

# Physical Training in Patients With Chronic Heart Failure Enhances the Expression of Genes Encoding Antioxidative Enzymes

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- OBJECTIVES** We sought to determine whether the benefit of training for vasodilation in the skeletal muscle vasculature of patients with chronic heart failure (CHF) is likely to be caused at the molecular level primarily by increased nitric oxide (NO) production or decreased inactivation of NO.
- BACKGROUND** Physical training reverses endothelium dysfunction in patients with CHF, mediated by increased NO bioactivity. Some animal studies support a mechanism whereby training results in increased vascular NO levels by sustained transcriptional activation of the endothelial NO synthase (eNOS) gene, presumably due to shear stress. The mechanism has not been addressed in patients with CHF.
- METHODS** The steady state transcript levels for eNOS and two other shear stress regulated genes (angiotensin-converting enzyme [ACE] and prostacyclin synthase [PGI2S]) were measured in samples of skeletal muscle from patients with CHF before and after 12 weeks of training. Transcript levels were measured in the same samples for two genes encoding antioxidant enzymes, copper zinc superoxide dismutase (Cu/Zn SOD) and glutathione peroxidase (GSH-Px). Untrained patients served as controls.
- RESULTS** As expected, training significantly enhanced peak oxygen uptake in the patients with CHF. Training did not increase steady-state transcript levels for eNOS, ACE or PGI2S. In striking contrast, training increased the expression of the antioxidative enzyme genes by approximately 100%.
- CONCLUSIONS** Our results do not support a model of benefit from training by increased eNOS expression. However, the data are entirely consistent with the alternative hypothesis, that reduced oxidative stress may account for the increase in vascular NO-mediated vasodilation. Insight into the mechanism may be relevant when considering therapies for exercise-intolerant patients with CHF. (J Am Coll Cardiol 2001;38:194-8) © 2001 by the American College of Cardiology

For patients with chronic heart failure (CHF), vascular endothelial function and, particularly, nitric oxide (NO)-mediated vasodilation are clearly enhanced by physical training (1-4). The molecular basis for this improvement is unclear, although animal studies support either of two (nonexclusive) mechanisms. One attractive hypothesis is that training induces NO production by increased expression of the gene encoding endothelial NO synthase (eNOS) (5,6). The eNOS promoter contains a cis-acting shear-stress response element (7), and, thus, its expression could be regulated directly by periodic increases in blood flow that occur during physical training. Alternatively, vasodilation could be enhanced indirectly after training by a distinct mechanism to decrease oxygen free radicals that otherwise can inactivate NO. For example, increased expression of the antioxidative superoxide dismutase gene has been reported in rats without heart failure after endurance training that

results in greater NO activity (8). Indeed, additional evidence indicates that increased eNOS expression might not be sufficient to explain the benefit of training. Depressed vascular endothelial function occurs in experimental heart failure despite an increase in eNOS gene expression and is attributed to increased vascular superoxide anion  $O_2^-$  production (9).

To address this issue for the first time in patients with CHF, we determined whether physical training affects the steady-state expression levels of eNOS or other genes that contain a shear-stress response regulatory cis-element (10) in skeletal muscle, including angiotensin-converting enzyme (ACE) and prostacyclin synthase (PGI2S). For comparison, we also analyzed transcript levels for copper zinc superoxide dismutase (Cu/Zn SOD) and glutathione peroxidase (GSH-Px), two antioxidative enzymes that metabolize oxygen free radicals and, thereby, increase NO activity indirectly. Our results do not support a model that patients with CHF benefit from training by sustained enhancement of eNOS expression due to vascular shear stress. However, the data are entirely consistent with the alternative hypothesis, that reduced oxidative stress could account for the increase in vascular NO-mediated vasodilation.

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Manuscript received May 23, 2000; revised manuscript received March 2, 2001, accepted March 23, 2001.

