SOD-1 expression in pig coronary arterioles is increased by exercise training

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Rush, James W. E., M. Harold Laughlin, Christopher R. Woodman, and Elmer M. Price. SOD-1 expression in pig coronary arterioles is increased by exercise training. Am J Physiol Heart Circ Physiol 279: H2068–H2076, 2000.—Coronary arterioles of exercise-trained (EX) pigs have enhanced nitric oxide (NO)-dependent dilation. Evidence suggests that the biological half-life of NO depends in part on the management of the superoxide anion. The purpose of this study was to test the hypothesis that expression of cytosolic copper/zinc-dependent superoxide dismutase (SOD)-1 is increased in coronary arterioles as a result of exercise training. Male Yucatan pigs either remained sedentary (SED, n = 4) or were EX (n = 4) on a motorized treadmill for 16–20 wk. Individual coronary arterioles (~100-μm unpressurized internal diameter) were dissected and frozen. Coronary arteriole SOD-1 protein (via immunoblots) increased as a result of exercise training (2.16 ± 0.35 times SED levels) as did SOD-1 enzyme activity (measured via inhibition of pyrogallol autooxidation; ~75% increase vs. SED). In addition, SOD-1 mRNA levels (measured via RT-PCR) were higher in EX arterioles (1.68 ± 0.16 times the SED levels). There were no effects of exercise training on the levels of SOD-2 (mitochondrial), catalase, or p67phox proteins. Thus chronic aerobic exercise training selectively increases the levels of SOD-1 mRNA, protein, and enzymatic activity in porcine coronary arterioles. Increased SOD-1 could contribute to the enhanced NO-dependent dilation previously observed in EX porcine coronary arterioles by improving management of superoxide in the vascular cell environment, thus prolonging the biological half-life of NO.

superoxide dismutase; microcirculation; endothelium; antioxidant enzymes; nitric oxide; endothelium-dependent dilation; oxidative stress

VASCULAR ENDOTHELIUM IS CAPABLE of detecting both chemical substances in the blood and physical forces imparted to the vascular wall. The endothelium integrates these signals into an output response, endothelium-mediated vascular control, which is an important component in the overall complex control of the coronary circulation (12, 19, 31, 45). One of the substances released by the endothelium, nitric oxide (NO), appears to exert a dominant influence in mediating endothelium-dependent vasodilation (3, 10, 35), a process that can be important in the control of coronary vascular resistance and flow in vivo (12, 19).

Aerobic exercise training increases both coronary blood flow and vascular transport capacities (18, 21, 34). These adaptations are consistent with the increased demand for flow and exchange of oxygen and metabolites that accompanies each exercise-training bout. These adaptations depend in part on training-induced modifications in the control and distribution of coronary vascular resistance (19, 20, 32, 34). For example, endothelium-dependent vasodilation is enhanced in coronary resistance vessels from exercise-trained (EX) pigs (31) and in coronary arteries of EX dogs (45). This adaptation appears to involve changes in the management of NO and the regulation of endothelial cell nitric oxide synthase (ecNOS), because the training adaptations are eliminated in the presence of NOS inhibitors (31, 45).

One possible mechanism for the increased NO-mediated vasodilatory responses of EX vessels is increased NO production, because ecNOS gene expression is upregulated in pig coronary arterioles (46) and dog aorta (39) after exercise training. The efficacy and biological half-life of NO depend not only on its rate of production (i.e., ecNOS activity) but also on its rate of destruction. The primary pathway for NO degradation is via interaction with superoxide anion (O$_2^-$) to form peroxynitrite (4, 7, 24). Therefore, a potentially important mechanism to prolong NO activity is to better manage the O$_2^-$ load in the vascular environment. The Cu/Zn-dependent superoxide dismutase (SOD)-1 is the principle O$_2^-$ scavenging enzyme in vascular cells. Indeed, inhibition of this enzyme in vascular tissue in vitro impairs relaxation to the endothelium-dependent vasodilator ACh, suggesting that the biological activity of NO depends on SOD activity (29). Furthermore, endothelium-dependent dilation of the coronary resistance bed is impaired when free radical production is artificially increased in the perfusate (41). Conversely, when O$_2^-$ management is facilitated by administration of exogenous SOD, O$_2^-$ scavengers, or antioxidants, endothelium-dependent dilation is improved, most no-

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tably in vessels in which endothelial function is compromised by cardiovascular disease risk factors (25, 30, 33, 43). In addition to its indirect antivasodilatory function through destruction of NO, O$_2^-$ is also a powerful direct contracting factor for vascular smooth muscle (14). This combination of effects emphasizes the need to effectively manage O$_2^-$ as one way to protect or enhance endothelium-dependent dilation.

