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[Original Article]

Administration of insulin-like growth factor-1 inhibits the overdevelopment of chloroquine-induced vacuoles in muscle fibers in experimental rimmed vacuolar myopathy

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【ABSTRACT】

Objective: We investigated the effect of insulin-like growth factor-1 (IGF-1) on the histological (e.g., rimmed vacuoles and muscle fiber atrophy) and molecular properties of the denervated muscles of chloroquine-treated rats. *Methods:* The left hind-legs of adult male Wistar rats were denervated by ligation of the sciatic nerve. The rats with and without chloroquine treatment were given saline or IGF-1 intraperitoneally at a daily dose of either 2 or 8 mg/kg for 8 days. *Results:* In the denervated soleus muscles from chloroquine-treated rats, the number of rimmed vacuoles was significantly reduced in response to either low- or high-dose IGF-1. IGF-1 administration did not affect the muscle fiber atrophy. Although proteasome and ubiquitin immunostaining, and the mRNA levels of muscle-specific ubiquitin ligases and autophagy-related genes were significantly increased in denervated muscles from both rats, those levels did not change in response to either low or high-doses of IGF-1. *Conclusion:* IGF-1 suppressed rimmed vacuolar formation in muscle fibers in an animal model of chloroquine-induced myopathy.

Key words: chloroquine myopathy, rimmed vacuoles, ubiquitin, proteasome, IGF-1, denervation

INTRODUCTION

The lysosomotrophic drug chloroquine mediates autophagic protein degradation in the autophagy-lysosome system and induces accumulation of sequestered materials in autophagosomes and lysosomes by interrupting lysosomal protein degradation[1]. Skeletal muscles treated with chloroquine show formation of rimmed vacuoles and dense granular bodies within autophagosomes and lysosomes[1-3]. We previously demonstrated the presence of rimmed vacuoles in addition to severe atrophy in denervated muscles from chloroquine- treate d rats, but not in innervated muscles from chloroquine-treated rats or innervated and denervated muscles from the rats without chloroquine treatment[3]. Lysosomal cathepsin activity was increased in atrophic skeletal muscles from denervated chloroquine-treated rats, suggesting that the lysosomal autophagic process plays a pathophysiologic role in this degenerative process[3]. Further, protein or mRNA levels of autophagy-related elements (e.g., MAP-LC3[4]), lysosome-related elements (e.g., clathrin, alpha- and gamma-adaptin, mannose 6-phosphate receptor [M6PR], and Golgi-zone elements) increased in proportion to the number of rimmed vacuoles in these muscles[5, 6], suggesting that the autophagy-lysosome system also plays a central role in rimmed vacuolar formation. In another study, ubiquitin and proteasome immunostaining and the mRNA levels of

ubiquitin and ubiquitin ligases (e.g., muscle-specific Ring finger 1 [MuRF-1], and atrogin-1/muscle atrophy F-box protein [atrogin-1]) were significantly increased in denervated soleus muscles from rats without and with chloroquine treatment when compared with the contralateral, innervated muscles[7].

Further, ubiquitin and ubiquitin ligase mRNA levels were higher in denervated muscles from chloroquine-treated rats than from rats without chloroquine treatment. This suggests that proteasomes and ubiquitin levels were increased in denervated muscles from chloroquine-treated rats and that the ubiquitin-protease proteolysis pathway and the lysosomal proteolytic pathway may mediate muscle fiber destruction in this context[7]. Insulin- like growth factor-1 (IGF-1) is an endogenous hormone produced by numerous tissues, including skeletal muscles[8-10]. This hormone, delivered to cells by both endocrine and paracrine mechanisms, stimulates normal growth and development but can also stimulate muscle growth in pathologic states[8, 11, 12]. Muscle IGF-1 levels increase in response to injury and mechanical overload, along with formation of new fibers or growth of existing fibers[13, 14]. In addition, IGF stimulates satellites cell proliferation and differentiation during muscle regeneration and muscle growth[12], and stimulates muscle protein synthesis and suppression of proteolysis in vitro and in vivo [8), 10, 14, 15]. In animal models, overexpression of IGF-1 induces local skeletal muscle hypertrophy and attenuates age- related skeletal muscle atrophy, restoring and improving muscle mass and strength in mice[10, 16].

