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Vascular endothelial growth factor protects postganglionic sympathetic neurons from the detrimental effects of hydrogen peroxide by increasing catalase

Deborah H. Damon

Department of Pharmacology, University of Vermont, USA

Abstract

Aim—Vascular production of hydrogen peroxide is implicated in the development and progression of vascular disease. Hydrogen peroxide also promotes neuronal degeneration, which suggests that vascular hydrogen peroxide would promote degeneration of perivascular sympathetic nerves. Vascular cells also produce vascular endothelial growth factor, which could protect perivascular nerves from the detrimental effects of hydrogen peroxide. The aim of this study was to test these hypotheses.

Methods—The effects of hydrogen peroxide and vascular endothelial growth factor on neuronal survival and norepinephrine uptake were studied in cultures of rat postganglionic sympathetic neurons. Western analyses of catalase and growth associated protein 43 were performed and reactive oxygen species were measured using the fluorescent indicator 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester.

Results—Hydrogen peroxide (30 μ M) decreased the survival of postganglionic sympathetic neurons ($57.8 \pm 4.8\%$ of control) and decreased norepinephrine uptake into the neurons ($14 \pm 6\%$ of control). Hyperglycemia, which is known to increase hydrogen peroxide, also decreased survival ($31.4 \pm 12\%$ of control) and norepinephrine uptake ($25.3 \pm 11\%$ of control). Vascular endothelial growth factor reduced the effects of hydrogen peroxide ($94.3 \pm 12\%$ of control) and hyperglycemia ($83.5 \pm 23.6\%$ of control) on survival. Vascular endothelial growth factor increased catalase, a primary determinant of intracellular concentrations of hydrogen peroxide, and decreased hydrogen peroxide-induced increases in reactive oxygen species.

Conclusion—These results indicate that vascular endothelial growth factor protects postganglionic sympathetic neurons from the detrimental effects of hydrogen peroxide. Our data suggest that an increase in catalase is the mechanisms underlying this neuroprotection.

Keywords

sympathetic innervation; reactive oxygen species; hyperglycemia; vascular endothelial growth factor; catalase; neuronal protection

Introduction

The sympathetic nervous system is a major determinant of cardiovascular function. The sympathetic nervous system acts in part via release of neurotransmitters from postganglionic

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Address for correspondence: Deborah H. Damon, Ph.D., 89 Beaumont Avenue, Department of Pharmacology, University of Vermont, Burlington, VT 05405, USA, Phone: 802-656-2184, Fax: 802-656-4523, Deborah.Damon@uvm.edu.

Conflicts of Interest

There is no conflicts of interest.

sympathetic neurons innervating blood vessels. These neurons are critical for maintenance of blood pressure (19) and distribution of blood flow (22). In addition, increased release of neurotransmitters from perivascular sympathetic nerves contributes to the development and progression of many cardiovascular diseases, including hypertension (16,19), atherosclerosis (27) and heart failure (45). Paracrine signaling between vascular and neuronal cells is an important mechanism for controlling the development and function of perivascular sympathetic nerves (9,10,18,24,29,32,34,51), but little is known about how cardiovascular disease affects this signaling, or how this signaling contributes to cardiovascular disease.

Hydrogen peroxide (H_2O_2) is a reactive oxygen species (ROS) that is implicated in the development and progression of vascular diseases (1,2,5,8,11,12,20,33,39,47,48,49,54,55,56). H_2O_2 is membrane-permeant, stable, and diffusible, which suggests that vascular H_2O_2 could affect perivascular nerves. Little is known about the effects of H_2O_2 on perivascular nerves, but many studies indicate that H_2O_2 has detrimental effects on neurons (4,13,14,15,16,21,30,38,53). It is surprising, therefore, that the function of perivascular nerves is maintained or even enhanced in many cardiovascular and metabolic diseases in which vascular concentrations of H_2O_2 are increased. This suggests that perivascular nerves are resistant to effects of H_2O_2 .

Vascular endothelial growth factor (VEGF) is neuroprotective for many neurons (7,25,26,31,35,37,40,42,51). VEGF is also a paracrine modulator of perivascular sympathetic nerves (29,34,52). These findings suggest that VEGF may be a neuroprotectant for postganglionic sympathetic neurons and perivascular sympathetic nerves.

