Vascular-dependent effects of elevated glucose on postganglionic sympathetic neurons

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Damon DH. Vascular-dependent effects of elevated glucose on postganglionic sympathetic neurons. Am J Physiol Heart Circ Physiol 300: H1386–H1392, 2011. First published January 7, 2011; doi:10.1152/ajpheart.00300.2010.—Perivascular sympathetic nerves are important determinants of vascular function that are likely to contribute to vascular complications associated with hyperglycemia and diabetes. The present study tested the hypothesis that glucose modulates perivascular sympathetic nerves by studying the effects of 7 days of hyperglycemia on norepinephrine (NE) synthesis [tyrosine hydroxylase (TH)] release, and uptake. Direct and vascular-dependent effects were studied in vitro in neuronal and neurovascular cultures. Effects were also studied in vivo in rats made diabetic. In vivo, elevated glucose did not affect TH or NE uptake, but it increased NE release. Release from vascular sympathetic cultures grown in high glucose (HG; 25 mM) was less than that in control animals (5.39 ± 0.28%; n = 5; P < 0.05; unpaired t-test). In vivo, elevated glucose did not affect TH or NE uptake, but it increased NE release. Release from vascular sympathetic cultures grown in HG (1.8 ± 0.2%; n = 5) was greater than that from controls grown in LG (0.37 ± 0.28%; n = 5; P < 0.05; unpaired t-test). These data identify a novel vascular-dependent effect of elevated glucose on postganglionic sympathetic nerves that is likely to affect the function of perivascular sympathetic nerves and thereby affect vascular function.

THE SYMPATHETIC NERVOUS SYSTEM is a major determinant of cardiovascular function. The sympathetic nervous system acts in part via release of neurotransmitters from postganglionic sympathetic nerves innervating blood vessels. These nerves are critical for maintenance of blood pressure and distribution of blood flow (19, 21). Cardiovascular complications are a leading cause of morbidity and mortality in patients with diabetes (1). The present study tested the hypothesis that glucose modulates perivascular sympathetic nerves by studying the effects of 7 days of hyperglycemia on norepinephrine (NE) synthesis [tyrosine hydroxylase (TH)] release, and uptake. Direct and vascular-dependent effects were studied in vitro in neuronal and neurovascular cultures. Effects were also studied in vivo in rats made diabetic. In vivo, elevated glucose did not affect TH or NE uptake, but it increased NE release. Release from vascular sympathetic cultures grown in high glucose (HG; 25 mM) was less than that in control animals (5.39 ± 0.28%; n = 5; P < 0.05; unpaired t-test). In vivo, elevated glucose did not affect TH or NE uptake, but it increased NE release. Release from vascular sympathetic cultures grown in HG (1.8 ± 0.2%; n = 5) was greater than that from controls grown in LG (0.37 ± 0.28%; n = 5; P < 0.05; unpaired t-test). These data identify a novel vascular-dependent effect of elevated glucose on postganglionic sympathetic nerves that is likely to affect the function of perivascular sympathetic nerves and thereby affect vascular function.

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mitomycin-treated postganglionic sympathetic neurons. These cultures were then grown in neuronal medium.

Western analysis. Cells were pelleted in PBS and then lysed in enhanced RIPA buffer. Tissues were excised from adult rats and homogenized in enhanced RIPA buffer (50 mM Tris base, 150 mM NaCl, 10 mM EDTA, 0.25% deoxycholate, 1% Nonidet P-40 substitute, 10% glycerol, 1% protease inhibitor cocktail, 1 mM DTT, and 0.1% sodium dodecyl sulfate). Cell and tissue samples were diluted with an equal volume of 2× electrophoresis loading buffer, boiled for 5 min, electrophoresed on 4–20% gradient Tris-glycine polyacrylamide gels, and transferred to nitrocellulose membranes. The membranes were blocked with 3% nonfat dry milk in PBS containing 0.05% Tween (PBST) for 20 min at room temperature and then incubated overnight at 4°C in blocking solution containing the appropriate primary antibody. Unbound primary antibodies were then removed with three 5-min washes (PBST), and the membranes were incubated for 1 h at room temperature in PBST containing 3% nonfat dry milk and a 1:3,000 dilution of horseradish peroxidase-conjugated secondary antibody. Unbound secondary antibodies were removed with three 5-min washes (PBST). Horseradish peroxidase was then detected with enhanced chemiluminescence (Pierce) and documented on autoradiographic film. Signals were quantified densitometrically.

