Critical involvement of hydrogen peroxide in exercise-induced up-regulation of endothelial NO synthase

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Abstract

Objective: Recent studies from our groups have indicated that endothelial nitric oxide synthase (eNOS) expression is increased in cell culture by both shear stress and by hydrogen peroxide (H2O2). In vivo, exercise training, known to increase both endothelial shear stress and oxidative stress, also increases eNOS expression. It is unclear if H2O2 contributes to an increase in eNOS expression in response to exercise training.

Methods: To address this question, we generated mice overexpressing human catalase (hCat) driven by the murine Tie-2 promoter to specifically target this transgene to the endothelium (cat++).

Results: Vessels of cat++ expressed significantly higher levels of catalase mRNA and catalase protein and activity but normal levels of eNOS. Exercise alone had no effect on catalase expression in C57BL/6. Wild-type littermates of cat++ showed an increase in eNOS expression with 3 weeks of exercise (2.53 ± 0.42-fold) comparable to C57BL/6 (2.93 ± 0.45-fold). In striking contrast, 3 weeks of exercise had no effect on aortic (1.33 ± 0.32-fold) and myocardial (1.1 ± 0.2-fold) eNOS expression in catalase transgenic mice.

Conclusions: These data suggest that endogenous H2O2 plays a key role in the endothelial adaptation to exercise training by stimulating an up-regulation of eNOS.

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1. Introduction

Regular physical activity is an important factor in the prevention of cardiovascular diseases [1]. The effects of exercise on organ function are associated with an approximately 30% reduction of mortality in patients with coronary artery disease or heart failure [2]. Even daily walking of 2 miles reduces the mortality of nonsmoking retired men [3]. Exercise induces a reduction of heart rate and blood pressure, increases maximal myocardial oxygen uptake and leads to several physiological adaptations involving skeletal muscle, cardiac muscle, circulating blood volume and a variety of metabolic modifications [4–7]. In contrast, little is known about the mechanisms leading to the positive effects of exercise in the vasculature.

Several studies suggest an important role of endogenous production of nitric oxide (NO) in the beneficial effects of exercise [8]. In-vivo studies in animals and humans have shown that exercise results in an increased vascular
expression of the endothelial cell eNOS [9–12]. This potentially important adaptation to exercise training seems to require two intact endothelial nitric oxide synthase (eNOS) genes [13] and might contribute to the improvement of endothelial function in coronary conductance and resistance vessels of exercised coronary artery disease patients [14]. On the other hand, exercise is associated with increased vascular oxidative stress which is considered to be a pathogenic factor in cardiovascular disease [15].

It is not yet known how the induction of vascular eNOS by exercise is mediated. Increased shear stress potently stimulates an increase in eNOS expression. In addition, previous studies from our laboratory have shown that hydrogen peroxide (H₂O₂) increases the expression and activity of eNOS in endothelial cells [16–18]. The increase of eNOS activity in response to H₂O₂ comes along with a change of eNOS phosphorylation and is thought to be an acute cellular adaptation to an increase in oxidant stress [19,20]. This is relevant to exercise, which is associated with an increase in oxidative stress within the skeletal muscle and an increase in circulating levels of H₂O₂ and markers of lipid oxidation. We have also demonstrated that exercise increases expression of vascular extracellular superoxide dismutase (ecSOD) which could enhance the formation of H₂O₂ [21]. Given these considerations, we hypothesized that endogenously produced H₂O₂ would contribute to the up-regulation of eNOS expression caused by exercise training. Unfortunately, there is no reliable method to directly measure vascular H₂O₂ levels in vivo [22]. Thus, we generated transgenic mice with vascular-specific overexpression of human catalase (hCat) and compared the effect of exercise training on eNOS expression in these and wild-type animals.

2. Methods

2.1. Generation of transgenic mice

To target catalase gene expression to the endothelium, we generated a transgenic construct, in which human catalase (hCat) was inserted between murine Tie-2 promoter (2.1 kb) and a 10-kb Tie-2 intron fragment, designated as Tie-2 enhancer (Fig. 1A).

