Enhanced Generation of Reactive Oxygen Species in the Limb Skeletal Muscles From a Murine Infarct Model of Heart Failure

Hiroyuki Tsutsui, MD; Tomomi Ide, MD; Shunji Hayashidani, MD; Nobuhiro Suematsu, MD; Tetsuya Shiomi, MD; Jing Wen, MD; Kei-ichiro Nakamura, MD; Kazuhiro Ichikawa, PhD; Hideo Utsumi, PhD; Akira Takeshita, MD

Background—The generation of reactive oxygen species (ROS) is enhanced in the failing myocardium. We hypothesized that ROS were also increased in the limb skeletal muscles in heart failure.

Methods and Results—Myocardial infarction (MI) was created in mice by ligating the left coronary artery. After 4 weeks, the left ventricle was dilated and contractility was diminished by echocardiography. Left ventricular end-diastolic pressure was elevated after MI in association with an increase in lung weight/body weight and the presence of pleural effusion. The generation of ROS in the limb muscles, including the soleus and gastrocnemius muscles, which were excised after MI, was measured by electron spin resonance spectroscopy with 4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl (hydroxy-TEMPO). Overall, generation was increased, but it was attenuated in the presence of dimethylthiourea or 4,5-dihydroxy-1,2-benzenedisulfonic disodium salt in the reaction mixture, indicating increased generation of hydroxyl radicals originating from superoxide anion. Thiobarbituric acid–reactive substance formation was also increased in muscles after MI. Mitochondrial complex I and III activities were both decreased after MI, which may have caused the functional uncoupling of the respiratory chain and ROS production. Antioxidant enzyme activities, including superoxide dismutase, catalase, and glutathione peroxidase, were comparable between groups.

Conclusions—Skeletal muscle in post-MI heart failure expressed an increased amount of ROS in association with ROS-mediated lipid peroxidation. This supports the hypothesis that oxidative stress may cause (at least in part) skeletal muscle dysfunction in heart failure. (Circulation. 2001;104:134-136.)

Key Words: free radicals ▪ muscles ▪ heart failure ▪ antioxidants ▪ exercise

Exercise intolerance is a major limiting symptom in patients with congestive heart failure.1 It is well accepted that exercise intolerance correlates poorly with the degree of left ventricular dysfunction.2 Thus, the impairment of skeletal muscle perfusion and the intrinsic alterations in the muscle itself have been proposed to account for this phenomenon.3 Indeed, the decrease in oxidative enzymes, atrophy of fibers, and shift from slow-twitch to fast-twitch fibers have been demonstrated in patients with heart failure1,4 and an animal model of heart failure due to myocardial infarction (MI).5 However, causal or contributing factors responsible for these alterations have not been delineated.

Previous studies have shown higher plasma levels of thiobarbituric acid–reactive substances (TBARS) in patients with heart failure.6 Increased reactive oxygen species (ROS) may play an important role in the development of myocardial failure.7 Various factors, including catecholamines, angiotensin II, cytokines such as tumor necrosis factor–α, and nitric oxide, can enhance the generation of ROS. A recent study demonstrated that cytosolic oxidant levels were increased in the diaphragm from tumor necrosis factor–α transgenic mice.8 Oxidative stress has been shown to be related to exercise intolerance in patients with heart failure.9 Further, ROS are generated during repetitive muscle contraction, which is implicated in muscle fatigue after exercise.10 On the basis of these findings, it is conceivable to hypothesize that the generation of ROS is also increased in the limb muscles after heart failure. We quantified the amount of ROS by electron spin resonance spectroscopy combined with 4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl (hydroxy-TEMPO).7

Methods

Murine Model of Heart Failure

The study was approved by our Institutional Animal Research Committee, and it conformed to the animal care guidelines of the

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From the Departments of Cardiovascular Medicine, (H.T., T.I., S.H., N.S., T.S., J.W., A.T.) and Developmental Molecular Anatomy (K.N.), Graduate School of Medical Sciences, Kyushu University, and the Department of Biophysics, Graduate School of Pharmaceutical Sciences (K.I., H.U.), Kyushu University, Fukuoka, Japan.

Correspondence to Hiroyuki Tsutsui, MD, PhD, Department of Cardiovascular Medicine, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, 812-8582, Japan. E-mail prehiro@cardiol.med.kyushu-u.ac.jp

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American Physiological Society. MI was created in male CD-1 mice (5 to 8 weeks old with a body weight of 25 to 35 g) by ligating the coronary artery, according to methods described previously. To determine the effects of antioxidants on the amount of ROS, dimethylthiourea (DMTU; 50 mg/kg in sterile saline) was administered to MI mice by an intraperitoneal injection (MI+DMTU).

**Left Ventricular Function**

On the day of study, 4 weeks after surgery, in vivo left ventricular function studies, including echocardiography and pressure measurements, were performed.

**ROS and TBARS**

With the animals under deep anesthesia, the limb skeletal muscles, including the soleus and gastrocnemius muscles, were quickly excised and frozen in liquid nitrogen. ROS were quantified in tissues by electron spin resonance spectroscopy with hydroxy-TEMPO. All measurements were performed in 2 parallel runs, in the presence and absence of OH scavenger, DMTU, or the O2- scavenger 4,5-dihydroxy-1,2-benzenedisulfonic disodium salt (Tiron) as competitive reagents. TBARS were measured in the muscle through the biochemical assay.

