Hydrogen Peroxide Promotes Endothelial Dysfunction by Decreasing Nitric Oxide Bioavailability in Experimental Diabetes Mellitus

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Oxidative stress is the major pathophysiological mechanism that underlies the progression of both cardiovascular and metabolic diseases. The individual contribution of reactive oxygen species (ROS) to diabetes mellitus (DM)-related endothelial dysfunction is partially elucidated. While superoxide has been unequivocally involved in the progression of endothelial dysfunction, less it is known about the contribution of hydrogen peroxide (H_2O_2). The present study was purported to assess the amount of H_2O_2 generated in murine vessels in the presence of diabetes and to characterize its effects on vascular function, respectively. To this aim we isolated aortas from rats with streptozotocin-induced DM, measured the H_2O_2 production using the ferrous oxidation-xylenol orange (FOX) assay, and performed organ bath studies of vascular reactivity. Our data showed that in diabetic vessels: i) basal ROS production was comparable to the one generated after ex vivo stimulation with lipopolysaccharide and angiotensin II and ii) the amount of H_2O_2 generated in the vascular wall decreased relaxation via the impairment of NO signaling. In conclusion, metabolic abnormalities associated to diabetes elicit constant H_2O_2 overproduction with the subsequent impairment of NO signaling and endothelial dysfunction. Ongoing studies are addressing the sources of vascular H_2O_2 as well as the means to counteract its generation.

Keywords: hydrogen peroxide, endothelial dysfunction, experimental diabetes

It is widely accepted that the increased production of reactive oxygen species (ROS) plays a crucial role in endothelial dysfunction related to vascular pathologies and diabetes mellitus (DM) [1]. Indeed, the worldwide morbidity due to DM has rapidly increased mainly in developing countries, doubling the combined risk of cardiovascular events in patients with hypertension [2, 3]. The endothelium is the principal target of cardiovascular risk factors, including diabetes, its dysfunctionality underlying the development of chronic vascular inflammation [1,4]). Although low levels of ROS can play a physiological role in maintaining cardiac and vascular integrity [5], when generated in high amounts they play a pathophysiological role in cardiovascular dysfunction associated to diabetes [6].

Normally, ROS are produced in the vessel walls in a controlled and regulated manner. Under physiological conditions, low concentrations of ROS are produced in cells by respiratory chain from mitochondria [7], xantine oxidase [8], monoamine oxidase [9], NADPH oxidases [10] and arachidonic acid metabolizing enzymes including cytochrome *P*-450 enzymes [11]. They are controlled by endogenous antioxidants, namely superoxide dismutase [12], catalase [13], and glutathione peroxidases [14]. In 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 leading to accelerated atherosclerosis.

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Superoxide anion (O_2^-) is the main ROS whose role has unequivocally been ascribed to the progression of endothelial dysfunction in the setting of diabetes [15-18]. The metabolic abnormalities of diabetes are responsible for the increased O_2^- production that, in turn, causes the activation of the major pathways involved in the pathogenesis of complications: polyol pathway flux, increased formation of AGEs (advanced glycation end products), increased expression of the receptor for AGEs and its activating ligands, activation of protein kinase C isoforms, and overactivity of the hexosamine pathway [19]. Incubation of endothelial cells with high concentration of glucose activates protein kinase C and increases superoxide production [20]; yet, overproduction of superoxide in diabetes is not restricted to endothelial cells but occurs also in the smooth muscle layer [21]. According to the literature, the most relevant sources of superoxide are NADPH oxidase (NOX 2) and eNOS uncoupling [22].

However, the oxidative stress in the vasculature is contributed by other types of ROS, one of the most important being hydrogen peroxide (H_2O_2). In this study we assessed the amount of H_2O_2 generated in vasculature in the setting of experimental diabetes and its effects on vascular function, respectively.

Experimental part

Materials and methods

Study design and animal procedures

Wistar male rats were purchased from Cantacuzino Institute (Bucharest, Romania). Animals were housed

under standard conditions (constant temperature and humidity of 22.5 ± 2° C and 55 + 5%, 12-h light/dark cycle). Diabetes was induced by a single injection of streptozotocin (50 mg/kg STZ, IP). Age-matched control rats received an equal volume of vehicle (0.01 M citrate buffer, *p*H 4.5). Rats with a glycemia over 200 mg/dL (tail vein measurements) were considered diabetic. The duration of diabetes evolution was 1 month and glycemia and body weight were systematically monitored. Twenty-four hours prior to the experiment solid food was withdrawn with no limitation in water supply.

All experimental procedures used in this study were conducted in accordance with the Directive 2010/63/EU and the Romanian Law nr. 43/May 2014 concerning the protection of animals used for scientific purposes. The experimental protocol was approved by the Committee for Research Ethics of Victor Babes University for Medicine and Pharmacy of Timisoara, Romania.

All reagents used were of the highest quality available and were purchased from Sigma Aldrich.

