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NAD(P)H oxidase-induced oxidative stress in sympathetic ganglia of apolipoprotein E deficient mice

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Abstract

Superoxide anion ($O_2^{\bullet-}$) is increased throughout the arterial wall in atherosclerosis. The oxidative stress contributes to lesion formation and vascular dysfunction. In the present study, we tested the hypothesis that NAD(P)H oxidase-derived $O_2^{\bullet-}$ is increased in nodose sensory ganglia and sympathetic ganglia of apolipoprotein E deficient (apoE$^{-/-}$) mice, an established animal model of atherosclerosis. $O_2^{\bullet-}$ measured ex vivo by L-012-enhanced chemiluminescence was increased by 79±17% in whole sympathetic ganglia from apoE$^{-/-}$ mice ($n=5$) compared with sympathetic ganglia from control mice ($n=5$) ($P<0.05$). In contrast, $O_2^{\bullet-}$ was not elevated in nodose ganglia from apoE$^{-/-}$ mice. Dihydroethidium staining confirmed the selective increase in $O_2^{\bullet-}$ in sympathetic ganglia of apoE$^{-/-}$ mice, and revealed the contribution of both neurons and non-neuronal cells to the $O_2^{\bullet-}$ generation. We investigated the enzymatic source of increased $O_2^{\bullet-}$ in sympathetic ganglia of apoE$^{-/-}$ mice. The mRNA expression of gp91phox, p22phox, p67phox, and p47phox subunits of NAD(P)H oxidase measured by real time RT-PCR was increased ~3–4 fold in sympathetic ganglia of apoE$^{-/-}$ mice ($n=5$) compared with control ganglia ($n=5$). NADPH oxidase activity measured by lucigenin chemiluminescence was increased by 68±12% in homogenates of sympathetic ganglia from apoE$^{-/-}$ mice ($n=7$) compared with control ganglia ($n=7$) ($P<0.05$). The results identify sympathetic ganglia as a novel site of oxidative stress in atherosclerosis, and suggest that upregulation of NAD(P)H oxidase is the source of increased $O_2^{\bullet-}$ generation. We speculate that oxidative stress in sympathetic ganglia may contribute to impaired baroreflex control of sympathetic nerve activity.

Keywords: Sympathetic nervous system; Oxidative stress; Superoxide anion; Real time RT-PCR; NAD(P)H oxidase activity; Atherosclerosis; Apolipoprotein E; Mice

1. Introduction

Hypercholesterolemia and atherosclerosis are associated with increases in superoxide anion ($O_2^{\bullet-}$) and oxidative stress in the arterial wall (Mugge et al., 1991; Miller et al., 1998; Sorescu et al., 2002). Oxidative stress contributes to lesion formation and impairment of endothelium-dependent vascular relaxation in atherosclerosis (Mugge et al., 1991; Steinberg, 1997; Miller et al., 1998). Oxidative stress is not confined to blood vessels in cardiovascular disease. In hypertension and heart failure, markers of oxidative stress are present in the central nervous system (Zimmerman et al., 2004a; Gao et al., 2004; Lindley et al., 2004) and in sympathetic ganglia (Dai et al., 2004). Furthermore, administration of the superoxide dismutase (SOD) mimetic tempol, and targeted over-expression of SOD in brain reduce sympathetic nerve activity in hypertension and heart failure (Shokoji et al., 2003; Xu et al., 2004; Zimmerman et al., 2004a; Gao et al., 2004; Lindley et al., 2004). Taken together, these results indicate that

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oxidative stress in the nervous system is an important cause of excessive sympathetic nerve activity in hypertension and heart failure.

The possibility that oxidative stress may extend beyond the vascular wall to the peripheral nervous system in atherosclerosis remains unexplored. Markers of oxidative stress are present in the central nervous system of apolipoprotein E deficient (apoE$^{-/-}$) mice, an established animal model of atherosclerosis (Matthews and Beal, 1996; Pratico et al., 1999; Montine et al., 1999; Ramassamy et al., 2001). Based on our recent finding that atherosclerotic rabbits (Li et al., 1996) and apoE$^{-/-}$ mice (Meyrelles et al., 2000; Lazartigues et al., 2004) exhibit decreased baroreceptor and baroreflex sensitivity that are reversed by SOD and catalase, or by tempol, we propose that oxidative stress in sensory baroreceptor neurons and/or efferent sympathetic neurons may contribute to the baroreflex impairment.