These properties of IGF-1 suggest that it has therapeutic potential in the treatment of muscle wasting conditions, leading to investigation of the use of IGF-1 in animal models of human motor neuron diseases or muscular dystrophies[9, 14, 17]. Indeed, one study reported that muscle-specific overexpression of the IGF-1 gene slowed the rate of myofiber atrophy in denervated muscles, suggesting that IGF-1 is protective[18] and reduces atrophy of denervated myofibrils[19]. More recent studies have demonstrated that IGF-1 activates the phosphatidylinositol 3-kinase (PI3K)-Akt pathway and that inhibition of this pathway increased atrogin-1 mRNA expression and proteolysis. Thus, IGF-1 may induce muscle growth via the PI3k-Akt pathway, subsequent transcriptional inhibition of atrophy-related ubiquitin-ligase atrogin-1 and other atrogins (e.g., MuAF-1), and by degradation of myofibrillar proteins[8, 12, 15].

PI3k regulates both autophagy and apoptosis. IGF-1 activates the PI3k-Akt pathway, leading to stimulation of mammalian target of rapamycin (mTOR) signaling networks[20, 21]. mTOR plays a role in regular autophagy by inhibiting autophagy- regulated elements (e.g., Vsp34 and Beclin-1/Atp6). That mediate autophagosome formation[20-22]. Thus, activation of the PI3k-Akt pathway by IGF-1 inhibits the formation of autophagosome via mTOR Vsp 34, and Beclin-1[20-22].

MATERIALS AND METHODS

Animals. Forty adult male Wistar rats (initial body weight 200-250 g, 7 weeks of age) were anesthetized with ether. The rats were denervated by ligation of the left sciatic nerve midway between the popliteal fossa and sciatic notch, as previously described[3]. Chloroquine chloride (50 mg/kg) was injected intraperitoneally twice daily into 20 rats, beginning the day after denervation. The remaining 20 rats received saline injections instead of chloroquine (i.e., non-myopathic rats). Non-myopathic rats and chloroquine- treated rats were assigned to either untreated or IGF-1-treated groups. Treated rats were given IGF-1 (Somatomedin C, Astellas Pharma, Tokyo, Japan) intraperitoneally twice daily at a daily dose of either 2 mg (low-dose) or 8 mg/kg (high-dose). Untreated rats were given an identical volume of saline instead of IGF-1. The soleus muscles were obtained from the right (innervated) and left (denervated) legs from all animals 8 days after of IGF-1 or saline treatment. Muscles were then rapidly frozen in isopentane cooled in liquid nitrogen and then stored at -80°C until use.

All experiments were performed in accordance with the guideline for animal care and use of animals of Oita University (permission No. K017001). Rats were kept under standardized laboratory conditions in an

air-conditioned room. Food and water were provided *ad libitum*..

Histologic and immunohistochemical studies. Serial 10-μm thick transverse sections were cut using a cryostat. Frozen sections were stained using routine and histochemical methods as described previously[3]. Using Nikon Digital Sight DS-Fi-7 (Nikon, Tokyo, Japan) and Image J software, the number of fibers with rimmed vacuoles and dense granular bodies, and the diameter of muscle fibers at the light microscopic levels were determined in 200 muscle fibers per each muscle on hematoxyline-eosin-prepared specimens.