In the present study, we tested the hypothesis that VEGF protects postganglionic sympathetic neurons from the detrimental effects of H_2O_2 . In cultures of postganglionic sympathetic neurons, H_2O_2 decreased survival and norepinephrine (NE) uptake. Elevated glucose, which increases H_2O_2 , also decreased survival and NE uptake. The effects of H_2O_2 and elevated glucose on survival were reduced by VEGF. These data support the proposed hypothesis, and suggest that VEGF may be a mechanism for maintaining the function of perivascular sympathetic nerves when vascular concentrations of H_2O_2 are increased.

Materials and Methods

Animals

Sprague Dawley rats were used in this study. Male and female 3–4 day old rats were used to prepare cultures of postganglionic sympathetic neurons. The use of animals in this study was approved by the Animal Care and Use Committee at the University of Vermont and was in accordance with the National Institute of Health guidelines for use of animals in research.

Tissue Culture

Postganglionic sympathetic neurons were isolated from superior cervical ganglia of male and female Sprague Dawley neonatal rats. Ganglia were collected and dissociated for 10 minutes at 37°C in a collagenase/hyaluronidase solution (10 mg/ml bovine serum albumin, 4 mg/ml collagenase, 1 mg/ml hyaluronidase) and then for 10 minutes in trypsin (3 mg/ml). Dissociated neurons were resuspended in neuronal media (DMEM/F12 supplemented with 5% FBS, 10% NuSerum (Collaborative), 50 ng/ml NGF, and penicillin/streptomycin) and equal volumes were applied to collagen coated tissue culture dishes. Cells were allowed to attach overnight in a humidified 5% CO_2 environment maintained at 37°C. Non-neuronal cells were then growth arrested with mitomycin C (10 μ g/ml for 1 hour).

Neuronal survival

Numbers of neurons surviving were determined by counting numbers of phase-bright neurons in five microscope fields. Culture dishes were evenly divided into five regions. One field from each region was sampled. The five counts were averaged to obtain a representative number of neurons per field.

NE uptake

NE uptake was assessed using tritiated NE (Amersham). These assays were performed using low (5 mM D-glucose; 20 mM L-glucose) or high (25 mM D-glucose) glucose HEPES-buffered Krebs solution [122 mM NaCl, 3 mM KCl, 0.4 mM $\text{MgSO}_4 \cdot \text{H}_2\text{O}$, 1.2 mM KH_2PO_4 , 20 mM HEPES, 1.3 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1 mM ascorbic Acid, 10 μM pargyline, pH 7.4]. Cell cultures were preincubated at 37°C with 100 nM tritiated NE for 30 minutes, washed (6 $\times$ 5 minutes) and solubilized. Tritiated NE in all samples was analyzed using a Beckman LS6000IC Liquid Scintillation Counter and NE uptake (total cpm taken up by cells) was determined.

Western Analysis

Cells were pelleted in PBS and then lysed in enhanced RIPA buffer (50mM Tris Base, 150mM NaCl, 10mM EDTA, 0.25% deoxycholate, 1% Nonidet P 40 substitute, 10% glycerol, 1% protease inhibitor cocktail, 1mM DTT, 0.1% sodium dodecyl sulfate). Lysates were diluted with an equal volume of 2x electrophoresis loading buffer, boiled for 5 minutes, 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 minutes 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 minute washes (PBST) and the membranes incubated for one hour at room temperature in PBS-Tween containing 3% nonfat dry milk and 1/3000 dilution of horseradish peroxidase conjugated secondary. Unbound secondary antibodies were removed with three 5 minute washes (PBST). Horseradish peroxidase was then detected with enhanced chemiluminescence (Pierce) and documented on autoradiographic film. Signals were quantified densitometrically.

Measurement of Reactive Oxygen Species

Reactive oxygen species (ROS) were detected using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA; Invitrogen). Neurons were incubated with 1 μM CM-H₂DCFDA for 30 minutes in PBS. The cells were then washed to remove unincorporated dye. ROS in the neurons was visualized with a fluorescence microscope (Olympus BX50). Images were captured with a QImaging Retiga 2000R Digital Camera. Fluorescent intensity was measured using Image J software.

Statistical Analysis

Data are presented as means $\pm$ standard errors. One-sample or unpaired t-tests were performed to compare data. Differences were considered significant if p-values were less than or equal to 0.05.