SOD-1 mRNA has been shown to increase in response to stimuli similar to those that induce ecNOS expression. For example, flow/shear increases both ecNOS and SOD-1 mRNA expression in cultured vascular endothelial cells (11, 38) and in isolated perfused coronary arterioles (47). However, the vascular cell expression of SOD-1 in response to exercise training has not been previously evaluated. Increases in SOD-1 expression could enhance O$_2^-$ scavenging and therefore be complementary to increases in ecNOS expression in augmenting NO biological activity in EX coronary arterioles. Thus upregulation of SOD-1 could contribute to the decreased vascular resistance, enhanced endothelium-dependent dilation, and increased coronary blood flow observed in EX hearts.

The primary purpose of this study, therefore, was to test the hypothesis that aerobic exercise training increases SOD-1 expression in porcine coronary arterioles of ~100 µm diameter. In addition, we determined the effect of exercise training on the protein levels of two other antioxidant enzymes, the Mn-dependent SOD (SOD-2) and catalase, and a component of the superoxide-generating NAD(P)H oxidase, p67phox.

**METHODS**

**Animals and training.** Male Yucatan miniature swine (n = 8) were purchased from a commercial breeder (Charles River) and housed in an animal care facility in the Department of Veterinary Biomedical Sciences in a room maintained at 20–23°C, with a 12:12-h light-dark cycle. The pigs were 8–12 mo of age and weighed 30–37 kg at the time of study. All procedures involving the use of these animals were approved by the Animal Care and Use Committee at the University of Missouri and were in accordance with the “Guidelines for the Care and Use of Animals” of the American Physiological Society.

Sedentary pigs (SED) were confined to their pens during the training period (~16 wk) except for routine maintenance and feeding periods each day. Pigs from both treatment groups were fed at the same time each day. After a 1- to 2-wk period of familiarization with the treadmill, pigs in the aerobic exercise-training group began a progressive treadmill training program. During week 1, pigs ran at 0% grade, 3 mph for 20–30 min and at 5 mph for 15 min. The speed and duration of running were gradually increased such that during week 12 of training, the pigs ran for ~55 min/day, 5 days/wk. The training bouts after week 12 consisted of a warmup (2.5 mph, 5 min), a sprint (6–8 mph, 15 min), an endurance run (4–6 mph, 60 min), and a warmdown (2 mph, 5 min). Positive reinforcement was provided in the form of feeding after each training bout. The efficacy of training was assessed by the measurement of the heart weight-to-body weight ratio and by measuring citrate synthase activity (an index of mitochondrial enzyme activity) in the deltoid muscle, both of which have been shown to increase as a result of aerobic exercise training in this pig model of exercise training (20, 31, 32, 34, 46).

**Isolation of coronary arterioles.** After the ~16-wk training period, EX and SED pigs were sedated with ketamine (30 mg/kg im) and anesthetized with pentobarbital sodium (35 mg/kg iv). The pigs were intubated and ventilation was maintained with a Harvard large-animal respirator throughout tissue harvesting. After an intravenous infusion of heparin, a left thoracotomy was performed and the heart was rapidly excised and placed in Krebs bicarbonate buffer (4°C) previously aerated with 95% O$_2$-5% CO$_2$. Single coronary arterioles (~100 µm internal diameter, unpressurized) from the region of the left anterior descending artery were dissected with the aid of a Zeiss dissecting microscope. These samples were placed in prechilled RNase-free microcentrifuge tubes, frozen in liquid nitrogen, and stored at ~80°C until used for immunobLOTS, mRNA isolation, or SOD enzyme activity assays. Coronary arterioles dissected in this way were determined to be free from detectable cardiac myocyte contamination on the basis that the cardiac myocyte marker gene, cardiac β-myosin heavy chain (β-MHC), was shown by RT-PCR to be expressed in heart samples but not in dissected coronary arteriole samples (n = 6 per group, data not shown).