Immunofluorescence analysis for 20S proteasome and ubiquitin were performed as described previously[4]. Images were captured using a Carl Zeiss LSM5 Pasal-V3.2 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

Real-time reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from each specimen using acid guanidinium thiocyanate buffer (Isogen, Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions[23]. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using 200 units of Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Rockville, MD, USA) and 1 μg of oligo-(dT) 12-18 primer (Invitrogen, Tokyo, Japan). Gene-specific primers for real-time PCR were purchased from Takara Bio Inc. (Shiga, Japan) or Sigma-Aldrich Japan (Tokyo, Japan). The nucleotide sequences of the primers used in this study were as follows: the MuRF-1 mRNA (Accession No. NM 080903; sense, 5'-GGG AAC GAC CGA GTT CAG ACT ATC-3'; antisense, 5'-GGC GTC AAA CTT GTG GCT CA-3'); rat atrogin-1 32 mRNA (Accession No. NM 133521; sense, 5'-AGT GAA GAC CGG CTA CTG TGG AA-3'; antisense, 5'-TTG CAA AGC TGC AGG GTG AC-3'); rat ubiquitin-C mRNA (Accession No. NM 017314; sense, 5'-GGG CAT GCA GAT CTT TGT GAA-3'; antisense, 5'-ACC TCC AGG GTG ATG GTC TTG-3'); rat MAP1-LC3alpha mRNA (Accession No. RA021649; sense, 5'-GCA GCT GCC TGT CCT GGA TAA-3'; antisense, 5'-CAT AGA TGT CAG CGA TGG GTG TG-3'); the rat MAP1-LC3beta mRNA (Accession No. RA019913: sense, 5'-AGC TCT GAA GGC AAC AGC AAC A-3'; antisense, 5'-GCT CCA TGC AGG TAG CAG GAA-3'); the rat mTOR mRNA (Accession No. RA009998; sense, 5'-GCT TAT CAA GCA AGC GAC ATC TCA-3'; antisense, 5'-TCC ACT GGA AGC ACA GAC CAA G-3'); rat Vps34 mRNA (Accession No. 16191; sense, 5'-CAG TTC ATC CAG TCA GTT CC-3'; antisense, 5'-TCA CAC AGT AGC CAG CAC AG-3'; antisense, 5'-TCA CAC AGT AGC CAG CAC AG-3'); and the rat glycerol aldehyde 3-phosphate dehydrogenase (GAPDH) mRNA (Accession No. NM 017008; sense, 5'-GAC AAC TTT GGC ATC GTG GA-3'; antisense, 5'-ATG CAG GGA TGA TGT TCT GG-3'). Quantitative real-time RT-PCR was performed using a LightCycler 2.0 instrument (Roche Diagnostics, Mannheim, Germany) and software version 4.0. The reaction mixture consisted of 1:50 diluted cDNA (5 μl), 0.2 μM of each primer, 2 μl of LightCycler FastStart DNA Master SYBR Green I mix (Roche Diagnostics) and 4 mM of $MgCl_2$ in a total volume of 20 μl. Because the target sequences of these primers were located on different exons, only a proper RT reaction resulted in the amplification of a PCR product of the correct fragment size. Formation of expected PCR product was confirmed by agarose gel electrophoresis (2%) and melting curve analysis. The relative amount of mRNA expression was calculated by measuring the threshold cycle of each PCR product against that of GAPDH mRNA. All PCR runs were performed in triplicate.

Statistical analysis. Differences between control specimens and experimental specimens were evaluated using a two-way analysis of variance. Multiple comparisons were conducted using Turkey's honestly significant difference test. A p-value of < 0.05 was considered statistically significant.

RESULTS

Histologic study. Histologic findings of the innervated and denervated soleus muscles from non-myopathic rats and chloroquine-treated rats following an 8-day course of treatment with saline or IGF-1 administration are shown in Fig. 1. Briefly, most of the innervated

soleus muscles from all animals appeared normal, while denervated muscles showed moderate to severe muscle fiber atrophy. However, marked accumulations of rimmed vacuoles and dense granular bodies were observed in chloroquine-treated denervated muscles (either after saline treatment or IGF-1 treatment) but not in denervated muscles from rats that were not treated with chloroquine.

