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ENDOTHELIAL DYSFUNCTION IN DIABETES – CLASIC SOURCES OF VASCULAR OXIDATIVE STRESS (NADPH OXIDASES, eNOS UNCOUPLING AND XANTHINE OXIDASE)

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Abstract

Cardiovascular disease is the leading cause of disease / mortality worldwide. It is generally accepted that increased production of reactive oxygen species (ROS) has an important role in cardiovascular pathology, contributing to endothelial dysfunction and to the aggravation of atherosclerosis. Among all cardiovascular risk factors, diabetes mellitus is one of the most important. The worldwide prevalence of diabetes has increased rapidly even in developing countries, doubling the combined risk of cardiovascular events in patients with hypertension. In diabetes, increased reactive oxygen species (ROS) production leads to endothelial dysfunction, recognized by the presence of impaired vascular relaxation, increased vascular smooth muscle cells growth and hypertrophy, all together contributing to atherosclerotic plaque formation. On this basis, the vascular endothelium has emerged as a therapeutic target, with the aim to improve systemic metabolic state by improving vascular function. In this review we have focused on the most important sources of reactive oxygen species generated by vascular endothelium in diabetic patients (NADPH Oxidases, eNOS uncoupling, Xanthine oxidase). The importance of oxidative stress in mediating the vascular complications of diabetes is supported by studies showing that antioxidant therapy correct the vascular function in humans or in experimental models of diabetes. Therefore, understanding the physiological mechanisms involved in vascular disorders resulting from hyperglycemia is essential for the proper use of available therapeutic resources.

key words: *diabetes, oxidative stress / reactive oxygen species, NADPH Oxidase, xanthine oxidase, eNOS uncoupling.*

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Introduction

Cardiovascular disease is the leading cause of disease / mortality worldwide. It is generally accepted that increased production of reactive oxygen species (ROS) has an important role in cardiovascular pathology, contributing to endothelial dysfunction and that, to the aggravation of atherosclerosis. Among all cardiovascular risk factors, diabetes mellitus is one of the most important. The worldwide prevalence of diabetes has increased rapidly even in developing countries, doubling the combined risk of cardiovascular events in patients with hypertension [1]. The endothelium is the main target of cardiovascular risk factors, including diabetes, being the most involved in development of vascular inflammation and atherosclerosis [1,2]. Although low levels of reactive oxygen species (ROS) can play a physiological role in maintaining cardiac and vascular integrity [3], elevated levels of ROS play a pathophysiological role in cardiovascular dysfunction associated with diabetes [4]. Normally, ROS are produced in the vessel wall in a controlled and regulated manner. Under physiological conditions, low concentrations of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are produced in cells by respiratory chain from mitochondria [5], xantine oxidase [6], monoaminoxidase [9], NADPH oxidases [10] and arachidonic acid metabolizing enzymes including cytochrome P-450 enzymes [9]. They are controlled by endogenous antioxidants, like superoxide dismutase [10], catalase [11], and glutathione peroxidases [12]. Under pathological conditions, like diabetes, increased ROS production leads to endothelial dysfunction, recognized by the presence of impaired vascular relaxation, increased vascular smooth muscle cells growth and hypertrophy, all together contributing to atherosclerotic plaque formation.

Role of NADPH oxidases

NADPH oxidases (Nox) are enzymes present in the cardiovascular system, induced or activated by cardiovascular risk factors with a major contribution to vascular pathology. Moreover, ROS produced via Nox are important intracellular signaling molecules under physiological conditions [8]. Seven subtypes of Nox proteins were identified in mammalian cells: Nox1 - Nox5, Duox1 and Doux2. All these proteins differ in terms of activation, expression, interaction with other proteins and types of ROS formed [13]. Until now, the only relevant physiological function for Nox proteins is the production of ROS. In some systems there is a clear link between ROS produced via Nox and cellular function, like in immune response [14] or thyroid hormones (involved in signaling pathways that lead to hormone synthesis) [15].