**RESULTS**

Neurotransmitters from postganglionic sympathetic neurons innervating target organs produce the effects of the sympathetic nervous system. The primary neurotransmitter released by postganglionic sympathetic neurons is NE. The present study considered the direct and vascular-dependent effects of elevated glucose on postganglionic sympathetic neuronal function.

**Fig. 1.** Elevated glucose decreases tyrosine hydroxylase (TH) in postganglionic sympathetic neuronal cultures. A: representative Western analyses of TH and GAP43 in cultures of postganglionic sympathetic neurons grown for 7 days in low (LG; 5 mM) and high glucose (HG; 25 mM). B: quantitative analysis. Values are means ± SE. TH in neurons grown in HG was significantly less than that in LG neurons (*P < 0.05; 2-tailed 1-sample t-test; n = 4).

**Fig. 2.** Elevated glucose decreases uptake but does not affect norepinephrine (NE) release in postganglionic sympathetic neuronal cultures. Postganglionic sympathetic neuronal cultures were grown for 7 days in LG (5 mM; n = 6), HG (25 mM; n = 6), or HG plus 100 U/ml polyethylene glycol-catalase (HG + CAT; n = 4). Basal (A) and 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP)-stimulated norepinephrine (NE) release (B) and NE uptake (C) were measured. Values are means ± SE. NE uptake into neurons grown in HG was less than that into neurons grown in LG (*P < 0.05; 1-column t-test). NE uptake into neurons grown in the presence of HG + CAT was greater than that in the presence of HG (+P < 0.05; unpaired t-test).
elevated glucose on TH, the rate-limiting enzyme for the synthesis of NE, NE release, and NE uptake in postganglionic sympathetic neurons.

Direct effects of elevated glucose were studied in cultures of postganglionic sympathetic neurons. Neurons were grown for 7 days in low (LG; 5 mM) or high glucose (HG; 25 mM). These concentrations of glucose correspond to plasma glucose concentrations in normoglycemic and hyperglycemic animals and humans. Figure 1 shows the effects of elevated glucose on TH and GAP43 expression. TH is the rate-limiting enzyme in catecholamine synthesis and is thus a determinant of NE synthesis. GAP43 is a neuronal marker that was not reproducibly affected by the experimental conditions used in the present studies and is a marker of the amount of neuronal protein present in the samples. Representative (n = 4) Western analyses and quantitative analysis are shown. These data indicate that elevated glucose decreased TH. TH expression in neurons grown in HG was less than that in neurons grown in LG. The direct effects of 7 days of elevated glucose on NE release and uptake are shown in Fig. 2. Elevated glucose did not affect basal (Fig. 2A) or stimulated NE release (Fig. 2B), but it decreased NE uptake (Fig. 2C).

Many effects of elevated glucose are due to increased levels of reactive oxygen species (ROS) (3). I determined whether ROS contributed to the observed effect of HG on NE uptake by assessing the effects of HG in the presence of polyethylene glycol (PEG)-catalase. Catalase converts hydrogen peroxide to water and thus decreases levels of ROS (16). PEG-catalase reduced the effect HG on NE uptake (Fig. 2C).

Elevated glucose markedly affects vascular cells (3), and vascular cells markedly affect postganglionic sympathetic neurons (10–12, 23, 24). Thus elevated glucose may affect postganglionic sympathetic neurons indirectly by affecting vascular cells. Vascular-dependent effects of elevated glucose were assessed in sympathetic neurovascular cultures. In blood vessels, the primary targets of postganglionic sympathetic neurons are VSM. In the present study, these vascular cells were considered. In neurovascular cultures, postganglionic sympathetic neurons were grown in the presence of VSM derived from adult rat tail arteries. These VSM were chosen because they are representative of VSM from sympathetically innervated arteries (12). The sympathetic neurons were in direct contact or in close proximity to the VSM, and thus there was reciprocal contact (or proximity)-dependent communication as well as reciprocal exchange of soluble mediators.

The effects of elevated glucose on TH and GAP43 expression in sympathetic neurovascular cocultures were assessed. As noted above, in these cocultures, the neurons and VSM were in direct contact, and thus the samples analyzed would contain both neurons and VSM. Figure 3A shows representative Western analyses of TH and GAP43 expression in postganglionic sympathetic neuron cultures and tail artery VSM. These data indicate that VSM do not express detectable levels of TH and GAP43.

