SHORT REPORT

ABSTRACT: Clathrin-coated vesicles are involved in receptor-mediated intracellular transport pathways related to lysosomal proteolysis. Clathrin levels were significantly elevated in denervated soleus muscles from chloroquine- and saline-treated rats as compared with their contralateral, innervated muscles. No difference was found in the clathrin levels of the denervated muscles in both groups. The accumulation of autophagic vacuoles was marked only in chloroquine-treated muscles after denervation. These findings suggest that chloroquine does not inhibit intracellular trafficking of clathrin-coated vesicles during the overdevelopment of autophagic vacuoles. © 1998 John Wiley & Sons, Inc. *Muscle Nerve* **21:** 665–668, 1998
Key words: chloroquine myopathy; autophagic vacuoles; clathrin; lysosome; coated vesicle

EFFECT OF CHLOROQUINE-INDUCED MYOPATHY ON RAT SOLEUS MUSCLE SARCOPLASM AND EXPRESSION OF CLATHRIN

TOSHIHIDE KUMAMOTO, MD,* SHIN-ICHIRO NAGAO, MD, RIE SUGIHARA, MD, TOMOKO ABE, MD, HIDETSUGU UEYAMA, MD, and TOMIYASU TSUDA, MD

Third Department of Internal Medicine, Oita Medical University, Hasama 1-1, Oita 879-55, Japan

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Clathrin forms the coat of vesicles involved in three receptor-mediated intracellular transport pathways which (1) export aggregated materials from the trans-Golgi network for regulated secretion, (2) transfer lysosomal enzymes from the trans-Golgi network to the lysosomes, and (3) function in receptor-mediated endocytosis at the plasma membrane.[1,10,11] These vesicles have importance in the intracellular lysosomal proteolysis of various types of cells.[6,11]

Chloroquine, a lysosomotrophic agent, affects autophagic protein degradation in the lysosomal system, thereby inducing the formation of numerous autophagic vacuoles in the skeletal muscles.[4,5,13] The pathogenesis of this vacuole formation is not known. We previously reported that autophagic vacuoles accumulated markedly in chloroquine-treated soleus rat muscles after denervation but not in the corresponding contralateral innervated muscles.[5]

To clarify whether clathrin is involved in the overdevelopment of autophagic vacuoles, we made immunohistochemical and immunoblotting studies of innervated and denervated rat muscles after the induction of experimental chloroquine myopathy.

MATERIALS AND METHODS

As described elsewhere,[5] the left hind legs of 30 Wistar rats (200–250 g) were denervated by ligation of the sciatic nerve. Chloroquine chloride (50 mg/kg body weight) or saline was injected intraperitoneally into one of two groups of 15 rats twice daily, beginning the day after denervation. Soleus muscles from the right (innervated) and left (denervated) hind legs were obtained from both groups of rats on days 4, 8, and 16 after the initial injection.

A routine histochemical study was made on consecutive serial cryostat sections (10 μm thick) as described previously.[8] For immunohistochemical analysis, the sections were incubated for 1 h in 1:20 diluted mouse monoclonal anticlathrin antibody (Progen Biotechnik GmbH, Heidelberg, Germany). After incubation with alkaline phosphatase-conjugated goat antimouse immunoglobulin (Ig)M (Vector Lab. Inc., Burlingame, CA) for 30 min, the sections then were stained using an Alkaline Phosphatase Substrate Kit I (Vector Red) (Vector Lab, Inc.).

For Western blot analysis, proteins separated from homogenized muscle specimens in a 10% so-

*Correspondence to: Dr. Toshihide Kumamoto

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dium dodecyl sulfate (SDS)–polyacrylamide gel were transblotted electrophoretically on nitrocellulose sheets. The blotted proteins were incubated for 2 h with a 1:100 dilution of anticlathrin antibody. After incubation with a second antibody, the sheets were allowed to react with 4-chloro-1-naphthol. Scanning densitometry was used to determine the peak areas and to assess the relative amounts of protein in the immunoreactive bands. Data given as relative units were analyzed with the software NIH image (version 1.57) on a Macintosh 7100/66AV computer. Statistical analysis was done with the *t*-test.

