Vascular Pharmacology 56 (2012) 115–121

Contents lists available at SciVerse ScienceDirect

Vascular Pharmacology

journal homepage: www.elsevier.com/locate/vph

Activation of hexosamine pathway impairs nitric oxide (NO)-dependent arteriolar dilations by increased protein *O*-GlcNAcylation

Timea Beleznai [a,b], Zsolt Bagi [a,*]

[a] *Department of Pharmacology, University of Oxford, OX1 3QT, United Kingdom*
[b] *Division of Clinical Physiology, Institute of Cardiology, University of Debrecen, H-4032, Hungary*

ARTICLE INFO

Article history:
Received 25 July 2011
Received in revised form 23 October 2011
Accepted 28 November 2011

Keywords:
Hyperglycemia
Glucosamine
Hexosamine pathway
Diabetes mellitus
O-GlcNAcylation
Microvessel

ABSTRACT

We hypothesized that under high glucose conditions, activation of the hexosamine pathway leads to impaired nitric oxide (NO)-dependent arteriolar dilation. Skeletal muscle arterioles (diameter: ~160 μm) isolated from male Wistar rats were exposed to normal glucose (NG, 5.5 mmol/L) or high glucose concentrations (HG, 30 mmol/L, for 2 h) and agonist-induced diameter changes were measured with videomicroscopy. Western blots were performed to identify the vascular levels of protein *O*-linked-*N*-acetyl-glucosamine (*O*-GlcNAc) and phosphorylated endothelial NO synthase (eNOS). In arterioles exposed to HG, dilations to histamine were abolished compared to those exposed to NG (max: $-6\pm6\%$ and $69\pm9\%$, respectively), while acetylcholine-induced responses were not affected. Inhibition of NO synthesis with N^G-nitro-L-arginine methyl ester (L-NAME) reduced histamine-induced dilations in NG arterioles, but it had no effect on microvessels exposed to HG. Dilations to the NO donor, sodium nitroprusside and constrictions to norepinephrine and serotonin were similar in the two groups. In the presence of the inhibitor of hexosamine pathway, azaserine, histamine-induced dilations were significantly augmented in arterioles exposed to HG (max: $67\pm2\%$). Moreover, exposure of vessels to glucosamine (5 mmol/L, for 2 h) resulted in reduced histamine-induced arteriolar dilations (max: $26\pm3\%$). The level of protein *O*-GlcNAcylation was increased, whereas the P-eNOS (Ser-1177) was decreased in HG exposed vessels. These findings indicate that a high concentration of glucose may lead to glucosamine formation, which impairs histamine-induced, NO-mediated arteriolar dilations. We propose that interfering with the hexosamine pathway may prevent microvascular complications in diabetes.

1. Introduction

Hyperglycemia is recognized as the primary cause in the pathogenesis of diabetic complications, such as abnormal microvascular reactivity, including impaired endothelium-dependent relaxation (Fulop et al., 2007a; Wells et al., 2003). Elevated blood glucose concentration results in increased intracellular glucose levels in various cell types, such as endothelial cells, because they are unable to limit glucose intake (Brownlee, 2005; Dominiczak, 2003; Gugliucci, 2000). Increased glucose concentration may lead to augmented glycosylation of various proteins that are important in the regulation of normal cellular homeostasis (Fulop et al., 2007b). Glycosylation is considered to be one of the key mechanisms responsible for the long-term consequences of diabetes. The *O*-linked enzymatic attachment of *N*-acetyl-glucosamine (*O*-GlcNAc) on serine and threonine residues of nuclear and cytoplasmic proteins is a specific form of glycosylation, which is a highly dynamic post-translational modification process (Hart et al., 2007). An increased *O*-GlcNAcylation of proteins is considered to be a major contributor to the etiology of various human diseases, such as hypertension (Lima et al., 2009b) and diabetes mellitus (Fulop et al., 2007a).