Organ Culture

Aortic segments were isolated from the anesthetized rats, carefully cleared of connective tissue (to not damage the intimal surface), and cut into 2-3 mm wide rings (6-8 rings were obtained from the same animal). Some vascular samples from controls were dissected under sterile conditions, cleaned, and incubated at 37°C in EBM culture medium containing 0.1% BSA with either angiotensin II (Ang II, 100 nmol/L, Sigma-Aldrich) or lipopolysaccharide (LPS, 1 microlM/ml, Sigma-Aldrich), the classic inducers of *in vitro* endothelial dysfunction, and used for ROS measurements. In some rings, endothelium was removed by a short treatment with CHAPS (5 mg/mL dissolved in glucose solution 50g/L, 40 seconds).

Reactive oxygen species measurements

Hydrogen production was measured in rat aortic segments by the Ferrous iron xylenol orange OXidation method (FOX Assay, Sigma Aldrich). Peroxides oxidize Fe^{2+} to Fe^{3+} ions at acidic *p*H that forms a colored compound with xylenol orange (XO, 3,32 -bis[N,N-bis(carboxymethyl) aminomethyl]-o-cresolsulfonephthalein, sodium salt) that can be measured spectrophotometrically at 560 nm.

Organ Bath Experiments

Experiments on endothelial function were performed in aortic rings in the presence of diclofenac (10μmol/L). The concentration of phenylephrine, used for preconstriction, was adjusted to obtain a preconstriction level corresponding to 80% of the contraction elicited by KCl (80 mmol/L). Rings were contracted with cumulative doses of phenylephrine and relaxed to cumulative concentrations of acetylcholine (ACh) in the presence or absence of the endothelial NOS inhibitor N^{ω}-Nitro-L-arginine methyl ester (L-NAME, 10 μmol/L).

Statistics

All values are presented as mean ± SEM. Relaxations were calculated from individual dose-response curves. Statistical analysis was carried out by ANOVA or ANOVA for repeated measurements followed by Fisher LSD posthoc test. Values of $p < 0.05$ were considered significant.

Results and discussions

In experimental Ddabetes the arterial walls generate high amounts of H_2O_2 regardless the presence or the absence of endothelium

In the present study we focused on the role of H_2O_2 in vascular impairment from experimental diabetes. In streptozotocin-induced DM the basal level of this ROS is significantly increased as demonstrated by FOX assay (fig.1A). In order to investigate whether this effect is related to the endothelial layer we repeated the experiments in endothelium denuded arteries (treatment with CHAPS solution). The results (fig.1B) were similar with those obtained in intact endothelium samples, suggesting that the source of the vascular H_2O_2 is the entire arterial wall.

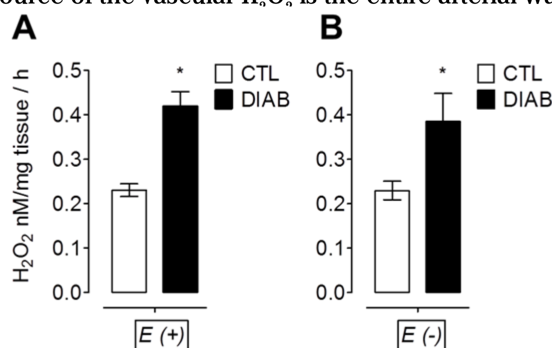

Fig. 1. Vascular generation of H_2O_2 in diabetic aortic rings (DIAB) *vs.* controls (CTL) determined by FOX assay in vessels with A) intact endothelium (E +) or B) denuded (E -) samples (n=6, *p<0.05)

H_2O_2 Generation In Diabetic Vessels Induces Endothelial Dysfunction Via The Impairment of NO Bioavailability

Vascular reactivity measurements were performed using the Schuler organ bath (Hugo-Sachs, Germany). The endothelium-dependent relaxation was significantly attenuated (fig. 2A). Also, contractility to L-NAME (Nω-Nitro-L-arginine methyl ester hydrochloride, 10 μM), the classic inhibitor of endothelial NO synthase (eNOS) was significantly increased in diabetic rings (fig.2B). Indeed, by inhibiting eNOS, less NO-mediated vasodilation could be elicited, with a subsequent increase in contractility. In order to elucidate whether the vascular reactivity changes are the consequences of H_2O_2 formation, experiments were repeated after incubation with PEG-catalase, the classic H_2O_2 scavenger. Indeed, incubation of the vascular segments with PEG-catalase prevented the vascular attenuation of acetylcholine–induced relaxation (fig.1A). This observation strongly incriminates the increased H_2O_2 production as being responsible for the vascular functional impairment and was further confirmed by the mitigation of ROS generation when the scavenger was present (fig. 2B).