IGF-1 treatment appeared to result in asignificantly decreased proportion of fibers with rimmed vacuoles and dense granular bodies in chloroquine-treated denervated muscles (18.8 ± 3.6 % with 2 mg/kg IGF-1, 17.6 ± 9.9 % with 8 mg/kg IGF-1, 35.7 ± 5.3 % in rats given an identical volume of saline instead of 2 mg/kg IGF-1; and

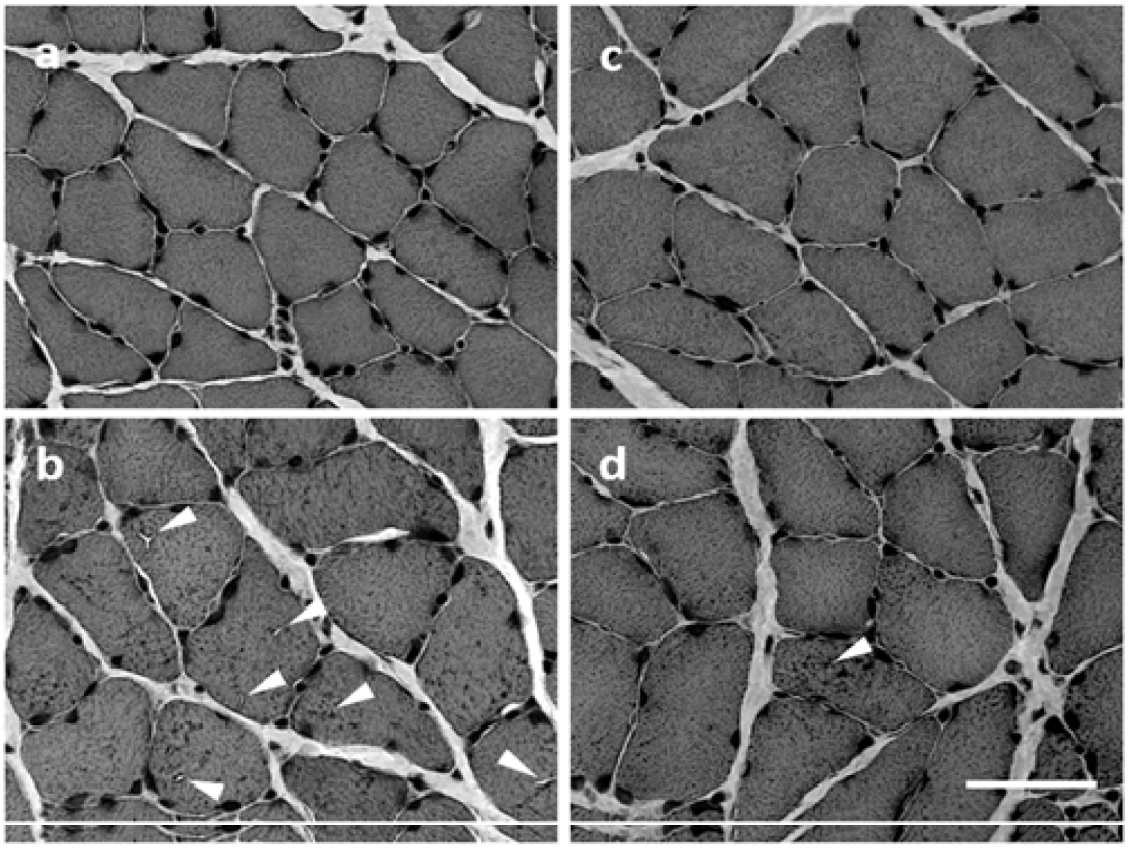

Fig.1. Cross-sections of denervated soleus muscles from rats without (a,c) and with (b,d) chloroquine treatment after administration of either saline (a, b) or IGF-1 (c, d). Arrowheads indicate rimmed vacuoles, H.E. staining, Bar=50μm.

