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Protein nitration is associated with increased proteolysis in skeletal muscle of bile duct ligation–induced cirrhotic rats

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Abstract

Cirrhosis is characterized by skeletal muscle wasting. In this study, the effects of nitric oxide production on skeletal muscle protein nitration and degradation in cirrhosis were investigated. Cirrhosis was induced by bile duct ligation (BDL) in Sprague-Dawley rats for 4 weeks. The BDL-induced cirrhotic rats and sham-operated rats were then injected daily with either saline or N^G-L-nitro-arginine methyl ester (L-NAME) for 7 days from week 4 to week 5, after which nitrite/nitrate, glutathione reduction, as well as protein nitration, ubiquitination, and degradation were assessed in skeletal muscle. Elevated muscular nitrite/nitrate concentrations, protein nitration, total ubiquitin conjugates, and degradation fragments of myosin heavy chain as well as diminished glutathione reduction levels were observed in BDL-induced cirrhotic rats as compared with controls. Administration of L-NAME for 1 week led to reduction of nitrite/nitrate levels; protein nitration was also decreased in the skeletal muscle. In addition, ubiquitination of muscular proteins and degradation of myosin heavy chain were significantly diminished after treatment of L-NAME. In conclusion, nitrosative stress occurred in the skeletal muscle of BDL-induced cirrhotic rats and may lead to increased proteolysis of muscle-specific structural proteins.

1. Introduction

Cirrhosis is known to be associated with increased cardiac output and peripheral vasodilatation with impaired response to vasoconstrictors, termed *hyperdynamic circulation* [1]. Excessive nitric oxide (NO) production may play an important role in these cirrhosis-associated cardiovascular abnormalities; inhibition of NO synthesis leads to increased splanchnic and peripheral resistance with restored responsiveness to pressor agonists [1]. In addition to inducing vasodilatation, NO modulates skeletal muscle function in several wasting disorders, such as heart failure, chronic obstructive pulmonary disease, and sepsis [2-6]. It is postulated that NO can induce nitration of skeletal muscle proteins, resulting in subsequent degradation by the ubiquitin-proteasome pathway, leading to skeletal muscle wasting observed in the catabolic disorders [7,8].

In cirrhosis, a progressive decrease in muscle mass has been estimated to develop in about 10% to 100% of patients [9-11]; and increased degradation of skeletal muscle protein through the activation of the ubiquitin-proteasome pathway has been observed [12]. Although NO overproduction in cirrhosis is well recognized, its relationship with skeletal muscle wasting has yet to be elucidated. Here, nitration of skeletal muscle proteins was analyzed in a rat model of biliary cirrhosis. In addition, the effects of inhibition of NO synthesis on skeletal protein degradation were determined.

2. Materials and methods

2.1. Animal model

The bile duct ligation (BDL) model used in this study has been previously described [12,13]. In this model,

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cirrhosis is associated with excessive NO formation [14]. Male Sprague-Dawley rats purchased from National Science Council Animal Center (Taipei, Taiwan) weighing 250 to 300 g at the time of surgery were used. Rats were anesthetized with phenobarbital (60 mg/kg body weight, intramuscularly), and the common bile duct was exposed and ligated by 2 ligatures using 3-O silk. The first ligature was made below the junction of the hepatic ducts, and the second ligature was made above the entrance of the pancreatic ducts. The common bile duct was then resected between the ligatures. Sham procedure involved a similar operation without ligation and cutting of the bile duct. Prophylactic benzathine benzylpenicillin (50 000 U) was administered intramuscularly during the postoperative period. After the surgery, vitamin K (8 mg/kg) was also administrated intramuscularly at weekly intervals. During the induction period, the rats were housed in plastic cages at 24°C, with a 12-hour light-dark cycle, and allowed to have free access to food and water until the time of experimentation. Mortality rate was about 20%, with most deaths occurring within the first 2 weeks. In all experiments, the authors adhered to the *Guiding Principles for the Care and Use of Laboratory Animals* issued by the Taiwan Government.

2.2. Experimental protocols

Four weeks after surgery, the animals (BDL and control) were randomly divided into 2 experimental groups, which received N^G-L-nitro-arginine methyl ester (L-NAME; 0.5 mg/[kg d]) or an equivalent volume of saline subcutaneously for 1 week (from week 4 to week 5). On the 36th day (24 hours after the last dose of L-NAME), the rats were anesthetized intraperitoneally with sodium phenobarbital; and intraarterial catheterization of the left femoral artery was performed for blood sampling. Blood was immediately collected, and the serum was frozen at −70°C until required. In addition, peripheral gastrocnemius muscles were rapidly dissected, weighed, and stored in liquid nitrogen at −70°C until required.