The NAD(P)H oxidase is the major source of $O_2^{\bullet-}$ in blood vessels and is upregulated in atherosclerosis (Griendling et al., 2000; Cai et al., 2003). NAD(P)H oxidase subunits have recently been identified in embryonic/neonatal sympathetic ganglion neurons at both the mRNA and protein levels (Tammariello et al., 2000; Hilburger et al., 2005). NAD(P)H-derived $O_2^{\bullet-}$ has been shown to initiate a cascade leading to apoptosis in cultured sympathetic neurons deprived of nerve growth factor (Tammariello et al., 2000).

The major aims of the present study were to determine if $O_2^{\bullet-}$ is increased in nodose sensory ganglia and sympathetic ganglia of adult apolipoprotein E deficient mice, and investigate the role of NAD(P)H oxidase as a source of the $O_2^{\bullet-}$.

2. Materials and methods

ApoE$^{-/-}$ mice were obtained from our own mouse colony maintained at the University of Iowa. The mice were derived from breeding pairs originally purchased from Jackson Laboratories. Genotypes of apoE$^{-/-}$ and control mice were determined by RT polymerase chain reaction (PCR) of DNA isolated from tail biopsies in the University of Iowa Transgenic Animal Facility. Mice were studied at 25–30 weeks of age (22–30 g, female and male). All experiments were conducted in accordance with the American Physiological Society's "Guiding Principles for Research Involving Animals and Human Beings" and were approved by the University of Iowa Animal Care and Use Committee. Control mice ($n=22$) and apoE$^{-/-}$ mice ($n=22$) were anesthetized with sodium pentobarbital (60 μg/g, ip). Nodose and sympathetic ganglia were harvested from apoE$^{-/-}$ and control mice for ex vivo measurement of $O_2^{\bullet-}$, mRNA of NAD(P)H oxidase subunits, and NADPH oxidase activity.

2.1. Measurement of $O_2^{\bullet-}$ in whole ganglia by L-012-enhanced chemiluminescence

$O_2^{\bullet-}$ production in nodose and sympathetic ganglia from individual apoE$^{-/-}$ ($n=5$) and control mice ($n=5$) was measured using the chemiluminescent dye 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione sodium salt (L-012) (Wako Pure Chemical Industries) as described previously (Miller et al., 1998; Daiber et al., 2004a, b). Both nodose ganglia from individual mice were analyzed together. Celiac, aortic-renal, and a portion of the lumbar sympathetic chain were pooled. The tissues were washed in phosphate buffered saline (PBS) and added to tubes containing L-012 (100 μM) in 1 ml PBS for signal reading in a luminometer (Zylux Corp.). $O_2^{\bullet-}$ was measured at 30 s intervals over 5 min and was expressed in relative luminescence units (RLU) per second per microgram tissue dry weight after subtraction of the background signal measured in tubes in absence of ganglia.

2.2. Cellular localization of $O_2^{\bullet-}$ generation by DHE fluorescence

$O_2^{\bullet-}$ was detected in whole nodose and aortic-renal ganglia ($n=8$) by dihydroethidium (DHE) fluorescence confocal microscopy as described previously (Bindokas et al., 1996; Miller et al., 1998; Zhang et al., 2003). When $O_2^{\bullet-}$ is present, DHE is oxidized to ethidium bromide which is trapped by intercalating with DNA. Immediately after harvesting, the ganglia were washed in PBS and incubated with the $O_2^{\bullet-}$ sensitive fluorogenic probe DHE (5 μM) for 45 min in dark conditions at room temperature. After incubation in DHE, the ganglia were again washed in PBS, coverslipped in a shallow concave-shaped microscopy slide, sealed with an enamel solution, and immediately examined by a laser scanning confocal microscope (BioRad LaserSharp2000) using an excitation wavelength of 488 nm and an emission wavelength of 510 nm. Serial sections of confocal images were obtained from the top to bottom of each ganglia at 10 μm steps. Fluorescence was quantitated in individual neurons (diameter 10–30 μm) using ImageJ software (v1.31 and v1.32, NIH).