Fig. 3. Elevated glucose does not decrease TH in sympathetic neurovascular cultures. A: representative Western analyses of TH and GAP43 in sympathetic neuronal cultures and tail artery vascular smooth muscle cultures (VSM). B: representative Western analyses of TH and GAP43 in SN grown for 7 days in LG and HG. C: quantitative analysis for data in B. Values are means ± SE; n = 4. D: representative (n = 4) Western analyses of TH and GAP43 in sympathetic/HEK-293 cocultures grown for 7 days in LG and HG.

Fig. 4. Elevated glucose increases NE release from sympathetic neurovascular cultures. Sympathetic neurovascular cultures were grown for 7 days in LG (n = 5), HG (n = 5), or HG + CAT (n = 2). Basal (A) and DMPP-stimulated NE release (B) and NE uptake (C) were measured. Values are means ± SE. NE release from the neurons grown in HG was greater than that from neurons grown in LG (*P < 0.05; unpaired t-test; n = 5).
of TH or GAP43 and that TH and GAP43 in the neurovascular cultures would be attributable to the postganglionic sympathetic neurons and not the VSM.

Elevated glucose did not affect TH or GAP43 expression in sympathetic neurovascular cultures (Fig. 3, B and C; n = 4; P > 0.05; 1-sample t-test). These data indicate that the effect of elevated glucose on neurons grown in the presence of VSM differs from that on neurons grown in the absence of VSM (Fig. 1), suggesting that the VSM altered the response of the neurons to glucose. Was this effect specific to VSM? Figure 3D shows representative Western analyses (n = 4) of TH and GAP43 in sympathetic neuron/HEK-293 cultures grown for 7 days in LG and HG. Elevated glucose markedly reduced TH and GAP43 in these cultures, suggesting that both VSM and HEK-293 cells altered the neurons response to glucose, but in markedly different ways.

The effects of HG on NE release and uptake in sympathetic neurovascular cultures are shown in Fig. 4. Elevated glucose did not affect basal NE release or NE uptake but increased DMPP-stimulated NE release. PEG-catalase did not inhibit the HG-induced increase in stimulated NE release (n = 2), which suggests that ROS did not contribute to this effect.

To assess the effects of elevated glucose on postganglionic sympathetic neurons in vivo, rats were made hyperglycemic with STZ. The effects of 7 days of hyperglycemia were then studied. Seven days after injection, STZ rats were hyperglycemic (blood glucose > 296 mg/dl) compared with control rats (blood glucose = 108 ± 5.4 mg/dl). Western analyses indicate that in vivo, 7 days of elevated glucose did not affect TH in cell bodies of postganglionic sympathetic neurons in superior cervical ganglia (Fig. 5A) or in sympathetic nerve fibers innervating femoral arteries (Fig. 5B). Representative analyses and corresponding quantitative analyses are shown. TH in the ganglia was normalized to GAP43, which is an index of the amount of neuronal protein in each sample. TH in the arteries was normalized to smooth muscle α-actin, which is an index of the amount of vascular smooth muscle protein in the sample.

In vivo effects of elevated glucose on femoral artery sympathetic nerves were also studied. Electrically stimulated NE release (Fig. 6B) from perivascular nerves of isolated femoral arteries from rats that had been hyperglycemic for 7 days (STZ) was greater than that from arteries of control rats. Basal release (Fig. 6A) and uptake (Fig. 6C) were not significantly different.

In some blood vessels, NE release from perivascular sympathetic nerves is inhibited by presynaptic α2-adrenergic receptors, and the function of these receptors can be inhibited by ROS (14). Thus HG-induced increases in ROS could increase NE release by inhibiting the function of presynaptic α2-adrenergic receptors. This does not appear to be the case in the present study, since the α2-adrenergic antagonist yohimbine (1 μM) did not affect stimulated NE release from femoral arteries of normoglycemic animals. Release in the presence of yohimbine (11.1 ± 0.3%) was not different from that in the absence of yohimbine (14.8 ± 2.6%; P > 0.05; unpaired t-test; n = 3).

**DISCUSSION**

The sympathetic nervous system is a major determinant of cardiovascular function. Sympathetic control of vascular and cardiovascular function is mediated in via perivascular sympathetic nerve fibers (4–6). Vascular complications are a major cause of morbidity and mortality in patients with diabetes (1), and studies suggest that perivascular nerves contribute to vascular complications of diabetes (8, 17). Elevated glucose causes many of the complications of diabetes (3, 15, 36). The present study considers how elevated glucose affects postganglionic sympathetic neurons and perivascular sympathetic nerves.