2.3. Assessment of protein nitration, ubiquitination, and degradation in skeletal muscle

Gastrocnemius muscles were homogenized in tissue protein extraction reagents (T-PER; Pierce Biotechnology, Chicago, IL) and then centrifuged at 10 000 rpm for 10 minutes. The protein concentrations in the supernatants were determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA) before use. Crude muscle proteins (50 μg) were electrophoretically separated on 7% to 10% gradient sodium dodecyl sulfate–polyacrylamide gels and transferred to a nitrocellulose membrane as previously described [12]. Membranes were then blocked for 1 hour in 5% (vol/vol) nonfat dried milk in TTNS (25 mmol/L Tris/HCl, pH 7.5, 0.1% Tween-20, 0.9% NaCl). To detect the expression of the ubiquitin-proteasome pathway, the crude proteins were incubated for 1 hour in Dakocytomation rabbit polyclonal antiubiquitin antibody diluted 1:1000 (Dako, Glostrup, Denmark). To determine myosin heavy-chain protein degradation, the extracted soluble fraction of muscle protein was incubated with antimyosin heavy-chain fast type diluted 1:500 (Sigma-Aldrich, St Louis, MO) as previously described [15]. Because previous studies reported that nitrotyrosine was easily reduced to aminotyrosine by heating in thiol-containing Laemmli buffer, muscle proteins were incubated in Laemmli buffer without heating and then incubated with a 1:1000 dilution of rabbit antinitrotyrosine polyclonal antibody (Upstate Biotechnology, Saranac Lake, NY) to assess muscle nitrotyrosine levels [16]. After washing 3 times with TTNS, the blots were incubated with secondary horseradish peroxidase–conjugated antibodies (Amersham Life Sciences, Piscataway, NJ). The blots were washed 4 times with TTNS for 20 minutes, incubated in enhanced chemiluminescence reagent (ECL, Amersham Life Sciences), and exposed on radiographic films (Eastman-Kodak, Rochester, NY). Finally, all measured protein levels were quantified by densitometry; and the percentage change relative to sham-operated rats was determined after normalization by α-tubulin determined with anti–α-tubulin antibody diluted 1:500 (Sigma-Aldrich).

2.4. Analysis of nitrite/nitrate and glutathione

Muscle nitrate (NO_3^-) and nitrite (NO_2^-) were measured by a commercially available assay (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's protocol. Skeletal muscle reduced glutathione (GSH) levels were used as a marker of oxidative stress in the muscle and were measured using a commercially available kit (Cayman Chemical).

2.5. Statistical analyses

Data were expressed as the mean ± SEM, and the difference was determined by Mann-Whitney *U* nonparametric test (for comparisons between 2 groups) or Kruskal-Willis 1-way analysis (for comparisons among more than 2 groups). All data were analyzed by SPSS V 6.0 software (SPSS, Chicago, IL). *P* values <.05 were considered significant.

3. Results

3.1. Weight and biochemical change

Rats typically developed cirrhosis 4 weeks after BDL, as previously reported [12]. The mean body weight and gastrocnemius mass of sham-operated rats were greater than those of BDL-induced cirrhotic rats ($P < .05$, Table 1). The total serum bilirubin and alanine aminotransferase (ALT) values were significantly higher in cirrhotic rats as compared with the sham controls, and albumin concentration was lower in the cirrhotic rats ($P < .05$; Table 1). However, with the exception of total bilirubin levels, no changes in

Table 1
Body weight, liver biochemistry, and skeletal muscle oxidative stress parameters in control and BDL rats with and without L-NAME administration

	Control with saline (n = 6)	Control with L-NAME (n = 6)	BDL with saline (n = 6)	BDL with L-NAME (n = 6)
Body wight (g)	470.0 ± 3.8	472 ± 4.0	419.5 ± 9.0*	407.1 ± 8.8
Gastrocnemius (g)	3.0 ± 0.1	3.1 ± 0.1	2.6 ± 0.1*	2.4 ± 0.2
Bilirubin (mg/dL)	0.1 ± 0.0	0.1 ± 0.0	8.9 ± 0.7*	7.2 ± 0.4†
Albumin (g/dL)	3.6 ± 0.2	3.7 ± 0.2	2.8 ± 0.2*	2.7 ± 0.1
ALT (IU/mL)	30 ± 1	32 ± 2	98 ± 35*	103 ± 23
Nitrate/nitrite (μmol/μg protein)	12.8 ± 1.6	11.6 ± 1.6	25.4 ± 1.8*	16.4 ± 1.4†
GSH (μmol/mg protein)	250.3 ± 5.4	245 ± 7.5	200.1 ± 5.1*	220.5 ± 4.3†