**ImmunobLOTS.** Single coronary arterioles were digested in a sample buffer containing 6 M urea, 2% SDS, 150 mM dithiothreitol, and 50 mM Tris-Cl (pH 6.9) and boiled for 5 min three times with intermittent agitation (vortex). Samples were loaded onto four 20% acrylamide gradient SDS gels (precast Mini-Gels, Bio-Rad) and electrophoresed. Proteins were transferred to polyvinylidene difluoride membranes (Amersham) after which the membranes were blocked for 1 h with 5% (wt/vol) nonfat milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 detergent (TBS-Tween). Membranes were then washed several times with TBS-Tween and incubated overnight with the primary antibody against the target protein: SOD-1, SOD-2, catalase, p67phox, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Polyclonal (rabbit) antibodies to SOD-1 and SOD-2 were obtained from Stressgen and were used at a 1:1,500 dilution, a polyclonal antibody to catalase was obtained from Chemicon and was used at a 1:5,000 dilution, a monoclonal antibody against p67phox was obtained from Chemicon and was used at a dilution of 1:5,000, and a monoclonal antibody against GAPDH was obtained from Amersham and was used at a dilution of 1:5,000. Blots were then washed several times in TBS-Tween and incubated for 2 h with an appropriate secondary antibody (either anti-rabbit IgG for SOD-1, SOD-2, and catalase or anti-mouse IgG for p67phox and GAPDH) conjugated to horseradish peroxidase. After secondary antibody incubation, membranes were again washed several times in TBS-Tween, and the immunoreactive bands were detected using the enhanced chemiluminescence system (ECL, Amersham) and exposure to X-ray film (Amersham). The relative expression of target proteins was determined by scanning densitometry. Target protein density values were adjusted for solubilization and gel loading differences among the different samples by correcting to the intensity of the GAPDH signal within individual samples. The data were then adjusted such that the SED group had a mean value of 1 to easily illustrate the multifold effect of exercise treatment.