**Enzymatic Sources of ·O2**

Mitochondrial complex I, II, III, and IV activities were measured in the muscle. Endothelial nitric oxide synthase (eNOS) protein levels were determined by Western blot analysis with antibodies against human vascular eNOS.

**Antioxidant Enzyme Activities**

The activities of the scavenging enzymes, including total superoxide dismutase, catalase, and glutathione peroxidase, were measured in the muscle.

**Statistical Analysis**

All data are expressed as mean±SEM. *P<0.05 was considered statistically significant.

**Results**

**Animal Characteristics**

Echocardiographic and hemodynamic data demonstrated marked left ventricular dilatation and contractile impairment in the MI mice, with an increased lung weight and pleural effusion, thus confirming the occurrence of heart failure (Table). There were no differences in body or limb muscle weights.

We detected no evidence of skeletal muscle injury in MI animals at the ultrastructural level (data not shown). Myofibrillar organization was maintained, mitochondria exhibited clearly defined cristae, and membrane structure was preserved at the sarcolemma.

**ROS and TBARS in Limb Muscles**

The rate of electron spin resonance signal decay, an index of the amount of ROS, was significantly (*P<0.01) larger in MI mice (n=6) than in sham animals (n=6; Figure, A). When DMTU (50 mmol/L) was added to the reaction mixture, it completely abolished the increase of signal decay in MI, indicating that ·OH contributed to the increase of signal decay. Tiron also attenuated an increase in signal decay rate, which implies the contribution of ·O2- to the production of ·OH. In the MI+DMTU mice (n=7), the rate of signal decay was normalized (0.012±0.001 minute^-1; *P<0.01 versus MI and P=NS versus sham), which indicated that the in vivo administration of DMTU during MI prevented the production of ·OH. In parallel to an increase in ·OH, MI mice had a 1.5-fold increase in TBARS formation (Figure, B).

**Enzymatic Sources of ·O2**

Enzymatic activities of complexes I (89±13 versus 41±5 nmol · min^-1 · mg protein^-1; *P<0.05) and III (655±61 versus 370±74 nmol · min^-1 · mg protein^-1; *P<0.01) were decreased in MI. In contrast, those of complexes II (110±14 versus 172±36 nmol · min^-1 · mg protein^-1; P=NS) and IV (778±142 versus 514±15 nmol · min^-1 · mg protein^-1; P=NS) remained unchanged. eNOS protein levels in MI remained unchanged (82±12% of sham values; P=NS).

**Antioxidant Enzyme Activities**

There were no significant differences in the activity of total superoxide dismutase (7.9±0.8 versus 7.9±0.9 U/mg protein), catalase (104±18 versus 96±14 nmol/mg protein), or glutathione peroxidase (10.7±0.5 versus 10.1±0.1 U/mg protein) between sham and MI mice.
Discussion
The present study demonstrated that the limb skeletal muscle from mice with heart failure after MI has increased generation of ROS and preserved antioxidant enzyme activities, which might play an important role in the metabolic abnormalities and exercise intolerance commonly seen in patients with heart failure.

The production of ROS was increased in the skeletal muscle homogenates obtained from a murine model of heart failure, and increased ROS were identified as ·OH originating from ·O2− (Figure, A) and were associated with a concomitant increase in the oxidation of lipids (Figure, B). These results are consistent with previous studies showing that oxidative capacity was reduced and O2 use was inadequate in the skeletal muscle mitochondria from patients with heart failure.4 In this respect, our results indicate that ROS are increased in cardiac and skeletal muscle in the setting of heart failure.

ROS are produced via several mechanisms, including mitochondrial oxidases, NAD(P)H oxidase, and NOS. This study demonstrated that skeletal muscle mitochondria in the setting of heart failure were associated with a decrease in complex I and III activities. As has been shown in failing hearts,11 these defects in electron transfer may lead to ROS production. Although eNOS protein levels remained unchanged in our model, inducible NOS has been shown to be increased in the skeletal muscle obtained from patients with heart failure.12 NO can produce ·O2−, and NO and ·O2− react to the powerful oxidant peroxynitrite, which may also contribute to increased ROS. Moreover, NAD(P)H oxidase could be another important mechanism of ·O2− production in heart failure.

Several possible factors might be involved in the increased ROS in heart failure. First, an impaired oxygen or substrate delivery to the muscle could lead to hypoxia and reoxygenation and the resultant generation of ROS. However, impaired oxygen delivery cannot be the sole cause of these derangements because the metabolic abnormalities are detected even in the presence of adequate blood flow. Second, various neurohumoral factors including catecholamines, angiotensin II, and cytokines can activate the generation of ROS.3

Our previous studies demonstrated that DMTU treatment of MI mice prevented myocardial oxidative stress in association with left ventricular remodeling.7 In the present study, we observed that DMTU also prevented ROS production in the skeletal muscles. Although ROS and lipid peroxides have been shown to impair biological tissue function and structure, a direct link between ROS and skeletal muscle dysfunction has yet to be established. ROS may play an important role in the muscle atrophy commonly seen in heart failure patients through the induction of apoptosis. In addition, ROS impair myoplasmic Ca2+ homeostasis and inhibit oxidative energy production in the mitochondria,13 both of which may contribute to muscle contractile dysfunction.

Oxidative stress could be the mechanistic basis for muscle fatigue and reduced exercise tolerance in heart failure patients.10 This notion is supported by a positive correlation between ROS and exercise intolerance in these patients.9 An attempt to attenuate oxidative stress would improve, to some extent, the exercise capacity of patients with heart failure.

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