2.3. mRNA expression of NAD(P)H oxidase subunits

Total RNA was extracted from all the sympathetic ganglia collected from 5 mice in each group using RNeasy Mini Kit (QIAGEN Inc., CA). mRNA quality was estimated by running a 1% agarose gel in MOPS buffer. Two micrograms of total RNA was used to perform the RT reaction using SuperScript III (Invitrogen, CA) for the first strand cDNA synthesis. mRNA expression for NAD(P)H oxidase subunits of gp91phox, p22phox, p47phox and p67phox were quantified by real time quantitative PCR with SYBR Green using an ABI Prism

7700 (Applied Biosystems) sequence detection system, as described previously (Ma et al., 2004). 18s ribosomal RNA was taken as an internal control. The specific products of the NAD(P)H oxidase subunits gp91phox, p22phox, p47phox and p67phox were validated by running dissociation curves or DNA gel. All primers were synthesized by Integrated DNA Technologies (Coralville, IA).

Primers	gp91phox	p22phox	p47phox	p67phox
GenBank	BC_071229	NM_007806	NM_010876	NM_010877

2.4. NADPH oxidase activity

NADPH oxidase activity in sympathetic ganglia from 4 apoE$^{-/-}$ mice and 4 control mice was measured by lucigenin chemiluminescence (Kitada et al., 2003). Briefly, sympathetic ganglia from an individual mouse were pooled and homogenized for 5 s in cell lysis buffer (added with PMSF) with an Ultrasonic Cell Disrupter. The homogenate was centrifuged for 10 min at low speed of 1000×*g* to remove the debris and unbroken tissue, and the protein level of supernatant was read with Sunrise Absorbance Microplate Reader (Tecan, CA). Supernatant (25–50 μg protein) was added to glass scintillation vials containing 5 μM lucigenin in 1 ml of PBS. The chemiluminescence value was recorded at 30 s intervals over 5 min after 15 min dark adaptation. The background was determined by measurement in the absence of homogenate and then subtracted from the reading obtained in the presence of homogenate. $O_2^{\bullet-}$ production was measured after addition of NADPH (100 μM) into the incubation medium as a substrate in absence and presence of diphenyleneiodonium (DPI, 10 μM), a flavoprotein inhibitor of NAD(P)H oxidase. Chemiluminescence was reported as relative light units per second normalized to protein concentration.

2.5. Data analysis

For measurement of $O_2^{\bullet-}$, pairs of ganglia from apoE$^{-/-}$ and control mice were studied together side-by-side under identical conditions enabling levels of $O_2^{\bullet-}$ in apoE$^{-/-}$ ganglia to be expressed as a percentage of the level measured in control ganglia, and quantitative comparisons between the paired ganglia. The normalized levels of $O_2^{\bullet-}$ in apoE$^{-/-}$ and control mice were compared by paired *t*-test. Differences in absolute DHE fluorescence intensity (lucigenin chemiluminecence counts) and NADPH oxidase activity between apoE$^{-/-}$ and control neurons were analyzed by unpaired *t*-test. NADPH oxidase activity measured before and after adding the NAD(P)H oxidase inhibitor DPI were compared by paired *t*-test. Data are expressed as means ± SE except for mRNA expression which is expressed as means ± SD. Significant differences were defined at $P<0.05$.

3. Results

3.1. $O_2^{\bullet-}$ generation in nodose sensory ganglia and sympathetic ganglia