Results

Postganglionic sympathetic neurons innervating blood vessels produce the effects of the sympathetic nervous system on vascular function. It is well known that paracrine factors affect the function of perivascular nerves (9,10,18,24,29,32,34,51). H₂O₂ is a stable, diffusible reactive oxygen species, is produced by vascular cells, and is increased in vascular

disease (1,2,5,8,11,12,20,33,39,47,48,49,54,55,56). Thus, H₂O₂ is likely to be a paracrine modulator of perivascular sympathetic nerves in animals and humans with vascular disease. In the present study, we consider the effects of H₂O₂ on postganglionic sympathetic neurons.

The effects of H₂O₂ were studied in cultures of postganglionic sympathetic neurons. H₂O₂ or vehicle was added once and the cells were then incubated for 72 hours. Figure 1A shows phase contrast images of control and H₂O₂-treated (30 μ M) cells. These images suggest that H₂O₂ promoted degeneration of the neurons. Quantitative analysis of neuronal survival is shown in figure 1B. These data indicate that 30 and 100 μ M H₂O₂ reduced the survival of postganglionic sympathetic neurons, and suggest that diseases associated with increased vascular concentrations of H₂O₂ would promote degeneration of perivascular nerves. Vascular H₂O₂ is increased in animal models of hypertension (49) and diabetes (2), but in these models, the function of perivascular sympathetic nerves is maintained or enhanced (23). This suggests that these nerves are protected from the detrimental effects of H₂O₂. VEGF A is produced by vascular cells (29,34,36,52) and protects many neurons (7,25,26,31,35,37,40,42,51), which suggests that this growth factor would also protect postganglionic sympathetic neurons. Figure 1 indicates that this is the case. VEGF A (250 ng/ml) reduced the effects of H₂O₂ on neuronal survival (figure 1A and B).

The effects of VEGF on cells are mediated by VEGF binding to multiple receptors. Studies of Li et al. (2008) and Poesen et al. (2008) suggest that VEGF activation of VEGFR1 is neuroprotective. Data in figure 1C suggest that VEGFR1 mediates VEGF protection of postganglionic sympathetic neurons. VEGF B (250 ng/ml), which selectively activates VEGFR1, protected these neurons as well as VEGF A.

Hyperglycemia is a pathologically relevant condition that induces ROS-mediated neuronal dysfunction and neuropathy (2,15,51,53). As noted above, sympathetic neurovascular transmission is maintained or enhanced in hyperglycemic animals (23) and humans (6), suggesting that perivascular sympathetic nerves are protected from the detrimental effects of hyperglycemia. We hypothesized that VEGF A mediates this protection. Figure 2 shows the effects of hyperglycemia and VEGF A on the survival of cultured postganglionic sympathetic neurons. Neurons were grown for seven days in low (LG; 5 mM) or high (HG; 25 mM) glucose in the absence or presence of VEGF A. These concentrations of glucose correspond to plasma glucose concentrations in normoglycemic (LG) and hyperglycemic (HG) animals and humans. Hyperglycemia decreased the survival of the neurons, but VEGF A prevented this effect. The role of ROS in mediating the effects of high glucose was also evaluated. PEG-catalase (100 units/ml), which decreases ROS levels, also prevented the effect of hyperglycemia on neuronal survival.

To determine if H₂O₂ (30 μ M), elevated glucose and/or VEGF A (250 ng/ml) affected neuronal function as well as neuronal survival, norepinephrine (NE) uptake was also assessed (Figure 3). These data indicate that H₂O₂ and elevated glucose decreased NE uptake, and that VEGF A prevented this effect. For both H₂O₂ and high glucose, the decreases in NE uptake were comparable to the decreases in neuronal survival. Furthermore, in three experiments, H₂O₂ did not affect NE uptake normalized to cell number. Normalized uptake (cpm $\times 10^{-4}$ /cell number per microscope field) in H₂O₂-treated cells (1.07 ± 0.44) was not significantly different from that in control cells (0.91 ± 0.45). These data suggest that the effects of elevated H₂O₂ and glucose on NE uptake were attributable to decreased neuronal survival.