**mRNA isolation.** Single coronary arterioles were digested in a lysis buffer containing (in mM) 500 LiCl, 10 EDTA, 5 dithiothreitol, and 100 Tris-Cl, with 1% lithium dodecyl sulfate (LiDS) (pH 8.0), using vigorous agitation (vortex). mRNA was isolated from the lysate using the Dynabeads oligo(dT)$_{25}$ paramagnetic polystyrene bead technology (Dynal), modified as previously reported in detail (46).
RT-PCR. First-strand cDNA synthesis was carried out using the isolated mRNA as template and oligo(dT)12–18 to prime the RT reaction. This was done with the SuperScript preamplification system (GIBCO-BRL) using modifications previously reported in detail to yield a 20-μl final cDNA sample (46). A 5-μl aliquot of cDNA was used to perform a PCR reaction in a 50-μl total volume containing (in mM) 50 KCl, 20 Tris-HCl (pH 8.4), 4 MgCl₂, 0.2 dNTP, and 0.2 each primer and 2.5 U Taq DNA polymerase (Promega). SOD-1 primers were based on a reported sequence for human SOD-1 (40), were designed to amplify a 375-bp product, and were as follows: sense, 5′-GATATGCAGCATGGAAGGTTGTA-3′; antisense, 5′-CAATTCACACACCGGCAACCA-3′. The RT-PCR protocol was standardized for small differences in starting material amount, efficiency of the reactions, and recovery by coamplifying SOD-1 with GAPDH in the same reaction tube. GAPDH primers were the same as those previously used in our laboratory (46, 47): sense, 5′-CAACCATGGCAATCTCATG-3′; antisense, 5′-TCTAGACGCGTGAGTGTC-3′. GAPDH is a constitutive gene, the expression of which is believed to be altered in vascular tissue by exercise training (39). PCR was initiated with a 5-min denaturation step (94°C) and a 5-min annealing step (63°C) followed by cycles of 72°C (elongation, 2 min), 94°C (1 min), and 63°C (1 min). The final step was a 10-min 72°C elongation. The PCR-amplified products were separated by 1.5% agarose gel electrophoresis and visualized with ethidium bromide staining. For semiquantitative PCR, the number of cycles was 25 (determined to be in a linear range for label incorporation into both SOD-1 and GAPDH PCR products, data not shown), and the PCR reaction was spiked with 10 μCi of α-[32P]dCTP (3,000 Ci/mmol). The PCR products were separated by agarose gel electrophoresis as described above, and the bands for SOD-1 and GAPDH for each sample were separately excised from the gel, dissolved in a chaotrope solution (QX1, Qiagen), and mixed with 10 ml of scintillation fluid. Radiolabel incorporation was evaluated by liquid scintillation counting. The SOD-1 to GAPDH (SOD-1/GAPDH) ratio of radiolabel incorporation was calculated for each individual arteriole, and the data were adjusted such that the SED group had a mean value of 1 to easily illustrate the multifold effect of exercise treatment. In an adjacent study, the same type of semiquantitative RT-PCR analysis was conducted to assess catalase mRNA levels in SED and EX arterioles. PCR conditions were identical to those outlined above for SOD-1, and aliquots of the same cDNA preparations used for the SOD-1 analysis were used in the catalase studies. The primers used for catalase PCR were based on a reported mRNA sequence for human catalase (2), were designed to amplify a 342-bp PCR product, and were as follows: sense, 5′-CGATGTGCATGCAGGACAATCAG-3′; antisense, 5′-GCTTCTACGACTGGTGATCC-3′. SOD enzyme activity assays. The SOD activity assay was based on the previously established method of inhibition of autoxidation of pyrogallol in alkaline solution (23). Pools of arterioles (3 arterioles/pool) from SED or EX pigs were homogenized in 200 μl of 50 mM Tris-cacodylic acid (pH 8.2) containing 1 mM diethylenetriaminepentacetic acid (DTPA) using a Potter Elvehjem homogenizer immersed in ice water and then subjected to a 15-s burst of sonic oscillation. Homogenates were extracted in ethanol-chloroform (6:2.5:37:5, vol/vol) to inhibit Mn-dependent SOD and make the assay specific for Cu/Zn-dependent SOD as has been previously validated (37). An 800-μl aliquot of 50 mM Tris-cacodylic acid and 1 mM DTPA at pH 8.2 that had been equilibrated with room air at 25°C was mixed with either 100 μl of arteriole homogenate or, for blanks, 100 μl of ethanol-chloroform-extracted homogenization buffer. The reaction was initiated by adding 100 μl of 2 mM pyrogallol (for a final concentration of 0.2 mM and a final reaction volume of 1 ml), and the increase in absorbance at 420 nm over time was followed (25°C reaction temperature). SOD activity was assessed as the degree of inhibition of the pyrogallol autooxidation rate. The autooxidation rate measured under the indicated assay conditions was 0.0169 ± 0.0002 OD/min, n = 14, assessed over the first 3 min of reaction. This assay system yielded proportional results when between 50 and 150 μl of arteriolar homogenate were used, and the reaction rate was linear for up to 10 min. One unit of SOD activity is defined as the amount of activity that inhibits the autooxidation rate of pyrogallol by 50%. Because the vessel dimensions diameter (D) and length (L) were measured and matched across treatment groups, SOD activity measurements were corrected to the calculated total surface area (SA) of arterioles used in each pool of arterioles (SA = π × D × L). The small amount of tissue precluded more traditional types of standardization such as to total tissue weight or protein content.

Data analysis. All values are presented as means ± SE. Between-group differences were determined by unpaired Student’s t-tests. Because multiple arterioles from each pig were used in the analyses, the data from immunoblot and PCR analyses were analyzed in each of two ways: using the number of arterioles as n, or averaging values among arterioles of an individual animal and using the number of animals as n. It was found that both methods of analysis yielded the same conclusions regarding significant differences between SED and EX groups. Thus the data presented were analyzed using n as the number of arterioles, as indicated. Differences were considered significant if P < 0.05.

Results

Efficacy of training. Heart weight-to-body weight ratio increased ~15% as a result of aerobic exercise training (Table 1). This was solely due to higher heart weight in the EX group, because body weight was not significantly different between EX and SED (P = 0.26, Table 1) pigs. In addition, citrate synthase activity of the deltoid increased ~40% as a result of the aerobic exercise-training regimen (Table 1). Previous studies in our laboratory have demonstrated that the outlined aerobic exercise-training program also results in prolonged running endurance to exhaustion and a relative bradycardia compared with SED controls (20, 31, 32, 34).