2.1.1. Plasmid construction

By PCR, a Sse83871 restriction site was added to the 5′ site and a MluI site was added to the 3′ site of hCat cDNA. The following primers were used: sense (5′ GAGAGACCTGAGGAGCAAACCGACGCTATG); antisense: (5′ GAGAGACCGTGCAAGTGATGACCGG-GTTAC). The resulting 1.7-kb Sse83871-hCat-MluI fragment was confirmed by restriction analysis and was sequenced by cycle sequencing with dye terminators. Sse83871-hCat-MluI fragment was isolated and inserted downstream of the Tie-2-promotor (a generous gift from T. Schläger, M.-Planck Institut, Bad Nauheim) and upstream of the Tie-2-enhancer. Plasmid (PK), confirmed by restriction analysis, comprised pBluescriptII SK(+) backbone (Stratagene, La Jolla, CA), 2.1-kb Tie-2-promotor, 1.7-kb hCat-cDNA, 250 bp SV40 poly A signal and 10-kb Tie-2 enhancer. Sal I restriction sites used for linearization of the plasmid and Sse83871/MluI sites added to catalase cDNA for ligation are indicated. PCR primers used for genotyping PCR are indicated by arrows. (B) PCR identifying 11 of 44 founder animal harbouring the transgene. A 470 bp transgene specific fragment is amplified in transgenic animals. Plasmid-DNA (100 ng) of the construct served as control (K).
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cDNA, 250-bp SV40 poly A signal and 10-kb Tie-2 enhancer (see Fig. 1).

2.1.2. Microinjection

The Tie-2-promotor-hCat-Tie-2 enhancer construct was excised from the vector, purified and microinjected in fertilized F2-eggs of F1 (C57BL/6×C3H/He) mice. Microinjected eggs were transferred into the oviducts of pseudopregnant mice and allowed to develop to term.

2.1.3. PCR

Eleven of 44 founder mice were identified harboring the transgene by PCR of genomic DNA isolated from tail biopsies at 4–5 weeks of age. Tail DNA was extracted after proteinase K (0.5 mg/ml) digestion by subsequent isopropanol precipitation and purification. Subsequent genotyping PCRs were performed using transgene specific primers, i.e, sense primer at the 3′end of Tie-2 promoter (5′-GGGAAGTGCAGAACAG) identifying a 470 bp transgene specific fragment (Fig. 1B).

By southern blot, identifying transgenic DNA (not shown), and by competitive PCR, using an internal standard fragment of 364 bp, the transgene copy numbers of different founder lines were estimated. One line showed approximately 100-fold higher catalase expression compared to other lines. Animals of this line were excised from the vector, purified and microinjected in fertilized F2-eggs of F1 (C57BL/6×C3H/He) mice.

2.1.4. Ethical statement

Permission for this study was provided by the regional government (AZ 23.05-220-3-94/00 and 23.05-230-3-65/99), and the experiments were performed according to the guidelines for the use of experimental animals as given by “Deutsches Tierschutzgesetz” and to the “Guide for the care and use of laboratory animals” of the US National Institutes of Health.

2.1.5. Competitive RT–PCR

Catalase mRNA was measured in hearts of cat++–mice in comparison to cat–mice by competitive RT–PCR specific for human, transgenic mRNA as well as murine, native mRNA. An internal deleted standard of 444 bp was constructed by linker-primer–PCR, cloned into pCRII-TOPO Cloning Vector, (Invitrogen, Karlsruhe) and confirmed by restriction analysis. The standard was in vitro transcribed into cRNA, (RNA Transcription Kit, Stratagene, Amsterdam) and quantified spectrophotometrically.

Total RNA was isolated from mice tissues with “Rneasy Mini Kit”, (Qiagen, Hilden, Germany). In competitive RT–PCR experiments, 500 ng of total RNA was incubated in separate reactions with defined amounts of an internal deleted catalase cRNA standard, reverse transcribed into cDNA (Superscript™ first-strand system for RT–PCR, Invitrogen) and amplified by PCR; sense-primer: 5′-GGTTTCTTTCTTGTTCAGTG, antisense-primer: 5′-CGTAGGACAGTTTCACA. A 554-bp fragment results for the native RNA, a 443 bp-fragment for the standard RNA. Fragments were separated through agarose gel electrophoresis, and the optical density of each fragment was measured (Gel Doc 1000, Bio-Rad, Münich, Germany). The respective RNA amounts were calculated using double logarithmic plots of quotients of standard to native PCR fragment density and determining the equivalence point.

