/R can be studied in cultured cells, in which case the consecutive events are referred to as hypoxia and reoxygenation (H/R). ROS generation is an important factor in cell damage caused by H/R (Terui et al., 2004a). An immediate effect of H/R may be the ROS-induced activation of pro-apoptotic/inflammatory signaling in the cell (Terui et al., 2004a) or a direct damage to cellular organelles (Terui et al., 2004b; Droge, 2002). Excessive ROS, such as superoxide anion or hydrogen peroxide can damage the cell membrane, oxidize proteins, DNA and lipids, leading to activation or loss of important cellular processes, including apoptosis or proliferation (Droge, 2002; Ozaki et al., 2003). Yet, cells are not completely defenseless to H/R-induced ROS. Oxidative stress is counteracted by a number of enzymatic anti-oxidant systems (Cu/ZnSOD, MnSOD, catalase, glutathione peroxidase), non-enzymatic anti-oxidants (vitamin C, vitamin E) (Suski et al., 2011) and regulatory proteins such as the redox factor-1 (Ref-1) and STAT3 (Ozaki et al., 2002; Haga et al., 2003), many of which are induced by oxidative stress at the transcriptional level.

I/R-related events have also been studied in a variety of tissues and cells and in relation to a number of signaling pathways, also involving p66Shc. Carpi and co-workers provided evidence for a link between post-ischemic reperfusion and a reduction in ROS-induced myocardial injury in hearts without p66Shc (Carpi et al., 2009). In p66Shc$^{-/-}$ hearts, structural components of cardiomyocytes, such as lipids and proteins, were less prone to oxidant damage. Cardioprotection and antioxidant effects of p66Shc deletion were comparable to and not additive with those afforded by other antioxidant or protective interventions. The authors established a direct link between mitochondrial dysfunction and myofilament oxidation that is likely to contribute significantly to several forms of contractile impairment (Carpi et al., 2009).

The beneficial effect of p66Shc ablation during renal proximal tubule cells I/R was also demonstrated (Arany et al., 2010). By knocking down p66shc or inhibiting its Ser36 phosphorylation or cytochrome *c* binding site, the authors managed to attenuate ROS production and consequently prevent mitochondrial depolarization as well as kidney injury (Arany et al., 2010).