SOD-1 protein in coronary arterioles. A single band at 17 kDa was detected in immunoblots for SOD-1 using coronary arterioles (Fig. 1). Exercise training

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<th>Table 1. Indexes of efficacy of the aerobic exercise training program</th>
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Values are means ± SE; n = 4 per group. Heart wt/body wt, heart weight-to-body weight ratio. Significantly greater than corresponding values for sedentary group. *P < 0.05 and tP < 0.01.
resulted in an ~120% increase in SOD-1 protein levels in coronary arterioles compared with SED (Fig. 1) animals. There appeared to be no systematic effect of exercise training on the expression level of GAPDH detected in immunoblots (Fig. 1; ratio of mean GAPDH band density; EX/SED = 1.04, n = 12 per group). Vessels for SOD-1 immunoblots had passive internal diameters of 125 ± 7 and 136 ± 7 μm for SED and EX groups (not significantly different, P = 0.15), respectively, with n = 12 per group (3 individual vessels from each of 4 pigs from each group).

SOD-1 enzyme activity in coronary arterioles. SOD-1 activity assessed in vitro by inhibition of pyrogallol autooxidation was increased by ~75% in coronary arterioles of EX pigs compared with SED pigs (Fig. 2). Vessels of similar dimensions were used from each of the treatment groups: D = 164 ± 21 vs. 157 ± 21 μm, and L = 2,038 ± 132 vs. 2,006 ± 146 μm, for SED versus EX groups, respectively, with n = 12 per group (3 individual vessels from each of 4 pigs from each of the SED and EX groups).

SOD-1 mRNA in coronary arterioles. As predicted from the known SOD-1 mRNA sequence (40), the primer pair used to amplify cDNA for SOD-1 produced a PCR product of 375 bp in size (Fig. 3). This band matches the size predicted from the primer pair chosen, and its identification was confirmed by direct sequencing (at the Core Facility, Dept. of Microbiology and Immunology, Univ. of Missouri) after gel purification of the 375-bp PCR product (obtained using the Qiagen system). The gel-purified band shared 100% homology to the pig and 91% homology to the human SOD-1 gene over 300 sequenced bases. Positive identification of the GAPDH PCR product was by the size criterion; a previous study in our laboratory also di-

Fig. 1. Immunoblot analysis of exercise-training effect on superoxide dismutase (SOD) protein expression in coronary arterioles. Top: luminogram of a representative immunoblot (1 of a total of 3 performed) for Cu/Zn-dependent SOD (SOD-1) and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using 4 individual arterioles each from sedentary (SED) and exercise-trained (EX) pigs. One arteriole is shown per lane. One arteriole from each of 8 pigs used in the study is represented (SED lanes 1–4, EX lanes 1–4). A single immunoreactive band (shown) was detected in luminograms at 17 kDa for SOD-1, and a single band at ~37 kDa was detected for GAPDH. Bottom: effect of exercise training on relative SOD-1 protein expression (data corrected to GAPDH expression and standardized such that mean of SED values is 1; see METHODS). Data are expressed as means ± SE. This bar graph represents composite data from all 3 immunoblots; n = 12 arterioles per group (3 arterioles from each of 4 pigs from each group). †Significantly greater than SED; P = 0.005.

Fig. 2. SOD-1 enzymatic activity in coronary arterioles. Activity is normalized to a fixed arteriolar surface area (SA) arbitrarily chosen as 10^6μm^2. Data are expressed as means ± SE; n = 3 pools of 3 arterioles each per group (SED vs. EX). †Significantly greater than SED; P = 0.01.

Fig. 3. RT-PCR analysis of exercise-training effect on SOD-1 mRNA expression in coronary arterioles. Top: agarose gel of representative SOD-1 PCR products from experimental samples and negative and positive controls. 123 bp, 123-bp marker ladder. PCR was performed for 25 cycles (within linear range). No RT and no cDNA are negative-control reactions in which the enzyme RT or the cDNA aliquot, respectively, was left out of the appropriate reaction (see METHODS). Heart is a positive-control SOD-1 RT-PCR product generated using pig heart RNA as starting material. Bottom: effect of exercise training on relative SOD-1 mRNA expression (data corrected to GAPDH expression and standardized such that mean of SED values is 1; see METHODS). For quantitative purposes, PCR was performed for 25 cycles (within linear range), and quantification was by α-[32P]dCTP incorporation into PCR products. Data are expressed as means ± SE; n = 12 arterioles per group (3 arterioles from each of 4 pigs from each group). †Significantly greater than SED; P = 0.0006.
rectly sequenced the GAPDH product generated using pig arteriole cDNA and the same primers as in this study and confirmed its identity (46).