2.2. Exercise protocol

Mice were exercised following a previously established protocol [23]. Briefly, mice ran in a newly established self-build exercise wheel treadmill especially designed for mice. Five animals were studied simultaneously. Mice were initially trained three times for 10 min every other day for 10 days. The maximal velocity of the treadmill was 0.25 m/s. After this training, mice were exercised for 3 weeks at 5 days a week for 30 min at 0.25 m/s. Thus, 8 days of training corresponds to a total time of 10 days (2 days without training) and 15 days of training to a total time of 3 weeks (4 days without training). The training was executed during the active cycle of the animals. Nonexercised controls were exposed to the same noise and the vibration of the environment. All mice completed the exercise protocol without signs of exhaustion. There was no obvious difference in exercise performance between the different strains of mice. Previous experiments revealed that 3 weeks of exercise greatly increased the activity of citrate synthase in soleus muscle from 40.3±2.8 mU/mg protein to 141.0±18.0 mU/mg protein (n=7) [24]. Within 16 to 20 h after termination of the last training, mice were sacrificed by inhalation of carbon dioxide, and their aortas and hearts were immediately frozen in liquid nitrogen. The frozen tissues were taken to prepare total protein for Western blotting.

2.3. Preparation of 100×g supernatants

To prepare protein homogenates, mice tissues (thoracic aorta, left ventricle) were flash frozen in liquid nitrogen, homogenized, solubilized in lysis buffer (50 mM Tris–HCL, 1 mM DTT, 1 μM proteinase inhibitors) and centrifuged 10 minutes at 100×g. Supernatants were stored at −70 °C until used for either Western blots or determination of catalase activity.
2.4. Western blotting

Western blotting was performed as described previously [25]. Briefly, increasing amounts of total protein per lane were loaded and 0.5 μg of mouse catalase (Sigma) was used as standard. Blots were incubated with a polyclonal antibody to catalase (Calbiochem, Darmstadt, Germany). Western blotting for eNOS was performed using a monoclonal antibody directed against human eNOS (Transduction Laboratories). Blots were subsequently challenged with a horseradish peroxidase-conjugated antibody (antimouse IgG, Biorad, Munich, Germany, antirabbit IgG, Calbiochem). Blots were developed using ECL (Roche, Mannheim, Germany) and exposed to X-ray film. The autoradiographs were analyzed by densitometry (Geldoc, Bio-Rad). The transfer efficiency was detected by staining the gel with Coomassie after the blotting procedure. In none of the gels, we found visible blue bands. Comparative quantitative evaluation was performed only with signals appearing on same blot, and arbitrary units obtained under control conditions were set to 100%.

2.5. Determination of catalase activity

Catalase activity was measured by KMnO₄-reduction assay, as described by Cohen et al. [26]. 100 × g supernatants were incubated with 1.2 μmol of H₂O₂ for 3 min. After the reaction has been stopped with 240 μmol of H₂SO₄, 2.8 μmol of KMnO₄ was added. Residual KMnO₄ was immediately measured spectrophotometrically at 480-nm wavelength.

2.6. Immunohistochemistry

Different organs of the mice were removed, immediately fixed in Bouin’s fluid and embedded in paraffin wax. Eight-micrometer sections were stained for catalase (antibody dilute 1:100, antihuman, from rabbit; Calbiochem). Following incubation for 60 min at room temperature, the reaction was detected by an ABC system (Vector Elite Kit) using biotinylated second antibodies and PO-conjugated streptavidin. Before the reaction, all sections were digested for 30 min with 0.1% trypsin.

The H₂O₂ level was monitored with 5- and 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate bis(acetomethyl) ester (DCDHF-DA, Molecular Probes), which can be oxidized to the fluorescent compound 2′,7′-dichlorofluorescein [27]. Mouse aortic slices were incubated with 5 μM of DHCDF-DA for 30 min at 37 °C, rinsed three times with phosphate-buffered saline and visualized on a Leica TCS SP2 confocal microscope (excitation 488 nm; emission 525 nm).

2.7. Substances and solutions

All chemicals were obtained from Merck, Darmstadt, Germany, or from Sigma, Deisenhofen, Germany, in analytical grade. Stock solutions were prepared daily, diluted with Krebs buffer as required, kept on ice and protected from daylight until use. All concentrations indicated in the text and figures are expressed as final bath concentrations.

2.8. Statistics

All data were analyzed by standard computer programs (GraphPad Prism PC Software, Version 3.0, Analysis of Variance, ANOVA) and are expressed as mean values and standard error of the mean (S.E.M.). Significant differences were evaluated using either Newman–Keuls multiple comparison test following one-way ANOVA (expression of murine catalase after 10 and 15 days of exercise) or student’s t-test. A P value below 0.05 was considered as significant.