$O_2^{\bullet-}$ measured by L-012-enhanced chemiluminescence was significantly increased by 79 ± 17% in sympathetic ganglia of apoE$^{-/-}$ mice ($n=5$) compared with sympathetic ganglia from control mice ($n=5$) (Fig. 1). In contrast, $O_2^{\bullet-}$ levels were not significantly different in nodose ganglia of control ($n=3$) and apoE$^{-/-}$ mice ($n=3$) (Fig. 1). To determine the cellular source of $O_2^{\bullet-}$ production, we obtained optical sections through ganglia using DHE fluorescence confocal microscopy. DHE fluorescence was evident in both neurons and small non-neuronal cells in ganglia from control and apoE$^{-/-}$ mice (Fig. 2). Quantitation of fluorescence in neurons confirmed the chemiluminescence results in that $O_2^{\bullet-}$ was significantly greater in sympathetic neurons of apoE$^{-/-}$ mice ($n=8$) vs. sympathetic neurons of control mice ($n=8$) (Fig. 3). The increased $O_2^{\bullet-}$ in apoE$^{-/-}$ sympathetic neurons was apparent both when fluorescence was expressed in absolute units (+26%) and when it was expressed as a percentage of the signal measured in the paired control ganglion (+25 ± 9%). In contrast, and again consistent with the L-012 chemiluminescence measurements, $O_2^{\bullet-}$ was not elevated in nodose neurons of apoE$^{-/-}$ mice (Fig. 3). In fact, $O_2^{\bullet-}$ expressed in absolute fluorescence units was significantly lower in nodose neurons of apoE$^{-/-}$ mice compared with nodose neurons of control mice (Fig. 3).

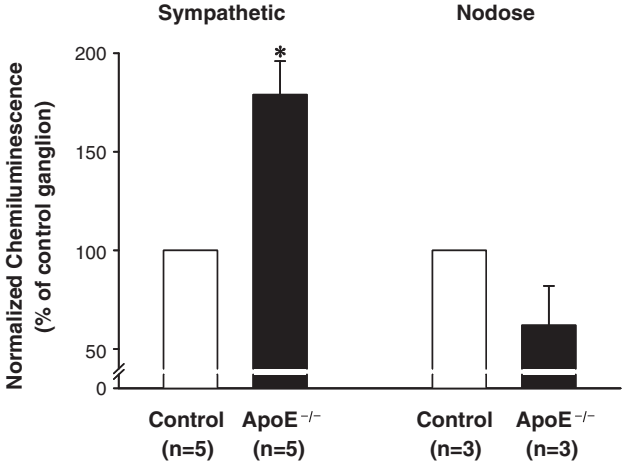

Fig. 1. $O_2^{\bullet-}$ generation measured by L-012-enhanced chemiluminescence in whole sympathetic ganglia of control ($n=5$) and apoE$^{-/-}$ ($n=5$) mice (left) and nodose ganglia of control ($n=3$) and apoE$^{-/-}$ ($n=3$) mice (right). Celiac, aortic renal, and a portion of the sympathetic chain were combined in individual mice. ApoE$^{-/-}$ and control ganglia were studied in pairs enabling $O_2^{\bullet-}$ in the apoE$^{-/-}$ ganglion to be expressed as a percentage of the level measured in the control ganglion. *Significant difference vs. control ganglia, $P<0.05$.

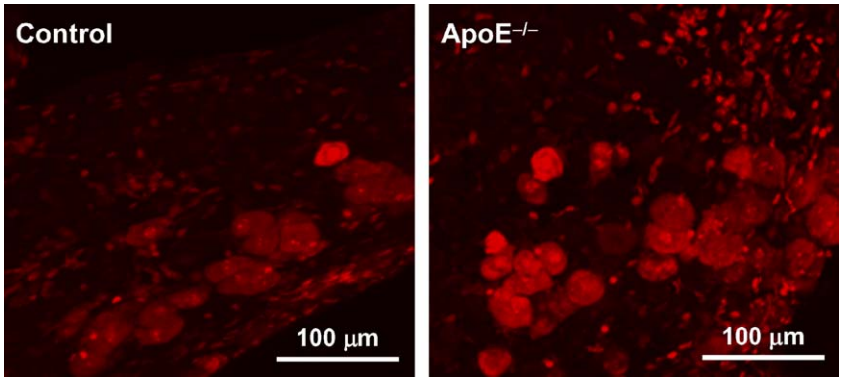

Fig. 2. Confocal images of DHE fluorescence in aortic-renal sympathetic ganglia from control mouse (left) and apoE$^{-/-}$ mouse (right). Note increased fluorescence in both neurons and non-neuronal cells of apoE$^{-/-}$ ganglion compared with cells in control ganglion.

3.2. mRNA expression of NAD(P)H oxidase subunits

The mRNA expression for each of the NAD(P)H oxidase subunits studied (gp91phox, p22phox, p47phox, and p67phox) was significantly increased by ~3–4 fold in apoE$^{-/-}$ sympathetic ganglia ($n=5$) compared with ganglia from age-matched control mice ($n=5$) (Fig. 4).