To eliminate the possibilities that PCR products were generated by amplification of potential contaminant genomic DNA from the arterioles or from nonspecific contamination of reagents, negative control RT-PCR reactions were performed by leaving out either RT from the RT reaction or cDNA from the PCR, respectively. As illustrated in Fig. 3, no PCR products were detected when either RT or cDNA was not included, confirming the specificity of the procedure for reverse transcribing SOD-1 and GAPDH mRNA present in the arteriole samples and amplifying the resultant cDNAs. A positive control for SOD-1 detection using RT-PCR was performed using pig heart muscle RNA as starting material (Fig. 3).

Semiquantitative PCR in which SOD-1 and GAPDH cDNAs were amplified in the presence of α-[32P]dCTP revealed an ~70% increase in the SOD-1/GAPDH ratio as a result of exercise training (Fig. 3). Randomly selected arteriole RT-PCR products from each of the SED and EX groups are illustrated in Fig. 3 (top). There appeared to be no systematic effect of exercise training on the expression level of GAPDH mRNA detected by RT-PCR (the ratio of mean GAPDH label incorporation during PCR; EX/SED = 0.83, n = 12 per group). This confirms earlier reports indicating no effect of exercise training on GAPDH expression in vascular cells (39). The increase in the SOD-1/GAPDH ratio is the result of increased SOD-1 expression in EX coronary arterioles (Fig. 3).

Samples for semiquantitative experiments were run for 25 rounds of PCR, which is in the linear range for amplification (data not shown). Vessels used for RT-PCR had passive internal diameters of 95 ± 4 and 91 ± 4 μm for SED and EX groups (not significantly different, P = 0.26), respectively, with n = 12 per group (3 individual vessels from each of 4 pigs from each of the SED and EX groups). SOD-2 and catalase protein in coronary arterioles. A single band at 22 kDa was detected in immunoblots for SOD-2 and a single band at 22 kDa was detected in immunoblots for catalase using coronary arterioles (Figs. 4 and 5). Exercise training did not significantly alter SOD-2 or catalase protein levels in coronary arterioles compared with SED (P = 0.49 and 0.18, respectively; Figs. 4 and 5), with n = 12 per group (3 individual vessels from each of 4 pigs from each of the SED and EX groups). SOD-2 and catalase protein in coronary arterioles. A single band at 61 kDa was detected in immunoblots for catalase using 4 individual arterioles each from SED and EX pigs. One arteriole is shown per lane, and 1 arteriole from each of the 8 pigs used in the study is represented (SED lanes 1–4, EX lanes 1–4). A single immunoreactive band (shown) was detected at 61 kDa. Bottom: effect of exercise training on relative catalase protein expression (data corrected to GAPDH expression and standardized such that mean of SED values is 1; see METHODS). Data are expressed as means ± SE. This represents composite data from all 3 immunoblots; n = 12 arterioles per group (3 arterioles from each of 4 pigs from each group). P = 0.177, no significant difference between groups.
ducted to assess catalase mRNA expression levels using the same cDNA preparations used for the SOD-1 measurements. There was no effect of exercise training on catalase mRNA levels in coronary arterioles (corrected catalase-to-GAPDH ratio 1.00 ± 0.06 vs. 1.08 ± 0.13, SED vs. EX, respectively, data not illustrated), with n = 12 per group (3 individual vessels from each of 4 pigs from each of the SED and EX groups). This result is consistent with the lack of effect of exercise training on the catalase protein level in coronary arterioles (Fig. 5).

*p67*phox Protein in coronary arterioles. A 67-kDa band was detected in immunoblots for p67*phox* (Fig. 6). Exercise training did not significantly alter p67*phox* protein levels in coronary arterioles compared with SED (P = 0.46, Fig. 6, with n = 8 per group (2 individual vessels from each of 4 pigs from each of the SED and EX groups). Arteriole samples used for p67*phox* immunoblots had passive internal diameters of 130 ± 6 and 134 ± 5 μm for SED and EX groups (not significantly different, P = 0.63), respectively, with n = 8 per group (2 individual vessels from each of 4 pigs from each of the SED and EX groups).