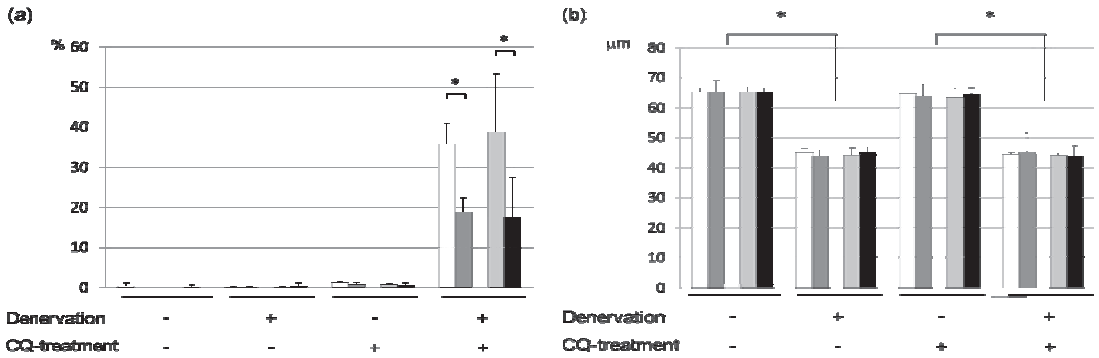

Fig. 2. (a) Frequency of muscle fibers with rimmed vacuoles and dense granular bodies in innervated and denervated soleus muscle from without and with chloroquine treatment after administration of either saline or IGF-1. (b) The mean diameters of the fiber in innervated and denervated soleus muscles from rats without and with chloroquine treatment after administration with either saline or IGF-1. Number of rats per group = 5:□, saline administration (identical volume of saline instead of 2 mg/kg IGF-1);■(dark gray), low-dose (2 mg/kg/day) IGF-1 administration; □(light gray), saline administration (identical volume of saline instead of 8 mg/kg IGF-1);■, high-dose (8 mg/kg/day) IGF-1 administration. *$p < 0.05$.

38.7 ± 14.5 % in rats given that instead of 8 mg/kg IGF-1) (Fig. 2a).

The mean diameters of muscle fibers in the denervated muscles of the rats without and with chloroquine treatment rats on day 8 (139.3 ± 5.5 μm and 130.6 ± 2.5 μm, respectively) were significantly smaller than those in the innervated muscles (192.5 ± 4.9 μm and 190.9 ± 3.6 μm, respectively (Fig. 2b).

On immunohistochemistry, 20S proteasomes and ubiquitin immunostaining were minimal in innervated muscles from rats without and with chloroquine treatment on all test days, whereas denervated muscles from both groups showed progressively stronger staining. Neither proteasomes nor ubiquitin immunostaining changed in the denervated muscles of saline- and chloroquine-treated rats after IGF-1 administration, although staining activity did tend to decrease in muscles from IGF-1-treated rats when compared with muscles from untreated rats.

Messenger RNA levels of ubiquitin, ubiquitin ligases and autophagy-related genes.

Ubiquitin ligase atrogin-1 and MuRF-1 mRNA levels were significantly elevated in the denervated soleus muscles of rats without and with chloroquine treatment after saline treatment when compared with the contralateral, innervated
muscles [4.3-fold and 3.5-fold in non-myopathic rats; 5.3-fold and 4.0-fold in chloroquine-treated rats] (Figs. 3a, b).

Treatment with low-dose IGF-1 resulted in significantly decreased atrogin-1 mRNA levels in the innervated and denervated soleus muscles of non-myopathic rats (0.4-fold in innervated muscles, 0.4-fold in denervated muscles) relative to muscles from animals treated with saline instead of IGF-1. In the denervated muscle of both rat groups, treatment with low-dose IGF-1 did not result in changes in atrogin-1 mRNA levels. Treatment with high-dose IGF-1 resulted in increased atrogin-1 mRNA levels in the denervated soleus muscles of non-myopathic rats (1.6-fold) relative to muscles from rats treated with saline instead of IGF-1. However, atrogin-1 mRNA levels tended to decline in the denervated muscle from chloroquine-treated rats compare with that in denervated muscle of non-myopathic rats, but these differences were not statistically significant (Fig. 3a).