#### Abbreviations and Acronyms

ACE	=	angiotensin-converting enzyme
cDNA	=	complementary DNA
CHF	=	chronic heart failure
Cu/Zn SOD	=	copper zinc superoxide dismutase
eNOS	=	endothelial nitric oxide synthase
GSH-Px	=	glutathione peroxidase
mRNA	=	messenger RNA
NO	=	nitric oxide
NT	=	nontrained group
PCR	=	polymerase chain reaction
PGI2S	=	prostacyclin synthase
RT	=	reverse transcription
RT/PCR	=	reverse transcription polymerase chain reaction
T	=	trained group
VWF	=	Von Willebrand factor

## METHODS

**Study population.** Fourteen training-competent patients with stable CHF of  $\geq 3$  months duration were recruited for the study. Patients with angina, significant obstructive lung disease or significant peripheral arterial disease were excluded. Four patients remained sedentary and served as controls. Trained (T) and nontrained (NT) patients were the same age (T:  $62 \pm 6$  vs. NT:  $66 \pm 6$  years), received the same treatment and had the same clinical characteristics. All patients were New York Heart Association functional class III. The T group included four patients with nonischemic and six patients with ischemic cardiomyopathies; the NT group included two patients with nonischemic and two patients with ischemic cardiomyopathies. Mean left ventricular ejection fraction was comparable between the two groups (T:  $34 \pm 9\%$  vs. NT:  $27 \pm 5\%$ ). Medical therapy for CHF consisted of furosemide and ACE inhibitors in all patients, digoxin in five patients, beta-adrenergic blocking agents in eight patients and long-acting oral nitrate preparations in one patient. All patients were clinically well-compensated at the time of the study, and their medical regimen did not change during the course of the study. The Ethical Review Board of the Albert Einstein College of Medicine approved the study. All patients gave written informed consent before the study.

**Training program.** Patients exercised on a semirecumbent bicycle (Tunturi 803, Turku, Finland) at a work load corresponding to 50% of peak aerobic capacity for 45 min four times a week for a total of 12 weeks. Peak oxygen uptake was measured at six weeks, and the workload was remeasured to correspond to 50% of peak oxygen capacity.

**Measurement of peak oxygen uptake.** Patients performed a symptom-limited maximal treadmill exercise test using a modified Naughton protocol. Leg fatigue or dyspnea limited exercise. Peak oxygen uptake was determined as the average in the last 30 s of exercise when the respiratory exchange ratio was  $>1.0$  using a breath-by-breath analysis

of expired gases (Medical Graphics System 2001, Minneapolis, Minnesota). The peak volume of oxygen at baseline was the average of two values that were within 10%. Thereafter, only one single test was performed at 6 and 12 weeks.

**Muscle biopsies.** Muscle biopsies of the vastus lateralis were performed using a needle (Bard Monopty, Covington, Georgia). Biopsy samples were plunged into liquid nitrogen immediately and transferred to a freezer at  $-80^{\circ}\text{C}$  until processed. Skeletal muscle biopsy could be performed at only two time points according to Institutional Review Board approval: 24 h before starting the training program and 48 h after the end of the training program.

**Reverse transcription polymerase chain reaction (RT/PCR) analysis of skeletal muscle samples.** RNA was prepared from tissue samples, complementary DNA (cDNA) generated by reverse transcription (RT) and specific products amplified by semiquantitative polymerase chain reaction (PCR), as described (11). All primers were designed to amplify fragments of cDNA spanning at least one intron (except those for Cu/Zn SOD, which lack introns). Polymerase chain reaction using mock RT reactions were conducted for all samples in order to exclude the presence of product derived from genomic DNA.

After PCR, products were separated by electrophoresis in 5% polyacrylamide nondenaturing gels. Phosphorimage analysis was performed with a Molecular Dynamics Storm 860 system (Sunnyvale, California) to quantify the relative levels of PCR products. Products were shown to accumulate linearly in accordance with cycle number and were directly dependent on the amount of RNA added to the RT reaction, determined by the ratio of products derived from samples using 0.25 or 0.5  $\mu\text{g}$  RNA. The eNOS, PGI2S, ACE, Cu/Zn SOD and GSH-Px transcript levels were normalized to Von Willebrand factor (VWF) transcript in each case to control for inherent small differences in vasculature content from independent biopsies. This is necessary because it is likely that vascular endothelial mass increases during physical training. Acute changes in VWF transcription may occur during each training session, but that is not relevant nor is it reflected in our analysis.

**PCR primers, product size and cycles used for semiquantitative RT/PCR.** The PCR primers, product sizes and cycles used for semiquantitative RT/PCR were as follows:

*eNOS*: 247 base pairs (bp), 26 cycles,

forward: 5'GAAGCTGCAGGTGTTTCGATG,

reverse: 5'ATGCAGAGCTCGGTGATCTC,

*ACE*: 401 bp, 24 cycles,

forward: 5'CACAGAGACCAGCAAGATTCTG,

reverse: 5'GCAGCCTGGTTGATGAGTTCC,

*PGI2S*: 324 bp, 28 cycles,

forward: 5'CAGCTTCCTCACGAGGATGA,

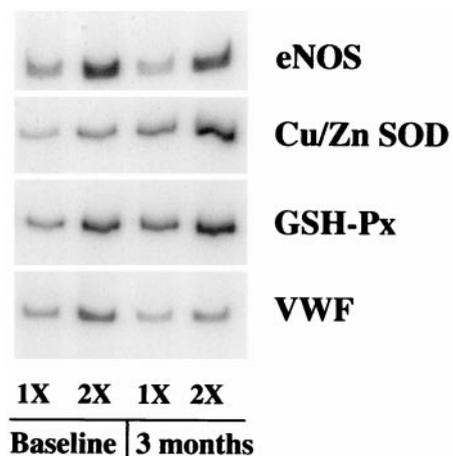
reverse: 5'CAGCCACTGCCTGCTTCTG,  
Cu/Zn SOD: 475 bp, 24 cycles,  
forward: 5'GCATCATCAATTTTCGAGCAGAAG,  
reverse: 5'TCTACAGCTAGCAGGATAACAGA,  
GSH-Px: 354 bp, 23 cycles,  
forward: 5'GGGGCCTGGTGGTGCTCGGCT,  
reverse: 5'CAATGGTCTGGAAGCGGCGGC,  
VWF: 562 bp, 22 cycles,  
forward: 5'TCCTCCAGTTTCCCAGCTTC, and  
reverse: 5'CTTCTCATTCCCATCCTCATCC.

**Statistical analysis.** Results are expressed as mean ± SEM. Differences between groups were assessed using a two-way analysis of variance with a Newman-Keuls adjustment for multiple groups. Significance was accepted at  $p < 0.05$ .

## RESULTS

The eNOS gene is regulated at the transcriptional level through a cis-element that responds to shear stress, and semiquantitative RT/PCR analysis of transcript levels is feasible using the limiting patient biopsy samples that preclude alternative analyses of protein or enzyme activities. For this study, 14 training-competent patients with CHF were enrolled and completed the program, while four patients remained sedentary and served as controls. All patients remained on steady doses of diuretics, ACE inhibitors and beta-blockers. As expected, training resulted in a significant improvement in peak oxygen uptake. Peak volume of oxygen increased after 12 weeks of training from  $14.5 \pm 2.4$  ml/kg/min at baseline to  $16.9 \pm 3.4$  ml/kg/min (17%,  $p < 0.05$ ). This increase was clinically significant since all patients volunteered the information that they felt better during the course of their daily activities.

For analysis of gene expression, RNA was prepared from vastus lateralis muscle biopsies taken from each patient before and after the training protocol, and steady-state eNOS transcript levels were measured using a semiquantitative RT-PCR assay performed under conditions that ensured a linear accumulation of product with respect to input RNA and cycle number. An example of data from one patient is shown in Figure 1, demonstrating the quantitative nature of the assay. Transcript levels were normalized in each case relative to those encoding VWF, as a marker for endothelial cells to account for inherent differences in vascular contribution within the biopsies. Therefore, we measured the relative amount of eNOS messenger RNA (mRNA) within a given amount of muscle vasculature for each patient. The data clearly demonstrate that there is no net increase in eNOS mRNA per unit vasculature after training (Fig. 2). The eNOS/VWF ratios were  $0.55 \pm 0.1$  and  $0.54 \pm 0.08$  before and after training, respectively. Similarly, there was no change in relative eNOS/VWF

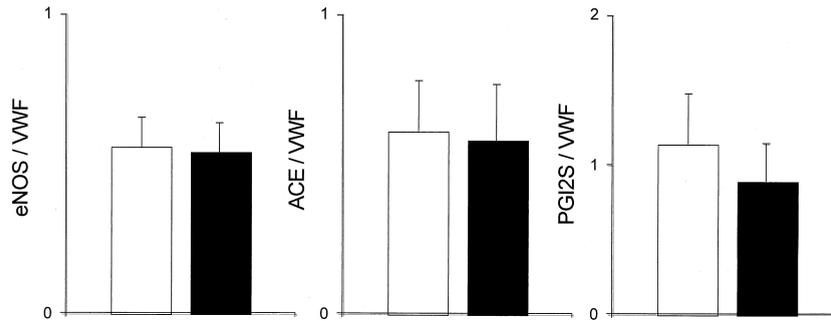


**Figure 1.** Shown are representative autoradiographs used to measure the relative abundance of transcripts in RNA isolated from vastus lateralis muscle at baseline and after 12 weeks of training. Reverse transcription reactions included either 0.25  $\mu$ g (1 $\times$ ) or 0.5  $\mu$ g (2 $\times$ ) total RNA. Polymerase chain reaction conditions were chosen to ensure that product derived from the 2 $\times$  sample was doubled compared with the 1 $\times$  sample, demonstrating that the assays are at least semiquantitative. Aliquots of the same reverse transcription reaction for each patient were used for each polymerase chain reaction using primers specific to the various gene products. Cu/Zn SOD = copper zinc superoxide dismutase; eNOS = endothelial nitric oxide synthase; GSH-Px = glutathione peroxidase; VWF = Von Willebrand factor.