3. Results

3.1. Characterization of catalase overexpression

Injection of the catalase-Tie-2-promotor construct resulted in a marked overexpression of catalase as evidenced by Southern blot (not shown), competitive RT–PCR (Fig. 2), Western blot and activity (Fig. 3). Immunohistochemistry performed in cross-sections of the aorta and the heart of cat++ showed that the overexpression occurred specifically in...
blood vessels (Fig. 4). This was also evident in the lung, liver and kidney (not shown) and was associated with a marked reduction of the H$_2$O$_2$ content in the aortic endothelial layer as measured using dichlorofluoresceine-fluorescence (in arbitrary units, $n=8$) from 3808$\pm$664 in cat$^-$ to 1973$\pm$244 in cat$^+$ (example given in Fig. 4, top panel). These data indicate that these mice specifically overexpressed catalase in vascular cells.

3.2. Effect of catalase overexpression on basal eNOS expression

To evaluate a possible effect of catalase overexpression on the expression of eNOS, we compared aortic eNOS protein expression in cat$^-$ and cat$^+$. Overexpression of catalase had no effect on basal protein expression of aortic eNOS (110$\pm$19% in cat$^+$ vs. cat$^-$, $n=5$). These data suggest that there is no change of basal eNOS expression in cat$^+$.

3.3. Effect of exercise on basal catalase expression

To evaluate a possible effect of exercise on the expression of aortic and myocardial catalase, we exercised C57BL/6 mice for both 10 days ($n=3$) and 3 weeks ($n=6$). Exercise had a small but nonsignificant effect on the expression of catalase in C57BL/6 mice both in the aorta and in left ventricular myocardium (Fig. 5). Thus, exercise...
itself does not change catalase protein content in the cardiovascular system.

3.4. Efficiency of exercise and its effect on eNOS expression in C57BL/6, catn and cat++

Three weeks of exercise significantly increased the heart weight/body weight ratio (in mg/g) of C57BL/6 mice from 5.48±0.22 (control, n=11) to 6.32±0.24 (exercise, n=12, \(P=0.0171\)), indicating efficient training. This was associated with a significant increase of eNOS expression in the aorta of 2.93±45.2-fold \((P<0.01)\) and the left heart ventricle of 1.88±33.1-fold \((P<0.05)\). A similar result was obtained in catn \((n=4, \text{Fig. 6})\), where 3 weeks of exercise increased eNOS expression by 2.52±0.464-fold in the aorta and by 1.9±0.37-fold in the left myocardial ventricle \((\text{Fig. 6})\). In striking contrast, there was no effect of exercise on eNOS expression in the aorta and the heart of cat++ \((\text{Fig. 6})\), although the heart weight/body weight ratio (in mg/g) increased from 5.15±0.16 to 6.04±0.22 \((n=8, P=0.0056)\). These data suggest that endogenous H\(_2\)O\(_2\) plays a key role in exercise-induced up-regulation of cardiovascular eNOS.

3.5. Effect of aminotriazole exercise-induced eNOS expression in cat++

Preliminary experiments indicated that 5 weeks of treatment with aminotriazole at a dosage of \(\approx 666 \text{ mg/kg body weight} \) tended to reduce the increase of aortic eNOS expression in response to 3 weeks of exercise \((P=0.3023, \text{data not shown})\). In striking contrast, aminotriazole treatment resulted in a significant increase of eNOS expression in response to 3 weeks of exercise in cat++ mice \((\text{Fig. 7B, } P<0.01)\) as compared to vehicle-treated cat++ mice \((\text{Fig. 7A, } P>0.05)\). These data show that the activity of catalase in cat++ mice is critically involved in the inhibition of exercise-induced vascular eNOS expression.

4. Discussion

In this study, we generated a new transgenic mouse line with vascular-specific overexpression of human catalase to investigate the importance of vascular H\(_2\)O\(_2\) for the increase of vascular eNOS expression induced by exercise. In striking contrast to the wild-type littermates, there was no increase of eNOS expression when cat++ mice were subjected to exercise. Based on these data, we suggest that
endogenously produced H₂O₂ contributes to the beneficial effects of exercise on endothelial function.

Our data strongly support the concept that H₂O₂ is critically involved in up-regulation of eNOS by exercise, as catalase prevented this response. The vascular specific overexpression of catalase was evident in both conductance and myocardial resistance vessels and associated with an increase of catalase mRNA, protein and activity, as well as a marked reduction of the endothelial steady-state concentration of reactive oxygen species including H₂O₂. Although vascular specific overexpression of catalase had no effect on basal expression of eNOS, it almost completely inhibited the increase of eNOS expression induced by exercise in the aorta and in left ventricular arterioles. Moreover, treatment of mice with the catalase inhibitor aminotriazole restored the up-regulation of aortic eNOS in response of cat++ to exercise, strongly supporting the concept that H₂O₂ is involved in this physiologic vascular adaptation to exercise training. Unfortunately, there is no reliable method to directly measure vascular H₂O₂ levels during exercise [22]. In addition, H₂O₂ is so fleeting that even rapid removal of vessels after an acute bout of exercise will unlikely reveal a change in its tissue level. This might be considered a limitation of our study; however, taken together, the effects of catalase overexpression and its reversal strongly support a role of H₂O₂ in regulation of eNOS expression during exercise.

