Carbon Dioxide Water Bathing Enhances Myogenin but Not MyoD Protein Expression after Skeletal Muscle Injury

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Abstract. [Purpose] We reported that carbon dioxide (CO₂) water bathing accelerates skeletal muscle regeneration; however, the underlying mechanism was unclear. MyoD and myogenin play roles in muscle regeneration, and the purpose of this study was to determine changes in MyoD and myogenin caused by CO₂ water bathing after injury. [Subjects] Sixteen female Wistar rats (n = 4 per group) were used. [Methods] The rats were divided into four groups: no-injury (NI), injury (IC), injury + tap water bathing (ITW), and injury + CO₂ water bathing (ICO₂). Muscle injury was induced by injection of bupivacaine hydrochloride into the left tibial anterior (TA) muscles. Tap water and CO₂ (1,000 ppm) water bathing were performed at 37 °C for 30 minutes once a day. The left TA muscles were removed 4 days after injury, and the expressions of MyoD and myogenin were measured. [Results] MyoD and myogenin were increased in the IC, ITW, and ICO₂ groups compared with the NI group. Although the MyoD level was similar in the IC, ITW, and ICO₂ groups, myogenin increased more in the ICO₂ group than in the IC and ITW groups. [Conclusion] CO₂ water bathing after muscle injury appears to induce an increase in the expression of myogenin.

Key words: Skeletal muscle injury, CO₂ water bathing, Myogenic regulatory factors

(This article was submitted Dec. 28, 2012, and was accepted Feb. 8, 2013)

INTRODUCTION

Carbon dioxide (CO₂) water bathing has a long history in European countries and has been used to treat cardiovascular disease since the Middle Ages. CO₂ water bathing is a kind of remedy with a wide spectrum of applications. CO₂ water bathing results in vasodilatation and an increase in blood flow^{1, 2)}. The vasodilatation is induced by CO₂, which diffuses into the subcutaneous tissue through the skin. CO₂ water bathing has been reported to have several positive physiological effects. For example, CO₂ water bathing enhanced VEGF mRNA levels, resulting in neovascularization in ischemic hindlimbs of mice³⁾. In our previous study, we investigated whether CO₂ water bathing accelerates skeletal muscle repair after injury⁴⁾.

Skeletal muscle has the ability to regenerate new muscle fibers after injury. Satellite cells are muscle stem cells and play an important role in skeletal muscle fiber repair after injury. Satellite cells proliferate, differentiate into myoblasts expressing muscle-specific proteins, fuse into myotubes, and finally mature into myofibers⁵⁾. Satellite cells are normally in a quiescent state, but they are activated when skeletal muscle fibers are injured. The activated

satellite cells proliferate, differentiate, and fuse to repair injured skeletal muscle fibers. Myogenic regulatory factors (MRFs), including MyoD, Myf5, myogenin, and MRF4, are transcription factors that control the expression of several muscle genes. The myogenic basic helix-loop-helix (bHLH) family of transcription factors are thought to play an important role in the development and regeneration of skeletal muscle⁶⁾, and MRFs regulate the complex phenomenon of myogenic differentiation.

As noted above, MRFs play an important role in skeletal muscle regeneration, but MyoD and myogenin were not increased by CO₂ water bathing at 2 weeks after injury in our previous study⁴⁾. Therefore, the mechanism underlying the acceleration of skeletal muscle regeneration induced by CO₂ water bathing was unclear. The expression of MRFs is observed in the early stage of regeneration after skeletal muscle injury. We hypothesized that CO₂ water bathing increases the expression of MyoD and myogenin in early stage of regeneration after injury, since the expression of MyoD and myogenin proteins was observed in a morphological study at 4 days after skeletal muscle injury⁷⁾. Thus, the purpose of this study was to examine the effect of CO₂ water bathing on the expression of MyoD and myogenin in injured muscles at 4 days after skeletal muscle injury.