The level of *O*-GlcNAc formation is regulated, in part, by the metabolism of glucose via the hexosamine biosynthetic pathway (HBP) (Buse et al., 2002; McClain, 2002; Ngoh et al., 2010; Patti et al., 1999; Ross et al., 2000). HBP is highly sensitive to changes in intracellular glucose concentration. Earlier studies in rodents have demonstrated that chronically elevated flux through the HBP leads to insulin resistance and glucose toxicity by hyperglycemia (Dias and Hart, 2007; Wang et al., 2007). There are two major nutrient inputs of glucose and glucosamine, and one rate-limiting enzyme glutamine fructose-6-phosphate amidotransferase (GFAT) in the HBP (Hawkins et al., 1997; Traxinger and Marshall, 1991). It has been shown that adipocytes and muscle cells exposed to chronic high glucose levels in the presence of insulin develop insulin resistance, which can be prevented when adipocytes are incubated with a the inhibitor of GFAT (Marshall et al., 1991; McClain et al., 2002). An excessive flux through the HBP, results in many of the phenotypic characteristics of diabetes (McClain, 2002); however, the functional consequence of *O*-GlcNAcylation in microvessels is not clear.

Abbreviations: HBP, hexosamine biosynthetic pathway; GFAT, glutamine fructose-6-phosphate amidotransferase; eNOS, endothelial NO synthase; *O*-GlcNAc, *O*-linked attachment of *N*-acetyl-glucosamine; OGT, *O*-linked *N*-acetylglucosaminyl-transferase.

* Corresponding author at: Department of Pharmacology, University of Oxford, Mansfield Road, Oxford, OX1 3QT, UK. Tel.: +44 1865 271627; fax: +44 1865 271853.
E-mail address: zsolt.bagi@pharm.ox.ac.uk (Z. Bagi).

1537-1891/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.vph.2011.11.003

It has been shown that hyperglycemia impairs endothelium-dependent vasodilation in diabetic patients (Beleznai et al., 2011) and healthy human subjects (Picchi et al., 2010). Previously, we demonstrated that in the presence of chronic hyperglycemia (experimental type 1 diabetes) (Bagi and Koller, 2003) or acute elevation of glucose concentration elicits reduction of NO-mediated dilations in skeletal muscle arterioles of the rat, due to the impaired synthesis of NO (Bagi et al., 2004). The endothelial NO synthase (eNOS) is classified as a constitutive and Ca^{2+}/calmodulin-dependent enzyme. In addition to changes in intracellular levels of Ca^{2+} a number of post-translational mechanisms have been proposed to regulate eNOS activity, including phosphorylation of eNOS (Fleming and Busse, 1999). Interestingly, it has been found that high glucose and glucosamine caused a reduction in insulin-induced eNOS activity as a result of increased O-GlcNAcylation in human coronary endothelial cells in culture (Federici et al., 2002). Whether increased activation of HBP and augmented *O*-GlcNAcylation by high glucose concentrations affects NO-mediated vasomotor responses in resistance arteries is not known.

In this study we have tested the hypothesis that under high glucose conditions activation of the hexosamine pathway leads to *O*-GlcNAcylation of eNOS. This mechanism may prevent phosphorylation-dependent activation of eNOS and leads to a diminished NO-dependent arteriolar dilation. Isolated skeletal muscle arterioles were exposed to high glucose concentrations and agonist-induced changes in diameter were measured with videomicroscopy before and after interfering with NO synthesis and HBP pathway. Phosphorylation of eNOS was also detected to provide evidence for changes in phosphorylation of eNOS at Ser-1177, known to be associated with activation of enzyme activity.

2. Material and methods

Experiments were carried out on male Wistar rats ($n = 22$, weighing ~300 g). The animals were housed in the animal care facility and were fed standard rat chow and drank tap water *ad libitum* with a 12-h light–dark cycle. All experimental procedures were in compliance with the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes. After overnight fasting, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg kg^{-1}) and the gracilis muscle was removed. The animals were then euthanized by an additional injection of sodium pentobarbital (150 mg kg^{-1}).

2.1. Measurment of diameter of isolated, cannulated and pressurized arterioles

With the use of microsurgical instruments and an operating microscope the gracilis muscle arteriole (~1.5 mm in length) was isolated and transferred into organ chambers containing two glass micropipettes filled with physiological salt solution (PSS), composed of (in mm) 110.0 NaCl, 5.0 KCl, 2.5 $CaCl_2$, 1.0 $MgSO_4$, 1.0 KH_2PO_4, 5.5 glucose and 24.0 $NaHCO_3$, equilibrated with a gas mixture of 10% O_2 and 5% CO_2, balanced with nitrogen, at pH 7.4. The vessels were cannulated at both ends and the micropipettes were connected with silicone tubing to a pressure servo control system (Living Systems Instrumentation, Burlington, VT, U.S.A.) to set the intraluminal pressure to 80 mm Hg. The temperature was set at 37 °C by a circulating bath temperature controller (Cole Parmer, Vernon Hills, IL, U.S.A.). Images were collected with a digital camera (CFW1310, Scion Corp., Frederick, MO, U.S.A.) connected to a microscope (Nikon, Eclipse 80i). The internal diameter at the midpoint of the isolated arteriole was measured offline by Image J software (NIH Image, Bethesda)(Bagi et al., 2002; Koller and Bagi, 2004).