* $P < .05$ vs saline-treated controls.
† $P < .05$ vs saline-treated BDL rats.

total body and muscle weight and in ALT and albumin levels were observed between BDL rats with and without L-NAME administration (Table 1).

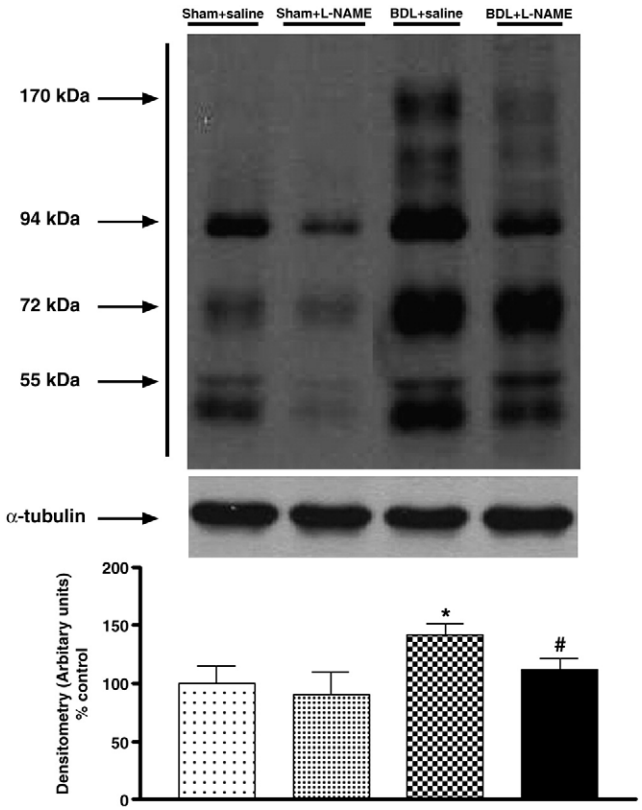

Fig. 1. Western blot analysis of total ubiquitin conjugates of skeletal muscle proteins (50 μg per lane) in sham-operated control and BDL-induced cirrhotic rats with and without L-NAME administration. Results represented the percentage change relative to sham-operated controls after normalization by α-tubulin and were expressed as mean ± SEM (vertical bars). *$P < .05$ vs saline-treated sham-operated controls; #$P < .05$ vs saline-treated BDL rats.

3.2. Nitrite/nitrate and GSH levels in skeletal muscle

Elevated NO_3^- and NO_2^- concentrations were observed in the skeletal muscle of BDL rats as compared with control rats ($P < .05$; Table 1). The GSH levels, the major nonenzymatic intracellular antioxidant, were significantly lower in cirrhotic rats as compared with control rats ($P < .05$, Table 1), indicative of oxidative stress in cirrhotic skeletal muscle. Administration of L-NAME to the BDL-treated rats resulted in reduced skeletal muscle NO_3^- and NO_2^-