3.3. NADPH oxidase activity

NADPH oxidase activity was significantly increased in sympathetic ganglia from apoE$^{-/-}$ mice compared with control sympathetic ganglia ($n=7$, $P<0.05$) (Fig. 5). The increased NADPH oxidase activity in apoE$^{-/-}$ ganglia was inhibited significantly from 8.9±1.6 to 6.3±1.4 RLU/s/μg protein by the NAD(P)H oxidase inhibitor DPI ($n=5$, $P<0.05$). However, DPI did not affect NADPH oxidase activity in control ganglia ($n=2$).

4. Discussion

The major new findings of the present study are that $O_2^{\bullet-}$ levels, mRNA expression of NAD(P)H oxidase subunits, and NADPH oxidase activity are significantly increased in sympathetic ganglia of apoE$^{-/-}$ mice as compared with sympathetic ganglia of control mice. $O_2^{\bullet-}$ was not increased in nodose ganglia of apoE$^{-/-}$ mice, indicating that the upregulation of NAD(P)H oxidase and associated oxidative stress are tissue-specific even among different peripheral ganglia. The results identify sympathetic ganglia as a novel site of oxidative stress in atherosclerosis, and suggest that increased expression of NAD(P)H oxidase is a major source of the $O_2^{\bullet-}$.

Numerous studies have demonstrated that NAD(P)H oxidase expression and $O_2^{\bullet-}$ are increased in the arterial wall of animals and humans with hypercholesterolemia and atherosclerosis (Mugge et al., 1991; Miller et al., 1998;

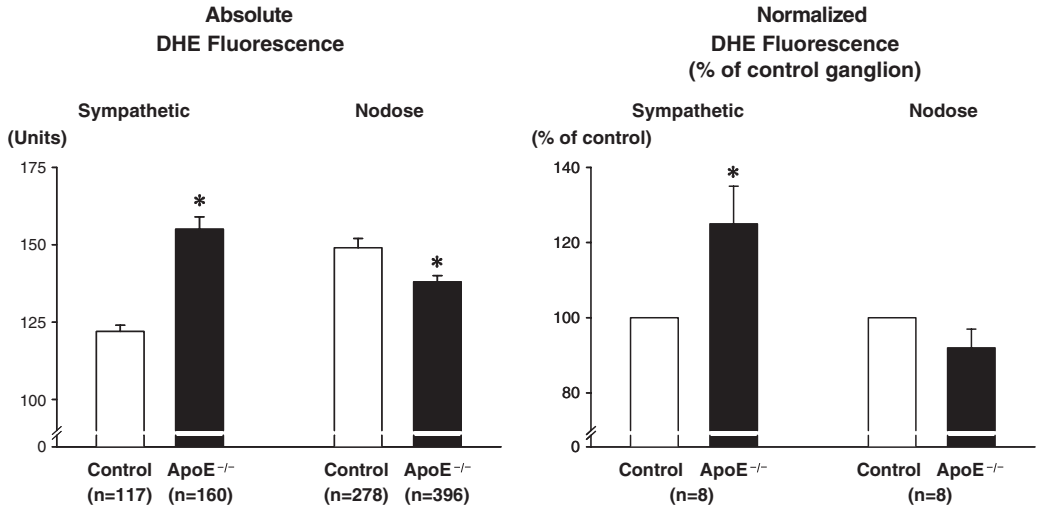

Fig. 3. DHE fluorescence intensity in sympathetic and nodose neurons from control and apoE$^{-/-}$ mice expressed in absolute units (left) and as a percentage of the average signal in paired control ganglion (right). $n=117$ neurons in 8 sympathetic ganglia from control mice; $n=160$ neurons in 8 sympathetic ganglia from apoE$^{-/-}$ mice; $n=278$ neurons in 8 nodose ganglia from control mice; and $n=396$ neurons in 8 nodose ganglia from apoE$^{-/-}$ mice. $O_2^{\bullet-}$ was increased in apoE$^{-/-}$ vs. control sympathetic neurons both when fluorescence was expressed in absolute units and when it was expressed as a percentage of the signal measured in the paired control ganglion. In contrast, $O_2^{\bullet-}$ was not elevated in nodose neurons of apoE$^{-/-}$ mice. *Significant difference between apoE$^{-/-}$ vs. control neurons, $P<0.05$.