In the innervated and denervated muscles from rats without chloroquine treatment, treatment with low-dose IGF-1 resulted in a significant decrease in MuRF-1 mRNA levels (0.6-fold in
innervated muscle, 0.6-fold in denervated muscle) relative to those muscles from rats treated with saline (Fig. 3b). In the innervated and denervated muscle of chloroquine-treated rats, treatment with low-dose IGF-1 tended to decrease MuRF-1 mRNA levels, but this difference did not reach the level of statistical significance. In chloroquine-treated rats, treatment with high-doses IGF-1 did not result in a significant change in MuRF-1 mRNA levels in denervated muscles but did result in a significant decrease in MuRF-1 mRNA level in innervated muscles.

Ubiquitin mRNA levels were also significantly higher in the denervated muscles of rats without and with chloroquine treatment (2.6-fold in non-myopathic rats, 2.6-fold in chloroquine-treated rats) when compared with the contralateral, innervated muscles of both groups (Fig. 3c).

Treatment with low-dose or high-dose IGF-1 did not result in any significant change in ubiquitin mRNA levels in the innervated or denervated muscles of rats without or with chloroquine treatment (Fig. 3c).

Messenger RNA levels of autophagy-related
genes (e.g., LC3alpha, LC3beta, mTOR and Vps34) were also elevated in denervated soleus muscles of rat groups without and with chloroquine treatment when compared with contralateral, innervated muscles, being 2.4 ($p < 0.05$), 4.2 ($p < 0.0001$), 2.2 ($p < 0.05$) and 2.5-fold ($p < 0.05$) in non-myopathic rats, and 2.8 ($p < 0.01$), 4.8 ($p < 0.05$), 2.8 ($p < 0.05$) and 4.6 ($p < 0.01$)-fold in chloroquine-treated rats, respectively, increased relative to innervated muscle from non-myopathic rats.

Treatment with low-dose or high-dose IGF-1 did not result in any significant change in the mRNA levels of LC3alpha, LC3beta, mTOR and Vps34 in the innervated or denervated soleus muscles of rats without or with chloroquine treatment.