Figure 4A demonstrates that VEGF A decreases levels of CM-H₂DCFDA fluorescence, an indicator of ROS, in postganglionic sympathetic neurons. CM-H₂DCFDA fluorescence was

measured five minutes after the addition of 100 μM H_2O_2 to cultures of neurons grown for seven days in the absence ($-\text{VEGF}$) and presence ($+\text{VEGF}$) of VEGF. Catalase catalyzes the conversion of H_2O_2 to water, thereby decreasing intracellular concentrations of ROS. Data in figure 2 indicate that increasing catalase promotes neuronal survival. We tested the hypothesis that VEGF decreased ROS levels and thereby protected postganglionic sympathetic neurons by increasing catalase. Figure 4B shows western analyses of catalase and GAP43 expression in cultures of postganglionic sympathetic neurons grown for seven days in the absence ($-\text{VEGF}$) and presence ($+\text{VEGF}$) of VEGF A (250 ng/ml). VEGF increased catalase in three independent experiments. The increases were variable and ranged from 181 to 2300% of control. Inhibition of catalase with aminotriazine (ATZ;3) reduced the neuroprotective effects of VEGF. Neuronal survival in the presence of 250 ng/ml VEGF A, 30 μM H_2O_2 and 1 mM ATZ was $76 \pm 8.3\%$ of that in the absence of ATZ ($p < 0.05$; one-tailed Wilcoxon signed rank test). Preliminary studies indicate that 100 nM ATZ completely inhibited the neuroprotective effect of VEGF.

Discussion

It is well established that H_2O_2 has detrimental effects on many neurons (4,13,14,15,16,21,30,38,53). Our studies indicate that this is also the case for postganglionic sympathetic neurons (Figures 1 and 3). It is estimated that intracellular H_2O_2 concentrations greater than 1 μM are pathological (50). It is also estimated that when applying exogenous H_2O_2 , intracellular concentrations are 1/10 of extracellular concentrations (50). Thus, the concentrations of H_2O_2 used in the present study are considered pathological. As noted in the results section, H_2O_2 was added once and effects of this addition assessed 72 hours later. A material safety data sheet indicates that the half-life of H_2O_2 in fresh water is between 8 hours to 20 days, it is possible that the concentration of H_2O_2 in our cultures decreased over time. The present studies do not allow us to determine the time course of the effects of H_2O_2 on the neurons; if transient or sustained increases in H_2O_2 produce the observed effects on neuronal survival.

There are likely to be multiple sources of H_2O_2 that would affect the function of postganglionic sympathetic neurons. All cells contain NADPH oxidases and superoxide dismutases that produce endogenous H_2O_2 . Thus, the neurons themselves are likely to be a source. In addition, because H_2O_2 is stable and diffusible, cells surrounding postganglionic neurons are likely to produce H_2O_2 that would act as a paracrine modulator of the neurons. In blood vessels, adventitial fibroblasts (8,20) as well as vascular smooth muscle (39) are likely to be sources of H_2O_2 that would affect perivascular sympathetic nerves. In cardiovascular disease in which vascular H_2O_2 is increased, it is likely that H_2O_2 would affect perivascular sympathetic nerve terminals but not cell bodies. It is possible that this would affect neuronal function without affecting survival or promote degeneration of nerve fibers without affecting the cell bodies. The experimental model used in the present studies did not allow us to selectively increase H_2O_2 in the nerve terminals and determine if this was the case. This possibility is currently under investigation.

In the neurons H_2O_2 would be degraded by multiple enzymatic and chemical reactions (12). The iron-dependent Fenton reaction converts H_2O_2 to OH^- . This highly reactive hydroxyl radical causes DNA, protein and lipid damage and thereby produces many of the detrimental effects of H_2O_2 . Catalase and peroxidases catalyze the conversion of H_2O_2 to H_2O and thus protect cells from the detrimental effects of H_2O_2 .

In vivo studies indicate that ROS are increased in blood vessels of hyperglycemic rats and humans, but perivascular nerve function appears to be maintained. For example inhibition of NE binding to α adrenergic receptors decreased blood pressure (6) in patients with diabetes.

This indicates that in these patients, NE released by functioning postganglionic neurons, including vascular neurons, affected blood pressure and blood flow. The discrepancy between our *in vitro* findings and the *in vivo* data led us to hypothesize that perivascular nerves are resistant to the detrimental effects of H₂O₂. Recent studies indicate that vascular-derived VEGF is a paracrine modulator of perivascular sympathetic nerves (29,34,52). Furthermore, it is well established that VEGF protects neurons (7,25,26,31,35,37,40,42,51). This led us to hypothesize that vascular-derived VEGF would protect perivascular sympathetic nerves. The data in figures 1 – 3 demonstrate that VEGF protects postganglionic sympathetic neurons and thus support the hypothesis. *In vivo* studies are required to further test this hypothesis.