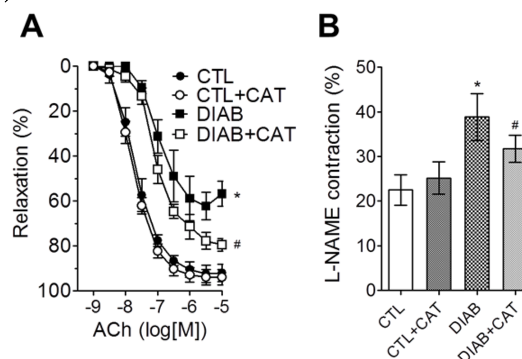

Fig. 2. Role of H_2O_2 in vascular function impairment. A) Acetylcholine-induced endothelium-dependent relaxation. n=6, *p<0.05 with and without diabetes, #p<0.05 with and without PEG-catalase (100 U/mL), B) Contraction to L-NAME (Nù-Nitro-L-arginine methyl ester hydrochloride), n=6, *p<0.05 with and without diabetes, #p<0.05 with and without PEG-catalase (100 U/mL)

Hydrogen Peroxide Generation In Diabetic Vessels Is Comparable To That Elicited by Ex Vivo Stimulation With Ang II And LPS

Two classic methods used to induce *ex vivo* endothelial dysfunction in murine models are using either angiotensin II (Ang II) or lipopolysaccharide (LPS). In both situations the amount of H_2O_2 is increased. In order to compare the amount of H_2O_2 in diabetic vessels with LPS and AngII stimulated segments, aortic segments were incubated either with Ang II or with LPS for 24 hours followed by H_2O_2 measurement (Fox Assay). The amount of H_2O_2 showed comparable values in all pathological vascular samples as compared to. controls (fig. 3).

In the present study we assessed the H_2O_2 generation and vasomotor function in aortic segments harvested from diabetic animals as compared to their normal controls. We reported an attenuation of relaxation in response to ACh, an effect that was partially mediated by the increased H_2O_2 generation that was responsible for the impairment in NO production. This last observation is supported by an excessive contractility to L-NAME recorded in organ bath studies.

Diabetes is associated with endothelial dysfunction which is known to promote abnormal vascular growth and atherosclerosis. It is known for years that cardiovascular

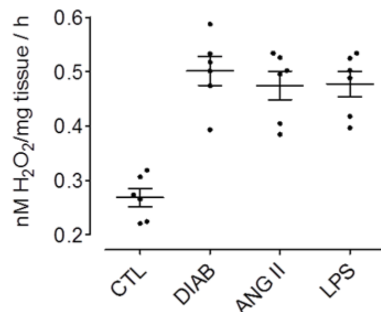

Fig. 3. Production of H_2O_2 in aortic segments in control (CTL), diabetic rings (DIAB) and, angiotensin II (ANG II, 100 nM) – and lipopolysaccharide (LPS, 1μg/mL) treated, respectively. FOX assay (n=6)

tissues can release a large amount ROS, including superoxide, H_2O_2, and nitric oxide [23]. Several enzymatic systems are currently considered responsible for the generation of reactive oxygen species (ROS) in development of endothelial dysfunction and neuropathy in diabetes, via limiting the bioavailability of nitric oxide (NO): NADPH oxidases, xanthine oxidase, mitochondrial respiratory chain and uncoupled endothelial NO synthase (eNOS) [24].

It is widely accepted that the main free radical with unequivocal role in the progression of endothelial dysfunction in diabetes is the superoxide anion (O_2^-). Indeed, it is the O_2^- that generates with NO the toxic peroxynitrite anion that further triggers eNOS uncoupling [25, 26]. NADPH oxidase is the major source of superoxide in cardiovascular system; in the setting of diabetes NADPH oxidase is activated via the protein kinase C pathway [27].

Hydrogen peroxide can be generated in cells by several cytoplasmic and mitochondrial enzymatic systems: NADPH oxidase 4, xanthine oxidase, monoamine oxidase, and the dysfunctional respiratory chain [28]. In physiological concentrations H_2O_2 has been recognized as a key signaling molecule, regulating of a wide array of biological processes. Accordingly, H_2O_2 has been reported to exert beneficial effects on vascular function by increasing the endothelial level of calcium with subsequent activation of eNOS, NO production, cyclic GMP level with the vascular smooth muscle relaxation, respectively [29, 30]. Unfortunately, in high concentrations it can promote inflammation and endothelial dysfunction, because is freely diffusible through cell structures and thus, may further potentiate the generation of superoxide [31]. Activation of most vascular NAD(P)H oxidases, xanthine oxidase and monoamine oxidases under pathological conditions, including diabetes, lead to the generation of H_2O_2 in large quantities, leading to detrimental consequences [32-34]. These concentration-dependent Janus-type functions of H_2O_2 are somehow similar to those reported for NO, i.e. vasodilation and protective effects at physiological concentrations but hypertrophic and pro-apoptotic when generated in large amounts [35].

Conclusions

We have demonstrated here that experimental diabetes is associated with a basal generation of hydrogen peroxide comparable to those elicited by *ex vivo* stimulation with angiotensin II and lipopolysacharride that, in turn, elicits NO signaling impairment and subsequent endothelial dysfunction. Further studies are required to tackle individual contribution of the vascular sources of H_2O_2 in order to mitigate its generation toward a minimal level that does not interfere with the vascular reactivity.

Acknowledgements:Research supported by the institutional grant code PII-C2-TC-2014.

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Manuscript received: 22.07.2015