**DISCUSSION**

The major findings of this study are that exercise training increases SOD-1 mRNA, protein, and enzymatic activity in coronary arterioles (100- to 160-μm diameter) and that this adaptation occurs in the absence of steady-state changes in the level of protein expression of other antioxidant enzymes such as SOD-2 and catalase or of the prooxidant p67*phox* component of NAD(P)H oxidase. The increased expression of SOD-1 is expected to better manage a given O2− load and thus both reduce the rate of biodegradation of NO by O2− in the coronary microvascular environment (4, 7, 24, 25, 30, 33, 43) and attenuate the direct vasconstrictor effect of O2− on vascular smooth muscle (14).

Together with increased ecNOS expression (46), this adaptation could be an important contributor to the observed increased sensitivity of endothelium-depend dent dilation of ~100-μm-diameter porcine coronary arterioles after aerobic exercise training (31).

Because of the coincidence of increases in both the mRNA and protein levels of SOD-1 observed, it is likely that the SOD-1 protein-expression level increased as a result of an increased amount of SOD-1 mRNA, rather than by a decreased SOD-1 protein-degradation rate. Whether SOD-1 mRNA was elevated because of increased expression or transcription of the SOD-1 gene or because of increased mRNA stability, however, cannot be determined from the current data.

The findings of this study indicate that exercise training specifically increases the expression of SOD-1 and that this is not coordinated with expression changes in other measured anti- or prooxidant enzymes in the fully adapted EX porcine coronary arteriolar circulation. The specific signals associated with exercise training that induce the upregulation of SOD-1 expression are not known, although recent results with cell culture and isolated perfused arterioles provide some possibilities. Exercise-training bouts are accompanied by increased coronary flow required to serve the increased metabolic needs of the myocardium (13, 15). Studies using cultured vascular endothelial cells have demonstrated that increased shear stress can increase the expression of SOD-1 mRNA (11). Using isolated perfused coronary arterioles, we recently demonstrated that levels of flow that elicit maximal flow-induced dilation increase SOD-1 mRNA expression in arterioles from SED pigs (47). Thus increased flow/shear associated with exercise training may play a role in the enhanced SOD-1 expression observed in coronary arterioles of EX pigs. The indictment of flow/shear as a possible mediator of the exercise-training effect on arteriolar SOD-1 expression does not eliminate the possibility that other signals associated with exercise training also contribute to the observed response. For instance, the cellular redox state may exert an influence on gene expression (22), and the vascular cell redox state is likely to be altered during exercise by exposure to superoxide generated by the surrounding myocardium or by the vascular cells themselves (1, 5, 14, 25, 29). Previous studies have also demonstrated that protein kinase C activation is a stimulus for SOD-1 expression (26). The proximal promoter region of the SOD-1 gene contains sequences known to bind transcription factors including nuclear factor (NF)-κB, activator protein (AP)-2, Sp1, Egr-1, and WT-1 (16, 17, 26). Therefore, stimuli that converge on these transcription factors could theoretically influence SOD-1...
gene expression. The extent to which these stimuli are involved in the exercise-induced response in coronary arterioles is currently unknown. To our knowledge, no previous studies have evaluated the responses of arterial SOD-1, SOD-2, catalase, or p67phox expression to exercise training, so there is currently no further basis for speculation regarding the mechanism of the effect.

The increased SOD-1 mRNA, protein, and enzymatic activity demonstrated here are complementary to recent observations by our group and others who have demonstrated that exercise training increases the expression of ecNOS mRNA in vascular endothelium (39, 46). The coordinated upregulation of these two genes in coronary resistance arteries may play a dual role in enhancing NO activity: increased production of NO via increased ecNOS expression, and decreased rate of NO destruction via increased SOD-1 expression and consequent better management of O2.

There is some precedent for the coordinated expression of these two genes as well as for some overlap in intracellular signaling pathways mediating the expression of these genes, because we demonstrated that expressions of ecNOS and SOD-1 mRNA are both up-regulated by exposure to elevated flow/shear in isolated perfused coronary arterioles from SED pigs (47). Regardless of the specific signaling pathways involved, however, the elevated expressions of ecNOS and SOD-1 are both expected to contribute to an increased efficacy and biological half-life of NO. In this way, the upregulation of expression of these enzymes is likely important in the observed increased sensitivity to endothelium-dependent dilation of coronary arterioles that results from exercise training, which is eliminated by inhibition of NO synthesis (31).