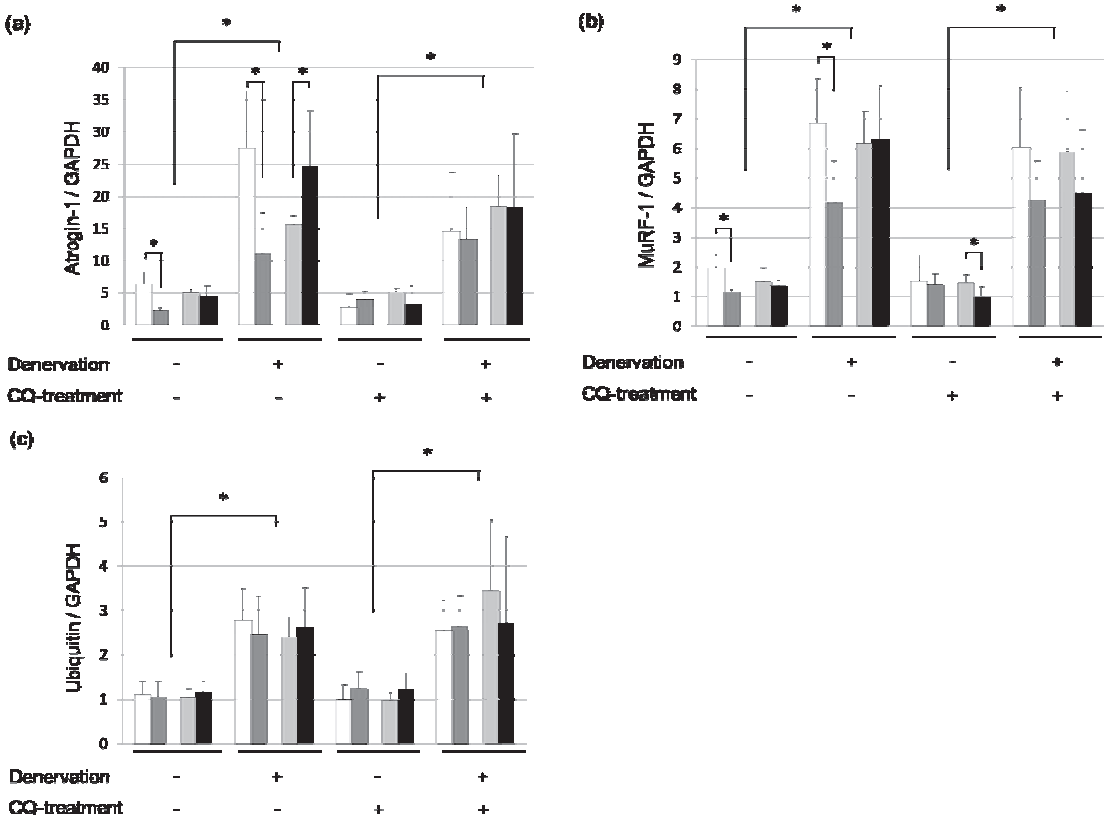

Fig. 3. Effects of IGF-1 on changes in atrogin-1/MAFbx (a), MuRF-1 (b), and ubiquitin (c) mRNA levels in innervated and denervated soleus muscles from rats without and with chloroquine treatment after administration of either saline or IGF-1. Number of rats per group = 5.□, saline administration (identical volume of saline instead of 2 mg/kg IGF-1); ▩ (dark gray), low-dose (2 mg/kg/day) IGF-1 administration; ▨ (light gray), saline administration (identical volume of saline instead of 8 mg/kg IGF-1);■, high-dose (8 mg/kg/day)IGF-1 administration. *$p < 0.05$.

DISCUSSION

The present study demonstrated that the appearance of rimmed vacuoles and dense granular bodies in the denervated muscle fibers from chloroquine- treated rats but not in the denervated muscle from rats without chloroquine treatment. This occurred despite the presence of severe muscle fiber atrophy in denervated muscles from both types of rats, as previously reported[3]. Furthermore, protein and/or mRNA levels of ubiquitin, proteasomes, and muscle specific ubiquitin-ligases (e.g., MuRF-1, atrogin-1) were upregulated in denervated soleus muscle from rats without and with chloroquine treatment when compared with contralateral, innervated muscles. The increase in proteasomes and ubiquitin in denervated muscles from chloroquine-treated rats, suggests that the ubiquitin-proteasome proteolysis pathway and the lysosomal proteolytic pathway may mediate muscle fiber destruction in the context of this myopath

A previous study demonstrated that IGF-1 decreased the expression of the ubiquitin ligases, atrogin-1 and MuRF-1[8, 15, 24], resulting in suppression of the ubiquitin-proteasome proteolytic pathway. This effect may contribute to the antiproteolytic action of IGF-1 in muscle, which is supported by the fact that IGF-1 induced a time- and dose-dependent reduction of atrogin-1 mRNA in the present study. However, the degradation rate of atrogin-1 mRNA was not affected by IGF-1 in denervated muscles of both rats, suggesting that the IGF-1 -induced reduction of atrogin-1 mRNA may occur at the transcriptional level. Indeed, an IGF-1 -dependent reduction in transcription of the atrogin-1 gene has been demonstrated in an in vitro model of muscle atrophy. This phenomenon involved inactivation of Foxo transcriptional factors by phosphorylation through the PI3k/Akt pathway[15, 24].