Our current studies do not identify a specific site or source of H₂O₂. High levels of shear stress, as encountered during exercise as a result of increased cardiac output, have been shown to stimulate vascular superoxide production, which would predispose to increased levels of H₂O₂ via dismutation [28]. Importantly, it is possible that H₂O₂ is produced either in adjacent tissues or in blood borne cells during bouts of exercise. In keeping with this concept, Ashton et al. [29] have...
shown that exercise acutely increases what appears to be a carbon-centered radical in the plasma of humans, using electron spin resonance spectroscopy. A variety of studies have indicated that exercise is associated with increased ATP synthesis in skeletal and myocardial muscle [30], and we have found a more than threefold increase of citrate synthase activity [24]. During ATP synthesis, the coenzyme Q radical can transfer its unpaired electron to molecular oxygen, which increases superoxide and subsequently the generation of H₂O₂ [31]. In contrast to superoxide, H₂O₂ is not charged and therefore able to freely diffuse within tissue. Thus, mitochondrial H₂O₂ likely contributes to increased vascular oxidative stress during exercise, particularly in myocardial and skeletal muscle arterioles where the diffusion distance is comparatively small. Furthermore, exercise increases heart rate, and this will almost certainly enhance the mechanical forces of blood flow, such as shear stress, pressure and cyclic strain on the vascular wall [1]. In conductance arteries, shear stress has been shown to increase endothelial superoxide generation [28]. Further studies have identified the endothelial NADPH oxidase as a major source of vascular superoxide induced by laminar and oscillatory shear [32].

Previous studies in our laboratories have shown that exercise not only induces the expression of eNOS but also that of ecSOD [13,21]. In addition, shear stress is a potent stimulus for expression of the endothelial cell Cu/Zn SOD [33]. The SODs not only reduce ambient levels of superoxide but, in doing so, generate H₂O₂. Thus, superoxide generated during exercise training may be more readily dismutated to H₂O₂. The two major enzymes which detoxify H₂O₂ are catalase and glutathione peroxidase. Of these, glutathione peroxidase is inhibited by NO in a concentration-dependent manner [34]. It is also known that exercise increases endothelial NO generation, suggesting a significant inhibition of glutathione peroxidase. Therefore, catalase may be more important for detoxification of exercise-induced generation of H₂O₂ because exercise also increases endothelial NO generation [13,21].

Regulation of eNOS expression is highly complex. A variety of factors, such as shear stress, lysophosphatidylcholine, cGMP analogs, lipoproteins, inhibitors of protein kinase C and different cytokines, are known to alter eNOS expression [35,36]. In addition, previous studies in endothelial cells have shown that H₂O₂ increases eNOS expression as well [16]. The mechanism underlying this up-regulation includes a calcium-dependent increase of calmodulin kinase II phosphorylation leading to activation of janus kinase II [17]. The latter tyrosine kinase phosphorylates other protein kinases, such as Ras, which directly activates transcription factors. Shear stress is also considered an important stimulus for eNOS expression. Recent studies have shown that the underlying signaling mechanism is completely different from that of H₂O₂, involving activation of the tyrosine kinase cSrc, which in turn initiates both eNOS transcription and eNOS mRNA stabilization via divergent pathways [18]. In recent studies, we have also found that mice with reduced cSrc are unable to increase eNOS expression in response to exercise training [37]. Taken together with our current data, it seems that both vascular H₂O₂ and cSrc are critical in allowing endothelial cells to increase eNOS expression during exercise training. The manner in which these signals interact in vivo remain poorly defined.

Our findings may have implications for the use of antioxidants and drugs that suppress the production of reactive oxygen species in vivo. While reactive oxygen species produced in large quantities clearly mediate cellular damage, it is clear that low levels of reactive oxygen species like H₂O₂ may have important signaling properties. Complete suppression of cellular production of reactive oxygen species would therefore likely be undesirable, as it might lead to loss of these signaling events and, in the case illustrated in this study, an inability to increase eNOS in response to exercise training.

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