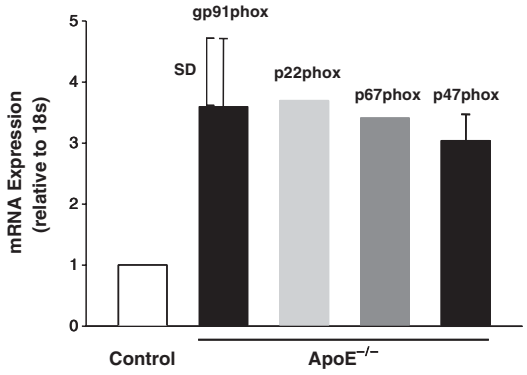

Fig. 4. Messenger RNA expression of NAD(P)H oxidase subunits in pooled sympathetic ganglia from control ($n=5$) and apoE$^{-/-}$ ($n=5$) mice. mRNA expression of gp91phox, p22phox, p67phox, and p47phox subunits of NAD(P)H oxidase were quantified by real time reverse transcription-polymerase chain reaction and were expressed relative to levels measured in tissues from control mice. These subunits were increased by ~3–4 fold in sympathetic ganglia of apoE$^{-/-}$ mice compared with control ganglia.

Sorescu et al., 2002; Cai et al., 2003; Guzik et al., 2004). A variety of hormonal and paracrine factors including angiotensin II, endothelin, platelet derived growth factor (PDGF), tumor necrosis factor alpha (TNFα), and thrombin contribute to the upregulation and activation of vascular NAD(P)H oxidases in atherosclerosis (Griendling et al., 2000; Cai et al., 2003). The renin-angiotensin system is particularly important. Hypercholesterolemia is associated with increases in circulating angiotensinogen, angiotensin II, and angiotensin AT_1 receptor expression (Nickenig et al., 1997; Daugherty et al., 2004). Furthermore, antagonists of the renin-angiotensin system reduce vascular oxidative stress and the severity of atherosclerotic lesions (Warnholtz et al., 1999; Hayek et al., 1999; Wassmann et al., 2002).

The systemic nature of the hormonal stimuli discussed above along with the recent observation that embryonic/neonatal sympathetic neurons express the NAD(P)H oxidase subunits gp91phox, p22phox, p40phox, p47phox, and p67phox (Tammariello et al., 2000; Hilburger et al., 2005) led us to hypothesize that oxidative stress in atherosclerosis may extend beyond the vasculature to peripheral ganglia important in cardiovascular regulation. Our results demonstrate that gp91phox, p22phox, p47phox, and p67phox are expressed in sympathetic ganglia of adult mice; and more importantly, that both NAD(P)H oxidase expression and $O_2^{\bullet-}$ levels in the ganglia are significantly increased in the atherosclerotic apoE$^{-/-}$ mouse. The increase in $O_2^{\bullet-}$ was significant both when measured by DHE fluorescence and by the more quantitative chemiluminescence method (Daiber et al., 2004a, b). Using a similar experimental approach, Dai et al. (2004) recently showed that $O_2^{\bullet-}$ is elevated in sympathetic ganglia of rats with DOCA-salt-induced chronic hypertension. The increase in $O_2^{\bullet-}$ in the ganglia from hypertensive rats was shown to be mediated by endothelin acting on ET_B receptors (Dai et al., 2004). In both our study and the study by Dai et al. (2004), the increase in $O_2^{\bullet-}$ was observed in both neurons and non-neuronal cells within the sympathetic ganglia.