levels in the untrained control patients ( $0.46 \pm 0.10$  vs.  $0.47 \pm 0.06$ ).

Because eNOS is regulated by shear stress, we analyzed transcript levels for ACE and PG12S, two similarly regulated genes (Fig. 2). Angiotensin-converting enzyme/Von Willebrand factor and PG12S/VWF mRNA ratios were not altered by physical training ( $0.61 \pm 0.17$  vs.  $0.58 \pm 0.19$ ;  $1.14 \pm 0.34$  vs.  $0.89 \pm 0.25$ , respectively), and, as expected, there was no change in relative levels for the control patients ( $0.47 \pm 0.09$  vs.  $0.51 \pm 0.07$ ;  $0.66 \pm 0.38$  vs.  $0.62 \pm 0.32$ ). We conclude that the steady-state transcript levels for shear-stress responsive genes are not altered in the vasculature of patients with CHF after a training program, and the data do not support a model of training-induced increases in steady-state levels of NO.

Therefore, we analyzed the relative transcript levels of genes encoding antioxidant enzyme by the same method. Transcript levels for these gene products were determined for each patient using the same RT reaction used to measure the shear-stress responsive genes (Fig. 3). In striking contrast with the previous results, mRNA ratios for both Cu/Zn SOD/VWF mRNA and GSH-Px/VWF were increased approximately 100% by physical training (Fig. 3) ( $0.69 \pm 0.09$  vs.  $1.47 \pm 0.22$ ;  $0.71 \pm 0.09$  vs.  $1.29 \pm 0.19$ , respectively). This increase per unit vasculature is highly significant ( $p < 0.05$ ), while, in the control sedentary patients, Cu/Zn SOD and GSH-Px gene expression did not change ( $0.63 \pm 0.09$  vs.  $0.66 \pm 0.17$ ;  $1.95 \pm 0.24$  vs.  $1.90 \pm 0.16$ , respectively). We conclude that, in patients with CHF, physical training affects the expression of antioxidative enzyme genes in the skeletal muscle vasculature.



**Figure 2.** Transcript levels of the shear-stress responsive genes endothelial nitric oxide synthase (eNOS), angiotensin-converting enzyme (ACE) and prostacyclin synthase (PGI2S) are unaltered in the skeletal vasculature of patients with chronic heart failure after 12 weeks of physical training. Shown in the graph, transcript levels are normalized to those from the Von Willebrand factor (VWF) gene to control for biopsy-dependent differences in vasculature. **Open bars** = pretraining; **solid bars** = posttraining. Data are presented mean  $\pm$  SEM. Note that the data allow quantitative comparisons pre- and posttraining for each individual gene but do not analyze relative expression of the various genes to each other.

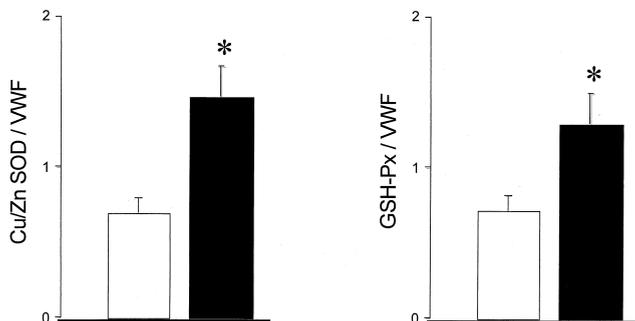
## DISCUSSION

**NO bioactivity can be regulated by changes in either the rates of NO inactivation or expression of eNOS.** Regulation of NO bioactivity is a complex process with important clinical relevance. Levels of reactive oxygen metabolites are increased in patients with CHF as evidenced by increased breath pentane extraction (12) and altered plasma levels of lipid peroxides (13), thiobarbituric reactive substances and thiols (14). Moreover, reactive oxygen metabolites may contribute to functional intolerance in patients with CHF since there is a significant correlation during maximal exercise between levels of malondialdehyde and peak oxygen consumption (15). The detrimental effects of increased levels of reactive oxygen metabolites on vascular endothelial function are indirectly demonstrated by the beneficial effects of antioxidant therapy with vitamin C on flow-dependent dilation in conduit arteries of patients with CHF (16).