2.2. Dilation to agonist

In the first series of experiments the gracilis muscle arterioles were exposed to PSS that contained 5.5 mmol/L glucose. During an incubation period of 1 h at 37 °C, spontaneous myogenic tone developed in the isolated arterioles in response to the intraluminal pressure of 80 mm Hg. Cumulative concentrations of the endothelium-dependent vasodilator histamine (1 nmol/L–10 μmol/L), acetylcholine (Ach, 1 nmol/L–1 μm/L) or endothelium-independent vasodilator sodium nitroprusside (SNP, 1 nmol/L–1 μmol/L) were administrated to the vessels and changes in diameter were measured. Furthermore, vasoconstrictors that primarily act on smooth muscle cells, such as norepinephrine (NE, 0.3 nmol/L–1 μm/L) and serotonin (5-HT, 0.1 nmol/L–1 μmol/L) were also applied. In a separate set of experiments the vessels were incubated with 30 mmol/L glucose or 5 mmol/L glucosamine (incubation time: 2 h) to investigate changes of agonist-induced arteriolar responses. As an osmotic control, mannitol (25 mmol/L) was administered and histamine-induced vasodilator response was reassessed. Histamine-induced dilations were also observed in the presence of inhibitor of NO-synthase, L-NAME (200 μmol/L, incubation for 20 min) with or without azaserine (20 μmol, incubation for 20 min), which is a glutamine analog and known to irreversibly inhibit fructose-6-phosphate amidotransferase (GFAT)(Liu et al., 2007). Azaserine was also employed in glucosamine exposed arterioles and histamine-induced responses were reassessed.

2.3. Immunoblots

Branches of femoral arteries were dissected from Wistar rats, cleared of connective tissue. Vessels were exposed to normal and high glucose concentrations as well as glucosamine and incubated for 2 h after which they were snap frozen in liquid nitrogen. After the addition of 20 μl RIPA buffer (containing protease and phosphatase inhibitors) arteries were homogenized and 20 μl of Laemmli sample buffer was added (from Sigma Inc.). Immunoblot analysis was carried out as described before (Jebelovszki et al., 2008).

Primary antibodies were used for detection of *O*-GlcNAc (dilution 1:1000, CTD110.6, Convance, USA) as well as for detection of protein expression of eNOS (anti-eNOS, Transduction, dilution 1:1000) and P-eNOS levels (anti-P-eNOS-Ser-1177, dilution 1:500, BD Bioscieses). Anti-β-actin IgG obtained from Abcam Inc was used as loading control. Corresponding horseradish peroxidase laballed secondary antibodies were used and signals were revealed with chemiluminescence and visualized autoradiographically. Optical density of bands was quantified by using NIH Image software.

2.4. Drugs

All salts and chemicals were purchased from Sigma-Aldrich Co. (St Louis, MO, U.S.A). All solutions were prepared in destilled water and on the day of the experiment and final concentrations are reported.

2.5. Data analysis

Statistical analyses were performed using GraphPad Prism Software (San Diego California USA) by two-way ANOVA repeated measures followed by Tukey's post-hoc test or Student's t-test as appropriate. Data are expressed as means + SEM. Agonist-induced arteriolar dilations were expressed as changes in arteriolar diameter as a percentage of the maximal dilation defined as the passive diameter of the vessel at 80-mm Hg intraluminal pressure in a Ca^{2+}-free medium. $P<0.05$ was considered statistically significant.

3. Results

3.1. Effect of high glucose concentration on arteriolar dilations

In isolated, pressurized (80 mm Hg) gracilis muscle arterioles of the rat, active arteriolar tone developed (~30%) in response to intraluminal pressure without the use of any vasoactive agent. In comparison with