The mechanisms underlying the neuroprotective effects of VEGF are less well established. Our data are the first to demonstrate that VEGF enhances catalase expression in neurons (Figure 4). Further studies, are required to determine if this effect is limited to postganglionic sympathetic neurons. Further studies are also required to determine the mechanisms underlying VEGF A induction of catalase. Since VEGF B, which selectively activates VEGFR1, also protects these neurons (figure 1C), it is likely that activation of VEGFR1 is required. Studies of Li et al. (2003) Lunn et al. (2009) suggest that activation of phosphatidylinositol 3-kinase/Akt signaling is also involved. In addition, the studies of Sampath et al. (1994) and Sampath and Perez-Polo (1997) suggest it is likely that VEGF increases catalase by increasing transcription of catalase mRNA.

Our data suggest that vascular-derived VEGF would oppose damaging effects of vascular-derived H₂O₂ on perivascular sympathetic nerve fibers. This is likely to be highly physiologically relevant in that perivascular sympathetic nerve fibers markedly affect vascular function. Further *in vivo* studies are required to determine how neuroprotective effects of VEGF affect sympathetic neurovascular transmission and vascular function.

Acknowledgments

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Figure 1. Hydrogen peroxide decreases survival of postganglionic sympathetic neurons in the absence but not in the presence of VEGF

A. Phase contrast photomicrographs of cultures of postganglionic sympathetic neurons grown in the absence and presence of H₂O₂ and VEGF. B. Cultures of postganglionic sympathetic neurons were grown for three days in the presence of the indicated concentrations of hydrogen peroxide (H₂O₂) in the absence (– VEGF; filled circles) and presence (+ VEGF; open circles) of 250 ng/ml VEGF. Numbers of live neurons were then counted as described in methods. * indicates that the numbers of cells surviving in the presence of VEGF were greater than that in the absence of VEGF (unpaired t-test). C. Postganglionic sympathetic neurons were grown for three days in the absence (control; open bar), in the presence of 30 μM H₂O₂ (filled bar), in the presence of 30 μM H₂O₂ + 250 ng/ml VEGF A, or in the presence of 30 μM H₂O₂ + 250 ng/ml VEGF B. Numbers of live neurons were then counted as described in methods. * indicates that survival in the presence of H₂O₂ was less than that in control.

Figure 2. Hyperglycemia decreases survival of postganglionic sympathetic neurons in the absence but not in the presence of VEGF

Cultures of postganglionic sympathetic neurons were grown for seven days in low glucose (5 mM; open bar), high (25 mM; HG; filled bar) glucose, high glucose + 250 ng/ml VEGF A, or in high glucose + 100 units/ml of PEG-catalase. Numbers of live cells were then determined. * indicates that the number of cells surviving in high glucose was less than that in cultures grown in LG ($p < 0.05$; unpaired t-test; $n = 6$).

A.

B.

Figure 3. Hydrogen peroxide and hyperglycemia decrease NE uptake into postganglionic sympathetic neurons in the absence but not in the presence of vascular endothelial growth factor
 A. Postganglionic sympathetic neurons were grown for three days in the absence of H₂O₂ (control; open bar) in the presence of the 30 μ M H₂O₂ (black bar) or in the presence of 30 μ M H₂O₂ + 250 ng/ml VEGF A (gray bar). NE uptake was then measured as described in methods. * indicates significantly different from control. B. Neurons were grown for seven days in low glucose (LG; open bar) high glucose (HG; black bar), or high glucose + 250 ng/ml VEGF A. NE uptake was then measured. * indicates significantly different from control. N's are indicated in parentheses.

Figure 4. Vascular endothelial growth factor (VEGF) increases catalase

A. H₂O₂-induced increases in CM-H₂DCFDA fluorescence (expressed as percent of maximum increase) in cultures of postganglionic sympathetic neurons grown for seven days in the absence and presence of VEGF. * indicates that the increase in fluorescence observed in the presence of VEGF was significantly less than that observed in the absence of VEGF.

B. Western analyses of catalase and GAP43, an index of neuronal protein, in postganglionic sympathetic neurons grown for seven days in the absence (–VEGF) and presence (+ VEGF) of VEGF.