Blunted NO-mediated vasodilation in patients with CHF could be caused by reduced NO synthesis or increased NO inactivation by reactive oxygen metabolites (17). Support for both mechanisms is provided by animal studies. For example, eNOS expression is reduced in the aorta of dogs

that underwent one month of left ventricular pacing, indicating that the resulting left ventricular dysfunction may impair vascular NO synthesis (18). In the same pacing model, exercising 2 h per day for 10 consecutive days increased expression of eNOS in the aorta (5). Since the promoter for the eNOS gene contains a shear-stress responsive element (19), a likely possibility is that periodic increases in shear stress associated with physical training can result in sustained upregulation of eNOS gene expression. In a rat model of heart failure, impaired vascular endothelial function was attributed to enhanced vascular production of superoxide anion (9), and expression of eNOS was increased in the aorta of rats with heart failure when compared with that of rats without heart failure. The recent finding that H<sub>2</sub>O<sub>2</sub> induces eNOS gene expression in endothelial cells and increases the stability of eNOS mRNA suggests that the increased oxidative stress that characterizes the syndrome of CHF may be, in part, responsible for eNOS production (20).

**Decreased inactivation of NO after training may benefit patients with CHF.** Our study is the first to investigate this complex issue in a clinical setting with respect to CHF patients and training. The data do not support a hypothesis that training benefits patients with CHF primarily by a mechanism of sustained increases in NO production. Steady-state transcript levels for the eNOS, ACE and PGI2S genes, which are all regulated at the transcriptional level by a shear-stress response element (21,22), were not altered in our patients after 12 weeks of physical training. Instead, we found significant changes in steady-state levels for genes encoding antioxidant enzymes, supporting the hypothesis that this mechanism may play an important role in providing benefit to patients with CHF. In fact, there was previously support for this mechanism from animal and human studies. In rats without heart failure, endurance training increases superoxide dismutase gene expression in skeletal muscle fibers with high oxidative metabolism (8). In healthy men, skeletal muscle superoxide dismutase activity correlates with maximum oxygen consumption attained during graded exercise (23). Presently, the mechanism by



**Figure 3.** Transcript levels of genes encoding antioxidant enzymes copper zinc superoxide dismutase (Cu/Zn SOD) and glutathione peroxidase (GSH-Px) are increased approximately 100% after 12 weeks of physical training. The Cu/Zn SOD and GSH-Px transcript levels were determined relative to Von Willebrand factor (VWF) complementary DNA in the same preparation of reverse transcribed RNA. **Open bars** = pretraining; **solid bars** = posttraining. Data are presented mean  $\pm$  SEM. \* $p < 0.05$  versus pretraining.

which physical training modulates antioxidative enzyme activity and gene expression is poorly understood. However, in cultured human aortic endothelial cells, shear stress modulates Cu/Zn SOD protein content, enzyme activity and gene expression (24). Whether periodic increases in shear stress associated with physical training are sufficient to upregulate Cu/Zn SOD expression in skeletal muscle remains to be demonstrated, but, based on our data, this is an intriguing possibility.

**Limitations of the study and relevance to therapy.** Our conclusions are limited by the size of the study because only patients with CHF that could tolerate an extended exercise program were eligible. We would like to document whether there is a linear relationship between peak aerobic capacity and changes in gene expression, but statistically meaningful relationships cannot be obtained given the small changes in peak capacity and the small patient sample. Also, it will be important in the future to investigate protein levels and enzyme activities for the genes we tested, which was not possible using the small sample materials obtained for each patient by biopsy. However, the data provide clear evidence that a beneficial training program in patients with CHF results in increased basal levels of antioxidant genes and not stress-responsive genes, including eNOS. Our conclusion is supported by recently obtained data that activation of NF- $\kappa$ B is reduced in vascular endothelial cells from patients undergoing the equivalent of physical training (unpublished data). This may be of relevance for devising alternative therapeutic strategies for this patient population, which is generally intolerant to exercise. Thus, increased NO bioactivity resulting from decreased levels of reactive oxygen metabolites and, thereby, reduced NO inactivation is most likely responsible for the beneficial effect of physical training on vascular endothelial function in patients with CHF.

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