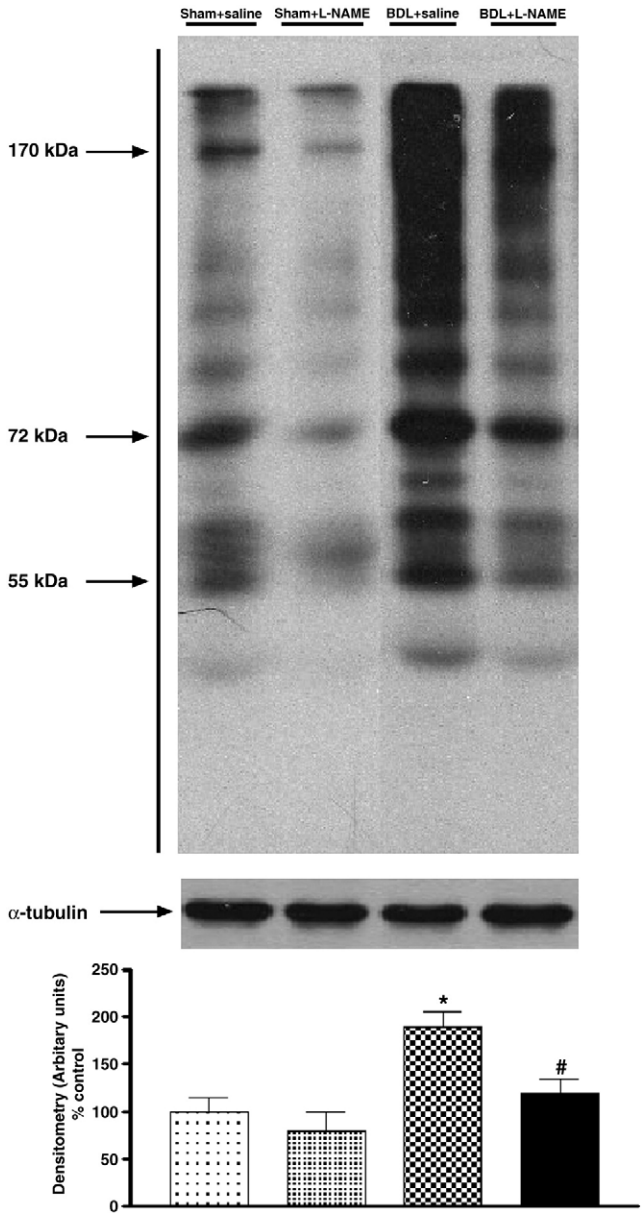

Fig. 2. Western blot analysis of degradation fragments of myosin heavy chain of skeletal muscle (50 μg per lane) in sham-operated control and BDL-induced cirrhotic rats with and without L-NAME administration. Results represented the percentage change relative to sham-operated controls after normalization by α-tubulin and were expressed as mean ± SEM (vertical bars). *$P < .05$ vs saline-treated sham-operated controls; #$P < .05$ vs saline-treated BDL rats.

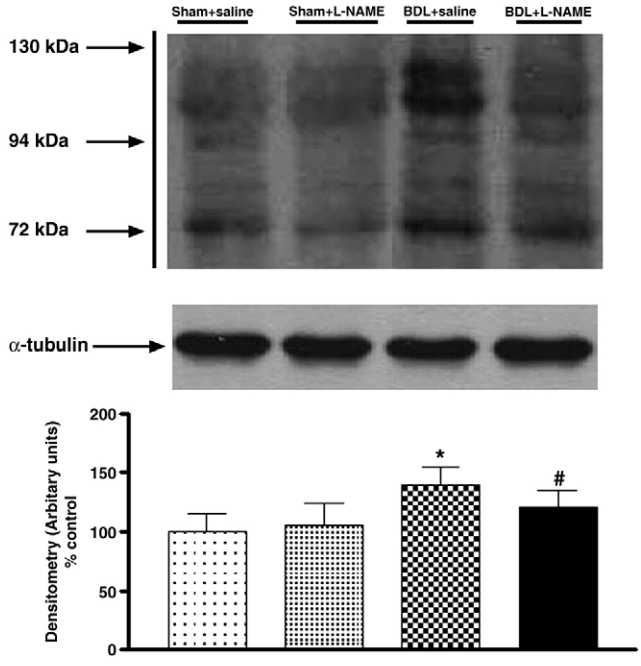

Fig. 3. Western blot analysis of nitrated proteins of skeletal muscle (50 μg per lane) in sham-operated control and BDL-induced cirrhotic rats with and without L-NAME administration. Results represented the percentage change relative to sham-operated controls after normalization by α-tubulin and were expressed as mean ± SEM (vertical bars). $^{*}P < .05$ vs saline-treated sham-operated controls; $^{\#}P < .05$ vs saline-treated BDL rats.

concentrations and elevated GSH levels as compared with BDL-treated control rats.

3.3. Myosin heavy-chain degradation and total protein ubiquitination and nitration

The total ubiquitin-conjugated protein levels from crude muscle lysates were 0.4-fold higher in BDL-induced cirrhotic rats than in the sham-operated controls (Fig. 1). In addition, myosin heavy-chain degradation was 0.9-fold higher in the skeletal muscle of the cirrhotic rats than in the controls (Fig. 2). Levels of nitrated proteins were also significantly (0.3-fold) elevated in the skeletal muscle of cirrhotic rats (Fig. 3). After administration of L-NAME, degradation of the myosin heavy chain as well as total ubiquitin conjugates and protein nitration of skeletal muscle were decreased by 30%, 60%, and 15%, respectively (Figs. 1-3).