4.2. p66Shc and ROS in hyperglycemia

As it was previously mentioned, at least a part of cellular damage resulting from hyperglycemic conditions is caused by enhanced ROS production. On the other hand, enhanced ROS production can be the result of the activation of intracellular signaling pathways, *e.g.* phosphorylation of p66Shc at Serine36. If hyperglycemic conditions induce oxidative stress, and p66Shc triggers apoptosis in response to oxidative stress, a link between these two events/processes should exist. There are data about an increased synthesis of p66Shc mRNA in mononuclear blood cells of patients with type 2 DM, suggesting that increased level of p66Shc may result from prolonged high level of glucose in blood of these individuals, which contributes to the plasma increase in the oxidative damage marker, 8-isoprostanein these diabetic patients (Pagnin et al., 2005). Another link between p66Shc and hyperglycemia is the fact that p66Shc is essential for glucose uptake in skeletal muscle. Glucose transporters are constitutively present or are activated and relocalized to plasma membrane after hormone stimulation or in response to high energetic demands during hypoxia or oxidative stress conditions (Foley et al., 2011). It has been established that p66Shc inhibits intracellular signaling cascades in response to tyrosine receptor kinase activation. Inhibition of members of MAP kinase family results in abnormal glucose transport due to affected actin network. ERK1/2-associated pathway is constitutively active in myoblasts lacking p66Shc, resulting in the up-regulation of glucose transporters synthesis. In contrast, p66Shc overexpression results in decreased glucose transporters levels and glucose transport is reduced by 50% (Natalicchio et al., 2009). These findings implicate p66Shc protein in the regulation of glucose transport into cells, but the evidence for p66Shc involvement in pathogenesis of hyperglycemia still needs to be strengthened. With this in mind, the link between hyperglycemia-associated ROS production and activation of p66shc phosphorylation pathway has been explored in different diabetes models. In STZ-induced diabetes in mouse models, a strong induction of p66Shc synthesis is observed. More $ONOO^-$ is produced in aorta epithelium of wild type diabetic mice that in $p66Shc^{-/-}$ mice. This leads to an increase in protein nitrosilation which is considered a marker for nitrosative damage. Additionally, ablation of p66Shc prevents NO depletion and keeps the proper vessels epithelium relaxation, which under hyperglycemic condition is impaired. Although ablation of p66Shc protein had no effect on the SOD1 and SOD2 levels in a STZ-induced animal model, it significantly contributes to increased HO-1 content and activity (Camici et al., 2007). Rota et al. hypothesized that the premature cardiac aging due to the loss of cardiac progenitor cells can be in part driven by p66Shc signaling, since it was prevented by its deletion. In cardiac progenitor cells without p66Shc, replication predominates while enhanced apoptosis and necrosis is observed in diabetic wild type cells. Cardiac progenitor cells proliferate and differentiate into myocytes preserving proper heart function in diabetic $p66Shc^{-/-}$ animals, because MAP kinases are not inhibited by this protein (Rota et al., 2006). These observations led to the conclusion that down-regulation of p66Shc associated signaling cascades might be a therapeutic target in hyperglycemia-induced heart failure (Messina and Giacomello, 2006). Independently, Malhotra et al. showed that cardiac muscle damage due to hyperglycemia is connected with increased Ser36 p66Shc phosphorylation (Malhotra et al., 2009). Thus the observation of reduced cardiac progenitor cells death in $p66Shc^{-/-}$ model (Rota et al., 2006) can be explained by decreased p66Shc associated ROS production. Besides p66Shc serine phosphorylation, hyperglycemia induces also translocation of the protein to mitochondrial fractions and formation of p66Shc-cytochrome c complexes. Moreover, a 40% increase in apoptosis rate due to up-regulation of Bax, p53 and caspase 3 cleavage was also observed. In contrast, transfection of rat ventricular myocytes with Ser36-mutated p66Shc protein prevents high glucose-induced oxidative stress and damage. These data was confirmed by *in vivo* experiments on Akita diabetic mice suggesting that hyperglycemia induced oxidative stress and damage is mediated by Ser36 p66Shc phosphorylation pathway (Malhotra et al., 2009). Hyperglycemia contributes to advanced glycation end products (AGEs) which can trigger specific receptors activating pathways causing further oxidative damage by increasing oxidative stress in cells (RAGE) or receptors that activate prosurvival pathways (AGER1), with cell fate depending on the balance between them. Cai and colleagues showed that some AGEs induce ROS production in cell which directly leads to an increase in Ser36 p66Shc phosphorylation and enhanced oxidative stress. Interestingly, this effect causes FKHRL factor inhibition, thus hyperglycemia additionally attenuates antioxidant defense through a p66Shc pathway, since FKHRL1 (FOXO3A) is a transcription factor responsible for SOD2 and catalase synthesis (Lam et al., 2006; Purdom and Chen, 2003; Pani et al., 2009). Overexpression of AGER1 stops ROS-induced p66Shc phosphorylation (Cai et al., 2008). Several pieces of evidence appeared suggesting a crosstalk between p66Shc and FKHRL1. Cells transfected with mutated Ser36 in p66Shc or with siRNA are resistant to hyperglycemia induced oxidative stress and to oxidant DNA damage (Chintapalli et al., 2007). In animal models, AGE treatment induces renal injury from which p66Shc KO mice are protected. In p66Shc WT animals increased oxidative stress involves elevated level of NFκB and induction of NOX4 (NADP(P)H oxidase4) (Menini et al., 2007), which again confirms a central role for p66Shc in a variety of signaling pathways that are altered in hyperglycemia.

5. Concluding remarks

The present review clearly confirms a series of clinically important observations, such as: (a) diabetes have a negative impact in the cardiovascular system, (b) mitochondria and oxidative stress are central players in the cardiac pathophysiology of hyperglycemia and (c) p66Shc is a novel hub for several cell and mitochondrial signaling pathways that can be modulated by hyperglycemia from an apparent normal function to a pro-oxidant, pro-apoptotic function. Considering the previous observations, therapeutic strategies involving mitochondrial-directed antioxidants including mitoQ, SkQ and others (Foley et al., 2011; Skulachev et al., 2010; Armstrong, 2008) can be designed. General antioxidants including vitamin E may have marginal effects only (Oliveira et al., 2004). The p66Shc signaling pathway may also present a good target to prevent mitochondrial degeneration during hyperglycemia. In this regard the use of hispidin, a PKCβ inhibitor, may present a good opportunity. Future research aimed at developing more selective inhibitors of p66Shc signaling of mitochondrial degeneration is clearly needed.

It is also clear that more work is needed in animal models of diabetes in order to understand whether “negative” p66Shc signaling occurs mostly in the more affected tissues or is instead a widespread phenomenon and if this is the case, what differences exist in the entire activation network. The next step is to understand if pharmacological strategies aimed at controlling diabetes alter the role of p66Shc in the diabetic tissues and if this has a clear

impact in the quality of life of patients. If so, a novel avenue of research can be pursued.

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