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SUBJECTS AND METHODS

Sixteen female Wistar rats (224–282 g) were used in this study. The rats were housed in a temperature controlled room, 22 °C, on a 12 h:12 h light-dark cycle, and were allowed free access to food and water. The rats were assigned to no-injury (NI, n=4), injury (IC, n=4), injury + tap water bathing (ITW, n=4), and injury + CO₂ water bathing (ICO₂, n=4) groups. The rats in the IC, ITW, and ICO₂ groups were anesthetized by injection of pentobarbital sodium (50 mg/kg); then, the left tibial anterior (TA) muscle was injured by injection of 0.3 mL of 0.5% bupivacaine hydrochloride (Marcaine, AstraZeneca, Osaka, Japan) using a disposable syringe with a 27-gauge needle. The needle was inserted into the mid-belly portion of the TA muscle and advanced longitudinally to the proximal portion. The solution was injected as the needle was withdrawn slowly as described previously⁴. One day after the TA muscle injury, rats in the ITW, and ICO₂ groups were immersed in tap water and CO₂ water (CO₂ concentration; 1,000 ppm), respectively, at 37 °C, for 30 minutes once a day for 3 consecutive days. CO₂ water containing a high concentration of CO₂ was made from high pressure CO₂ in a cylinder and tap water using an MRE-Spa (Mitsubishi Rayon Co., Ltd., Tokyo, Japan). Four days after injury, the rats were sacrificed by injection of an overdose of sodium pentobarbital and their left TA muscles were removed. The TA muscles were immediately frozen in isopentane cooled by liquid nitrogen and stored at – 80 °C until analysis. All procedures were approved by the Animal Care and Use Committee of Kibi International University.

For the analysis of the expression of MyoD and myogenin, the muscles were homogenized in Tris-HCl (pH 7.4). After centrifugation, the supernatant was collected as the measurement sample. The protein concentrations of these samples were measured using the Bradford method and a Coomassie Protein Assay Kit (Thermo Fisher Scientific K.K., Kanagawa, Japan). The samples were added to EzApply (ATTO, Tokyo, Japan), adjusted to a final protein concentration of 1 µg/µl, and then boiled at 95 °C for 5 minutes. We then applied 10 µg protein to a 12.5% polyacrylamide gel (ATTO, Tokyo, Japan). Electrophoresis was carried out at a constant current of 20 mA/gel for 60 minutes, and proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane using a HorizBLOT 2 M (ATTO, Tokyo, Japan) at a constant current of 2 mA/cm² for 60 minutes. Following blots, the PVDF membrane was then incubated for 60 minutes using EzBlot (ATTO, Tokyo, Japan). The membrane was then incubated with a monoclonal antibody for MyoD (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:2,000 with 0.1 M Tris-HCl (pH

7.5) or myogenin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:2,000 with 0.1 M Tris-HCl (pH 7.5) for 1 hour at room temperature. The membrane was washed 3 times in 0.1 M Tris-HCl with 0.1% Tween-20 and then reacted with anti-mouse IgG (Nacalai tesque, Kyoto, Japan) diluted 1:10,000 with 0.1 M Tris-HCl (pH 7.5) for 1 hour at room temperature. The membrane was washed 3 times further in 0.1 M Tris-HCl with 0.1% Tween-20 and then reacted with EzWestBlue (ATTO, Tokyo, Japan). Bands from western blots were quantified using the ImageJ software (NIH, Bethesda, MD, USA).

Data are expressed means ± SD and were analyzed by one-way ANOVA. When there were significant differences, the Tukey post hoc test was used to determine differences between the groups. Statistical analyses were performed using Excel Statistics 2008 (Social Survey Research Information Co., Ltd., Tokyo, Japan). Statistical significance was accepted for values of $p < 0.05$.

RESULTS

Our results are shown in Table 1. The MyoD level was significantly higher in the IC, ITW, and ICO₂ groups than in the NI group ($p < 0.01$). However, there were no significant differences among the IC, ITW, and ICO₂ groups. The myogenin level was significantly higher in the IC, ITW, and ICO₂ groups than in the NI group ($p < 0.01$). In addition, the myogenin level was significantly higher in the ICO₂ group than in the NI and ITW groups ($p < 0.05$), but there was no significant difference in its level between the IC and ITW groups.