Interestingly, we did not observe an increase in $O_2^{\bullet-}$ in nodose sensory ganglia from apoE$^{-/-}$ mice (Figs. 1 and 3). The neuronal $O_2^{\bullet-}$ measured in absolute DHE fluorescence units was actually significantly less in apoE$^{-/-}$ neurons vs. control neurons (Fig. 3). The decrease in $O_2^{\bullet-}$ in apoE$^{-/-}$ nodose neurons should be interpreted with caution considering the limitations in using DHE fluorescence for making quantitative comparisons. We do not know the reason for the selectivity of the increase in $O_2^{\bullet-}$ in sympathetic, but not nodose ganglia. In preliminary experiments, we have demonstrated that nodose neurons have the capacity to acutely generate reactive oxygen species during evoked electrical activity (Snitsarev et al., 2004). We speculate that differences in expression of NAD(P)H oxidase subunits, antioxidant (e.g. SOD) enzymes, and/or receptors for endothelin or angiotensin may account for the differential responses in the present study. Both endothelin and angiotensin are produced locally within sympathetic ganglia (Damon, 1998; Kushiku et al., 2001), but have not been shown to be generated within nodose ganglia to our knowledge. Receptors for both ligands (ET_B and AT_1) are expressed on sympathetic neurons and when occupied by ligand lead to increases in $O_2^{\bullet-}$ (Cai et al., 2003; Ma et al., 2004; Dai et al., 2004). Regional differences in oxidative stress have also been observed in the brain of apoE$^{-/-}$ mice (Matthews and Beal, 1996; Ramassamy et al., 2001).

The functional consequences of upregulation of NAD(P)H oxidase and increased $O_2^{\bullet-}$ generation in sympathetic ganglia of apoE$^{-/-}$ mice in vivo are not known. We speculate that oxidative stress in sympathetic ganglia may increase postganglionic sympathetic nerve activity and/or contribute to impaired baroreflex sensitivity. Preliminary experiments in our lab indicate that apoE$^{-/-}$ mice exhibit

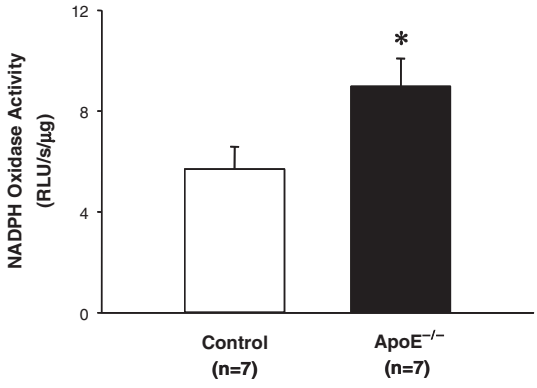

Fig. 5. NADPH oxidase activity in homogenates of sympathetic ganglia from control and apoE$^{-/-}$ mice. The enzyme activity was measured by lucigenin chemiluminescence with addition of NADPH (100 μM) as substrate. Chemiluminescence is reported as relative light units per second normalized to protein concentration. NADPH oxidase activity was significantly enhanced in sympathetic ganglia of apoE$^{-/-}$ mice. *Significant difference between apoE$^{-/-}$ vs. control mice, $P<0.05$.

upregulation of AT_1 receptors in sympathetic ganglia, increased excitability of their sympathetic neurons studied in culture, and baroreflex impairment in vivo (Ma et al., 2000; Meyrelles et al., 2000; Lazartigues et al., 2004). In a previous study, we found that transgenic mice expressing human renin and angiotensinogen (R^+A^+) exhibit increased AT_1 receptor expression in sympathetic ganglia and residual postganglionic renal sympathetic nerve activity after ganglionic blockade (Ma et al., 2004). The residual sympathetic activity in R^+A^+ mice was inhibited by losartan, and was not observed in control mice (Ma et al., 2004).

Shokoji et al. (2004) examined the role of $O_2^{\bullet-}$ in sympathetic nerves on nerve activity by directly applying the SOD mimetic tempol and the SOD inhibitor diethyldithio-carbamic (DETC) to renal postganglionic nerve fibers in anesthetized rats. Tempol decreased and DETC increased the sympathetic activity recorded at a distal site suggesting a sympatho-excitatory influence of $O_2^{\bullet-}$ (Shokoji et al., 2004). Several studies have provided compelling evidence that reactive oxygen species acting within the central nervous system acutely increase sympathetic nerve activity (Lin et al., 2003; Lu et al., 2004; Zimmerman et al., 2004b) and contribute to elevated baseline levels of sympathetic nerve activity in heart failure and hypertension (Shokoji et al., 2003; Xu et al., 2004; Zimmerman et al., 2004a; Gao et al., 2004; Lindley et al., 2004).

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