In the present study, the mean of diameter of fibers in

innervated and denervated muscle did not change in response to low-dose or high-dose IGF-1 treatment. Further, atrogin-1 and MuRF-1 mRNA levels in the denervated muscles from rats without chloroquine treatment significantly decreased in response to low-dose IGF-1 treatment but did not in response to high-dose IGF-1 treatment. In chloroquine-treated denervated muscles rats, atrogin-1 and MuRF-1 mRNA levels tended to decrease in response to low-dose IGF-1, but this difference did not reach the level of statistical significance. In contrast, atrogin-1 and MuRF-1 mRNA levels tended to increase in response to high-dose IGF-1 administration in denervated muscles from all animals. The one exception to this was a de acrease in MuRF-1 mRNA levels in the denervated muscles from chloroquine-treated rats. These results suggest that low-dose IGF-1 downregulates ubiquitin ligase, whereas high-dose IGF-1 has no effect on ubiquitin ligase. The mechanisms underlying this phenomenon remain unclear. Regardless, low-dose of IGF-1 over a period of 8 days may be insufficient to produce an effect in atrophic fibers in the denervated muscles from either rat type. Longer periods of IGF-1 treatment may be necessary to downregulate atrogin-1 and MuRF-1 expression in this myopathy, which is supported by studies that have reported that an 8-week course of IGF-1 protected against muscle fiber atrophy in dystrophic mice[9, 14].

Interestingly, our study demonstrated that in denervated muscles from chloroquine-treated rats, the number of muscle fibers with rimmed vacuoles and dense granular bodies was significantly decreased after IGF-1 administration when compared with saline-administrated animals. These results suggest IGF-1 administration may inhibite autophagosome formation or the lysosomal system.

Previous reports suggest that IGF-1 inhibits mTOR-mediated autophagy through the PI3k-Akt and Foxo pathway, thereby suppressing atrogin-1 and MuRF-1 transcription[15, 24, 25]. Activation of mTOR result in inhibition of autophagy-associated protein, such as Beclin-1 (Apg6), Apg14 and Vps34, and leads to decreased autophagosome formation[20-22, 25].

In the present study, mRNA levels of autophagosome-related genes, such as, mTOR, LC3alpha, LC3beta, and Vps34, were significantly increased in the denervated muscles of rats without and with chloroquine treatment relative to the contralateral, innervated muscles of either rat type. However, mRNA levels of those genes did not change in the denervated muscles of either rat type in response to IGF-1 treatment. That may be because IGF-1 affects these pathways at the transcription level or because accumulation of autophagosomes in the denervated, chloroquine- treated rats may be due to inhibition of autophagosome recycling related to a compromise in lysosomal formation.

In conclusion, these data suggest that IGF-1 treatment may suppress rimmed vacuole formation in the muscle fibers of some rimmed vacuolar myopathies, especially chloroquine myopathy and
therefore has the therapeutic potential to delay clinical progression of such disease.

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［原著論文］

インスリン様成長因子-１（IGF-1）は実験的クロロキン誘発縁取り空胞ミオパチーの筋線維における空胞の過剰形成を抑制する

熊本俊秀[1,2]、中村憲一郎[2]、仲野義和[2]、河村由子[2]、麻生泰弘[2]、迫　祐介[2]、木村成志[2]

【要　旨】

目的：脱神経処理したクロロキン投与ラット筋の病理所見および筋崩壊に関与する分子に対するIGF-1の影響を検討した。

方法：予め左側の坐骨神経を結紮（脱神経）したラットにクロロキン、または生理的食塩水（生食水）を各々投与した（各20匹）。そのうち両ラットの各10匹に連日IGF（2 mg/kg、8 mg/kg、各5匹）を投与し、残りの各10匹にIGF-1の代わりに同量の生食水を8日間、腹腔内に注射した。

結果：クロロキン投与ラットの脱神経したヒラメ筋でのみ多数の縁取り空胞（rimmed vacuole）を認め、少量および大量のIGF-1投与によりrimmed vacuoleを有する筋線維数は有意に減少した。IGF-1は筋線維の萎縮には効果はなかった。プロテアソームおよびユビキチンの免疫染色、筋特異的ユビキチンリガーゼおよびオートファジー関連遺伝子のmRNAレベルはクロロキン投与および非投与（生食水投与）の脱神経筋で有意に増加したが、少量および大量のIGF-1投与ではいずれも有意な変化はなかった。

結論：IGF-1は、クロロキン誘発ミオパチーの動物モデルの筋萎縮は抑制しないが、筋線維内のrimmed vacuoleの形成を抑制する。

キーワード：クロロキンミオパチー、rimmed vacuole、ユビキチン、プロテアソーム、IGF-1、脱神経

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