Postganglionic sympathetic neurons are modulated by the targets they innervate (10–12, 23, 24). In blood vessels, vascular cells affect survival (10, 23), growth (12, 24), and neurotrophin expression (11) of these neurons. Hyperglycemia markedly affects vascular cells (3), which suggests that hyperglycemia would affect vascular modulation of perivascular nerves. For example, work in this laboratory has shown that vascular-derived VEGF promotes the growth of perivascular nerves (24), and Natarajan et al. (27) have shown that hyper-
Elevated glucose is known to induce oxidative stress in neurons (34) and in vascular cells (3), and oxidative stress affects many cellular processes. The present studies suggest that ROS contribute to direct effects of HG on NE uptake in the neuronal cultures (Fig. 2C) but do not contribute to the effect of HG in the sympathetic neurovascular cultures (Fig. 4B). In addition, the present study found that inhibition of $\alpha_2$-adrenergic receptors did not affect NE release from isolated rat femoral arteries, which suggests that ROS-dependent inhibition of presynaptic $\alpha_2$-adrenergic receptors did not mediate the HG-induced increase in NE release from these arteries (Fig. 6B). Potential mechanisms underlying the effects of HG in the sympathetic neurovascular cultures and in the isolated femoral arteries are currently under investigation.
Elevated glucose also inhibits ATP-sensitive potassium (K_{ATP}) channels (26, 28), which if present could depolarize the nerves and increase NE release (7). Studies in rat tail artery suggest that this is not a primary mechanism underlying the glucose-induced increase in NE release shown in Fig. 6B. Acute (1 h) elevations of glucose, which would inhibit K_{ATP} channels, did not affect stimulated NE release from isolated rat tail arteries (P > 0.05; n = 4; data not shown).

It is well established that hyperglycemia has detrimental effects on many neurons and that neuropathy is a debilitating complication of diabetes (15, 34, 36, 38). Many studies have indicated that vascular sympathetic nerves remain functional in patients and animals with hyperglycemia and diabetes. Inhibition of NE binding to α-adrenergic receptors decreases blood pressure (8) in patients with diabetes. This indicates that in these patients, NE released by functioning postganglionic neurons, including vascular neurons, affects blood pressure and blood flow. Morphological analyses in humans and rats indicate that some, but not all, postganglionic sympathetic neurons are susceptible to hyperglycemia (30, 31, 33, 34). Vascular sympathetic neurons were not specifically identified in these studies, but additional evidence indicates that the function of this subset of postganglionic sympathetic neurons is maintained in hyperglycemic animals. Frisbee (18) reported enhanced activity of vascular sympathetic nerves in skeletal muscle of hyperglycemic obese Zucker rats, and Hart et al. (20) reported that arteries from hyperglycemic STZ-treated rats contained less but took up and released more NE than arteries from normoglycemic rats. Speirs et al. (35) reported that 3 mo of hyperglycemia did not affect the density or function of sympathetic nerves innervating rat tail arteries. Martinez-Nieves and Dunbar (25) found that acute inhibition of NE binding to α-adrenergic receptors increased blood flow in femoral arteries of rats that had been hyperglycemic for 5–6 wk, suggesting that NE was being released from postganglionic sympathetic neurons innervating these arteries. The persistence of sympathetic neurovascular function in diabetes suggests that perivascular sympathetic nerves are resistant to the detrimental effects of hyperglycemia. Little is known about the effects of hyperglycemia on perivascular sympathetic nerves and the mechanisms underlying the potential resistance of these nerves to hyperglycemia. The present studies demonstrate that glucose is likely to have direct and vascular-dependent effects on perivascular sympathetic nerves and suggest that vascular-dependent mechanisms maintain or enhance sympathetic neurovascular transmission. A recent study suggested that diabetes depresses synaptic transmission in sympathetic ganglia (9). Vascular-dependent increases in NE release may act to oppose decreased ganglionic transmission and thereby maintain sympathetic neurovascular transmission. Additional in vivo studies are warranted to further investigate the effects of hyperglycemia on sympathetic neurovascular transmission and how these contribute to the vascular and cardiovascular complications associated with diabetes.

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REFERENCES