DISCUSSION

In the present study, CO₂ water bathing increased the expression of myogenin, but not the expression of MyoD. This result indicates that CO₂ water bathing accelerates skeletal muscle regeneration via an increase in the expression of myogenin.

MyoD and myogenin are transcription factors belonging to the bHLH family of transcription factors. These proteins play important roles in satellite cell proliferation, differentiation, fusion, and the formation of myocytes. MyoD has a critical role in skeletal muscle regeneration via activation of satellite cells⁸. Cooper et al. observed that MyoD is expressed in myotubes with centrally located myonuclei at 5 days after cardiotoxin injection⁹. They also suggested that MyoD is required for proliferation and differentiation of activated satellite cells during the regeneration of skeletal muscle fibers. Myotube formation is delayed in regenerating skeletal muscle in the absence of MyoD¹⁰, indicating

Table 1. The expression of MyoD and myogenin

	NI (n=4)	IC (n=4)	ITW (n=4)	ICO ₂ (n=4)
MyoD (% of NI)	100 ± 13*	224 ± 16*	220 ± 23*	212 ± 20*
myogenin (% of NI)	100 ± 24*	174 ± 23*	171 ± 27*	226 ± 11*,#,†

Values are means ± SD, *, different from NI, $p < 0.01$. #, different from IC, $p < 0.05$. †, different from ITW, $p < 0.05$

that MyoD plays an important role in myotube formation in regenerating skeletal muscle. In experiments using knockout mice, MyoD and Myf5 were shown to have roles in the determination of the myogenic cell fate and the formation of myoblasts during embryogenesis, and myogenin and MRF4 were shown to have roles in the activation of muscle differentiation^{11, 12}. In addition, newborn mice deficient for both MyoD and Myf5 are devoid of myoblasts⁶, and myogenin ($-/-$) mice form myoblasts that fail to form myotubes¹³. In the present study, we observed an increase in MyoD in the groups with skeletal muscle injury. Activation of satellite cells and myoblast formations occurred normally in the injured skeletal muscle fibers in the groups with skeletal muscle injury, and the increase in MyoD was similar in the IC, ITW, and ICO₂ groups. These data suggest that CO₂ water bathing did not affect the expression of MyoD, indicating that CO₂ water bathing does not accelerate myoblast formation via activation of satellite cells. An increase in myogenin was also observed in the groups with skeletal muscle injury. Myotube formation occurred normally in the groups with skeletal muscle injury; however, the increase in myogenin was greater in the ICO₂ group than in the IC and ITW groups. This result indicates that CO₂ water bathing affects the expression of myogenin, and might accelerate fusion of myoblasts and formation of myotubes. In our previous study, CO₂ water bathing increased myonuclei in regenerating skeletal muscle fibers at 2 weeks after skeletal muscle injury. Taken together, these results indicate that CO₂ water bathing accelerates skeletal muscle fiber regeneration via increase in the expression of myogenin enhancing the fusion of myoblasts. Nitric oxide (NO) is a key signal responsible for satellite cell activation and hepatocyte growth factor (HGF), which is an activator of satellite cells, is released from the extracellular matrix after skeletal muscle injury¹⁴. Tatsumi et al. reported that activation of satellite cells and HGF release in stretched muscle were dependent on local NO production¹⁵. Irie et al. reported that CO₂ water bathing increased in phosphorylated NO synthase in ischemic skeletal muscle³, indicating that CO₂ water bathing promotes NO production in skeletal muscle. Although no measure of NOS was included in the present study, we speculate that NO production in skeletal muscle was promoted by CO₂ water bathing. NO might increase myogenin expression and promote skeletal muscle regeneration.

A number of factors are related to skeletal muscle regeneration. MyoD and myogenin are two factors regulating skeletal muscle regeneration. Further studies are needed to determine more clearly the mechanisms which accelerate skeletal muscle fiber regeneration in CO₂ water bathing.

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