Nox proteins from cardiovascular system are acting on different signaling pathways and ROS produced in this system (via Nox) are leading to cellular hypertrophy, inflammation and limit the bioavailability of nitric oxide (NO) [16]. ROS produced by NADPH oxidase are involved in G protein-receptor activation following stimulation of angiotensin 2 receptor type 1 (AT1) [17] and thrombin receptors [18]. Cardiovascular risk factors cause systemic oxidative stress and NADPH oxidase contributes to ROS formation in relation to almost all cardiovascular risk factors. Diabetes, obesity and metabolic

syndrome are situations with increasing systemic oxidative stress. Hyperglycemia, hyperinsulinemia and glycated proteins increase cellular ROS formation by a number of enzymes including NADPH oxidase, NOS and mitochondrial enzymes [19]. Hyperglycemia and increased free acid increase the NADPH oxidase activity in endothelial cells and vascular smooth muscle cells (VSMC) through pathways that include protein kinase C (PKC) [20].

Insulin increases the formation of ROS in adipocytes [21], fibroblasts [22], and VSMC [23]. Inhibition of Nox4 in adipocytes prevents the formation of ROS [24]. In the long term, insulin contributes to oxidative stress in cardiovascular system involving metabolic syndrome and increased free fatty acids. In people with type 1 diabetes NADPH oxidase expression is increased and may lead to eNOS uncoupling with subsequent endothelial dysfunction [25]. P47phox (a subunit of NADPH) is increased in endothelial cells in obese patients [26]. PPAR-γ agonists that increase insulin sensitivity improve vascular function in diabetic patients by reducing NADPH oxidase activity [27].

Hypercholesterolemia in diabetic patients activates PKC and increase the production of ROS by activation of endothelial Nox2 [28] and xanthine oxidase [29]. Oxidized LDL increases the expression of Nox2 [30] in mice. In humans, hypecholesterolemia increased Nox2 expression [31] and ROS generated in cells will be involved in LDL oxidation. Additional to lipid oxidation, NADPH oxidases contribute to: endothelial dysfunction, increase the expression of cytokines, matrix-metalloproteinases activation, and leukocyte adhesion [32]. Extremely important is that HDL can inhibit NADPH oxidase activation [33].

Partial uncouplig of eNOS

Decreased levels of NO in the vascular wall represent an early change induced by increased oxidative stress, characteristic for endothelial dysfunction. In this regard, ROS produced in excess reduce NO bioavailability and promote vascular cell injury [34].

Endothelial nitric oxide synthases (eNOS) has a homodimeric structure consisting of two identical subunits, each subunit being divided into two areas (reductase and oxygenase), connected by a sequence of amino acids containing a site for fixation of Ca^{2+}-calmodulin (CaM) [34]. In the presence of L-arginine and BH4 (tetra-hydro-biopterin), the two areas are "coupled" and eNOS is producing NO [35]:

$$\text{L-arginine} + O_2 + \text{NADPH} \xrightarrow{\text{BH4}} \text{L-citruline} + NO^{\cdot} + 2H_2O + NADP^+$$

NO has multiple biological implications, the most important being the physiological activation of soluble guanylate cyclase (cGMP) from the smooth muscle cells with the reuptake of the Ca^{2+} in sarcoplasmic reticulum and vascular relaxation.

In the absence of L-arginine or BH4, the two areas remain "partially uncoupled" and eNOS produces both NO and O_2^-. Consequence of "partial uncoupling" of eNOS is, on the one hand, decrease in NO production, and on the other hand, increased oxidative stress. Byproducts of the uncoupled eNOS (NO) and NADPH (O_2^-) combine rapidly and generate peroxy-nitrite ($ONOO^-$). This can oxidize essential cofactor BH4 to radical tri-hydro-biopterin (BH3-) or quinonoid 6,7 - [8H]-H2-biopterin (BH2). As

a consequence, eNOS is uncoupled from the formation of NO and eNOS is transformed into a dysfunctional enzyme generating O_2^- that contributes to vascular oxidative stress. An increase in eNOS expression in this case may aggravate the situation [36].