It is necessary to emphasize that the alterations (and lack of alterations) in gene and protein expression observed in the current study are likely to be steady-state adaptations reflecting a stable EX phenotype; i.e., the adaptations are likely complete and characteristic of the EX physiological state. The basis for this is that the training program used is of sufficient duration that the exercise-induced enhancements in muscle mitochondrial enzymatic capacity and coronary blood flow are stable and complete, and classical cardiovascular conditioning has occurred (20, 32, 44). Thus it is predicted that changes in gene expression due to exercise would also be complete. Therefore, even though we observed no changes in the level of SOD-2, catalase, or p67phox expression after ~16 wk of aerobic exercise training, it is possible that during the adaptation to the fully trained state, the expression of these proteins was changed at some point. Similarly, it is possible that the onset and/or magnitude of the adaptations we observed in SOD-1 expression was earlier in the exercise-training program than when we evaluated them. Nonetheless, we are interested in the stable adaptations that characterize the EX state, and the results of this study indicate that the enhanced endothelium-dependent, NO-dependent dilation and ecNOS expression in fully adapted EX coronary arterioles (31, 46) are accompanied by increased SOD-1 expression, but not with changes in SOD-2, catalase, or p67phox expression. We chose to examine p67phox expression as an index of the prooxidant enzyme response to exercise training because the NAD(P)H oxidase is quantitatively the most important source of superoxide in normal vascular cells (27, 28, 36). To our knowledge, this is the first demonstration of p67phox protein expression in coronary arterioles. Our results indicating no alteration in the expression of p67phox protein in coronary arterioles in response to exercise training suggest that the basal NAD(P)H oxidase activity and superoxide generation may not be altered in EX arterioles. These data do not, however, eliminate the possibility that the activity of NAD(P)H oxidase may be altered in a manner independent of the level of p67phox protein expression or that the activity of other oxidases that normally play more diminutive roles in vascular superoxide generation may be altered by exercise training.

A limitation of this study is that because we evaluated the SOD-1 mRNA, protein, and enzyme activity in arteriolar lysates, the adaptations cannot be assigned to a particular cell type. Whether the increased SOD-1 expression that accompanies exercise training occurs in endothelial cells, vascular smooth muscle cells, or both, it would still play a potentially important functional role in the enhanced endothelium-dependent dilation because NO and O2 exist in both cellular environments. In addition, the two molecules could interact in the interstitium. There is another isoform of SOD (SOD-3) that is an extracellular enzyme and can therefore react with interstitial O2 (42). We have not evaluated the expression of SOD-3 and therefore cannot make any statements regarding the importance of this enzyme in the management of O2 as it relates to interaction with NO and the control of endothelium-dependent dilation, or regarding possible adaptations in the expression of SOD-3 with aerobic exercise training. A further limitation of this study is that the possible altered expression of prooxidant enzymes other than the p67phox component of the NAD(P)H oxidase in coronary arterioles in response to exercise training has not been evaluated.

Speculation of functional impact. The possible functional impact of increased SOD-1 expression in the coronary arterioles of EX pigs has not been directly demonstrated in this study. The demonstrated increase in SOD-1 in the apparent absence of changes in other pro- and antioxidant enzymes suggests that the arterioles of EX animals would better manage a given superoxide load and that the biological half-life and efficacy of NO would be improved. If this is so, it would be expected that the NO-dependent relaxation response to a given stimulus would be increased in EX arterioles. This speculation is indirectly supported by previous work from our laboratory that demonstrates an improved NO-dependent dilatory response of coronary arterioles from EX versus SED pigs (31). It is therefore conceivable that increased levels of SOD-1 in coronary arterioles could contribute to the documented exercise-training-induced augmentation of flow-dependent dilation in both healthy individuals and those
with vascular disease (6, 8, 9, 45). This adaptation could also contribute to the well-known effect of exercise training in the primary prevention of a variety of cardiovascular diseases (1a).

In conclusion, a prolonged program of exercise training that leads to cardiovascular and muscular adaptations typical of increased aerobic capacity also results in increased expression of SOD-1 but does not alter SOD-2, catalase, or p67phox protein levels in coronary arterioles. It is expected that by more effectively managing O$_2^·$ in the vascular cellular environment, increased SOD-1 would prolong the biological half-life of NO$, an important component of endothelium-dependent vasodilation. By providing a more favorable environment to prolong the NO$-biological half-life, higher SOD-1 expression in EX coronary arterioles is expected to contribute to the enhanced endothelium-dependent NO-mediated dilation observed in these vessels compared with those of SED controls.

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REFERENCES