4. Discussion

In this study, elevated muscular levels of nitrate/nitrite and nitration of skeletal muscle proteins along with increased myosin heavy-chain degradation and total ubiquitin conjugates in BDL-induced cirrhotic rats were observed. Several studies have demonstrated that excessive NO production in cirrhosis originated from the vasculature [1]. Although this study did not address whether the up-regulation of nitrate/nitrite levels in skeletal muscle of BDL rats was derived from the vascular beds within the tissue or from blood contamination only, it was assumed that increased nitrate/nitrite levels might alter muscle metabolism within the local environment, regardless of its exact source.

Excessive production of NO in vivo exerts damaging effects on tissues through its diffusion-limited reaction with the superoxide anions to form peroxynitrite [17,18]. In the body, peroxynitrite further reacts with target molecules, resulting in nitration of amino acid tyrosine residues and formation of 3-nitrotyrosine proteins, which in turn mediate NO-induced tissue damage [17-19]. Nitrated proteins can induce proteasome-mediated degradation of the modified proteins [20]. Therefore, if this nitration process occurs in skeletal muscle structural proteins, increased protein degradation and subsequent muscle wasting would be expected.

In tumor-bearing and mechanically ventilated rat models of muscle wasting, increased 3-nitrotyrosine levels in skeletal muscle are associated with an activated ubiquitin-proteasome pathway [21]. On the contrary, changes in body weight and muscle mass can be obviated in mice implanted with tumor necrosis factor-α–over-expressing cells by the application of NOS inhibitors [22]. In cultured myotubes, muscle-specific protein degradation can also be reduced upon inhibition of protein nitration [23]. The present study revealed that application of the nonselective NOS inhibitor L-NAME to BDL-induced cirrhotic rats decreased skeletal muscle nitrite/nitrate levels as well as muscular protein ubiquitination and myosin heavy-chain degradation. Thus, in cirrhosis, excessive NO is involved in not only the development of hyperdynamic circulation, but also nitrosative stress in skeletal muscle, which is associated with increased proteolysis of muscle-specific structural proteins.

Myosin generally accounts for 40% to 50% of all myofibrillar proteins in skeletal muscle [24] and is the principle protein targeted by the ubiquitin-proteasome pathway in various models of muscle wasting [25-29]. In BDL-induced cirrhotic rats, reduced myosin heavy-chain degradation and total ubiquitin conjugates after the administration of L-NAME may indicate a stimulatory effect of NO on myosin proteolysis and the ubiquitin-proteasome system. Although muscle weight gain was not observed after administration of L-NAME for 1 week, inhibition of NOS activity might be beneficial for cirrhosis-associated muscle wasting as well as cirrhotic hyperdynamic circulation.

Previous studies have reported that NO has a toxic effect on hepatocytes; its inhibition may ameliorate hepatic damage [30]. Reduced liver function impairment may attenuate the muscle protein degradation associated with cirrhosis [9-12]. However, improvements in hepatic function, as indicated by blood ALT and albumin values, were not observed after administration of L-NAME in BDL-induced cirrhotic rats for 1 week. Although it is not likely that attenuated muscle protein proteolysis in cirrhotic rats would be due to reduced liver injury, the effects of prolonged L-NAME administration have yet to be explored.

A recent in vitro study of cultured skeletal muscle cells reported that both NO and peroxynitrite activate nuclear factor–κB and stimulate ubiquitin conjugation of muscle-specific proteins, resulting in their subsequent degradation [23]. In addition to activating nuclear factor–κB, it has been also proposed that nitrosative stress influences several signaling molecules including p38 mitogen-activated protein kinase, c-Jun-N-terminal kinase, and muscle-specific F-box protein, all of which are known to be involved in the regulation of ubiquitin-proteasome pathway [8]. Whether reactive nitrogen species stimulate muscle proteolysis through these signaling proteins in BDL cirrhotic rats requires further studies.

In summary, BDL-induced cirrhosis increased muscular levels of reactive nitrogen species, including nitrite/nitrate levels and protein nitration. Suppression of NO production by L-NAME attenuated protein ubiquitination, degradation, and nitration in the skeletal muscle of cirrhotic rats. Thus, nitrosative stress not only occurred in skeletal muscle, but also contributed to skeletal muscle wasting in cirrhosis, which might provide a new potential target for preventing muscle wasting in cirrhosis.

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