The cofactor BH4 is highly sensitive to oxidation by $ONOO^-$. Diminished levels of BH4 promote O_2^- production by eNOS ("eNOS uncoupling"). This transformation of eNOS from a protective enzyme to a contributor to oxidative stress has been observed also in patients with cardiovascular risk factors, including diabetes. In many cases, supplementation with BH4 has been shown to correct eNOS dysfunction in animal models and patients. In addition, folic acid and infusions of vitamin C are able to restore eNOS functionality, most probably by enhancing BH4 levels as well [37].

In diabetes, superoxide produced by the NADPH oxidase may react with NO released by the endothelial nitric oxide synthase (eNOS), thereby generating peroxynitrite ($ONOO^-$), leading to eNOS uncoupling and therefore eNOS-mediated superoxide production [38]. The reaction is mediated by PKC [39]. PKC activation can increase the expression of eNOS [40], and PKC inhibitors can reduce the expression of eNOS [41].

Xanthine oxidase

Increased production of ROS, especially $O2^-$, has been suggested to be the main cause of endothelial dysfunction [42]. Multiple evidences suggest that endothelial dysfunction, manifested by decreased vasodilator response, is associated with cardiovascular events. These adverse effects are attributed to the loss of vaso-protective properties of NO [42]. There is a major interest to understand the mechanisms leading to increased production of ROS and consecutive endothelial dysfunction in atherosclerosis.

Xanthine Oxido-Reductase (XOR) is an intracellular enzyme involved in the catabolism of purines. Its specific role is to catalyze the reduction of hypoxanthine and xanthine to uric acid [43]. XO exists in two forms, xanthine dehydrogenase (XDH) and xanthine oxidase (XO) [44]. In basal conditions, XOR exists as XDH. XDH can be converted to XO by sulfhydryl or proteolytic oxidation with generation of uric acid and superoxide O_2^- [43]. Multiple evidence suggests that ROS via XO activity plays a role in heart muscle disease [44]. Treatment of human mammary artery with allopurinol (XO inhibitor) causes a highly significant reduction in superoxide production, suggesting that this enzyme is an important source of ROS in vascular lesions [45]. Increased XO in the endothelial cells causes a reduction in NO bioactivity with an inverse relationship between endothelial XO activity and endothelium-dependent relaxation in patients with chronic heart failure [46].

Xanthine oxidase plays an important role in the generation of free radicals in diabetes. Inhibition of this enzyme should prevent oxidative stress in diabetes [47]. Allopurinol inhibits xanthine oxidase in vivo, and it is used in clinical practice [48]. Treatment of patients with allopurinol prevented glutathione oxidation and lipoperoxidation in human type 1 diabetes [47]. Inhibition of xanthine oxidase has proved effective in improving endothelial vasodilator function in hypercholesterolemic, but not hypertensive patients [49]. Recently, it

was reported that allopurinol protects against endothelial dysfunction in diabetic patients with mild hypertension [50]. In type 1 diabetes the liver releases xanthine oxidase to the plasma. The enzyme binds with glucosaminoglycans to the blood vessel, inducing local oxidative stress and tissue damage [47].

Conclusions

Alterations in vascular function are considered to be factors closely related to the development of cardiovascular complications in patients with diabetes. The most notable characteristic of endothelial dysfunction associated with diabetes is the vascular NO reduction. Multiple mechanisms are involved in this effect, but increased oxidative stress seems to be the first alteration that triggers several others. The importance of oxidative stress in mediating the vascular complications of diabetes is supported by studies showing that antioxidant therapy correct the vascular function in humans or in experimental models of diabetes. Therefore, understanding the physiological mechanisms involved in vascular disorders resulting from hyperglycemia is essential for the proper use of available therapeutic resources.

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