RESULTS

Histological and histochemical findings for the innervated and denervated soleus muscles from both

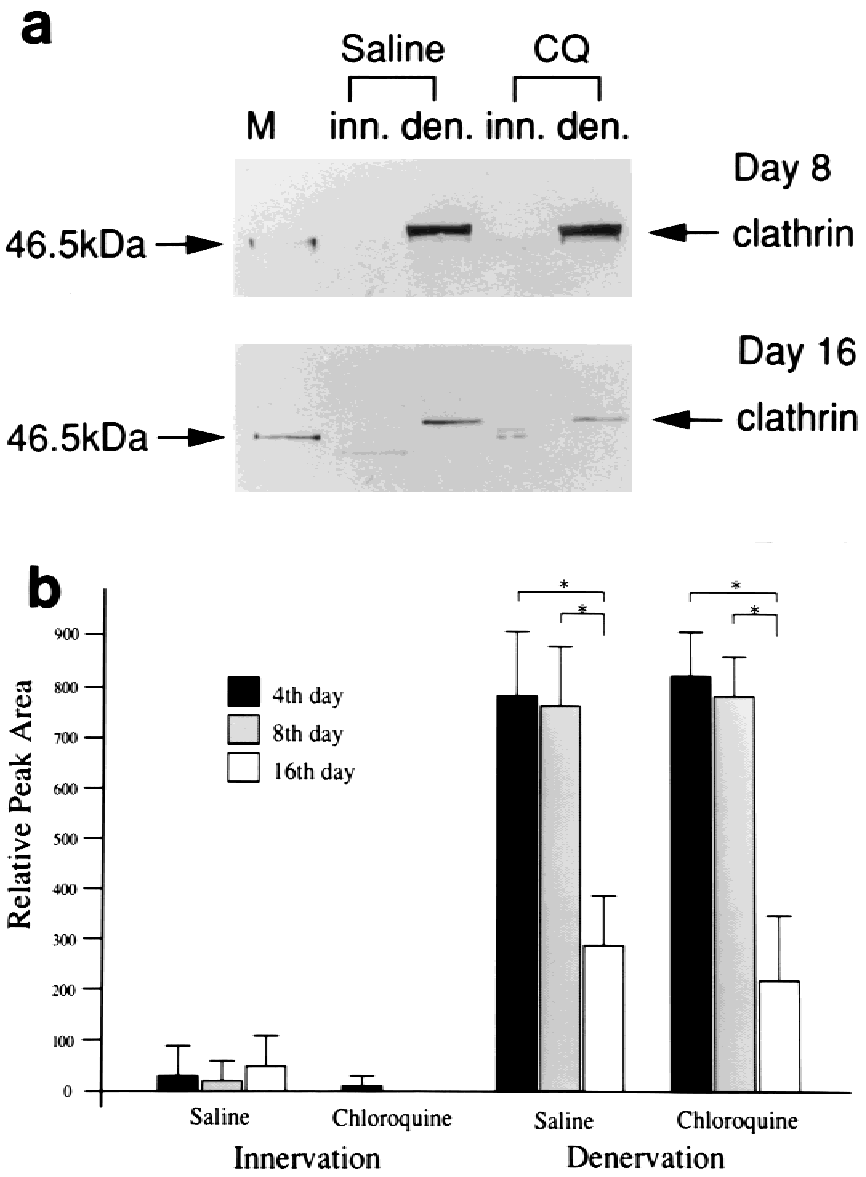

FIGURE 1. (a) Western blot analysis of clathrin in innervated and denervated soleus muscles after 8 and 16 days of saline or chloroquine treatment. A clathrin band of approximately 180-kDa is strongly expressed in the extracts from denervated muscles of both experimental groups, and, to a lesser extent, in the innervated chloroquine-treated muscles. M, molecular size marker; inn, innervation; den, denervation; CQ, chloroquine. **(b)** Densitometric quantification of clathrin expression in innervated and denervated rat soleus muscle on days 4, 8, and 16 of saline or chloroquine treatment. Scanning densitometry showed a significance on all the test days; the intensity of the clathrin-immunoreactive band being significantly higher in the saline- or chloroquine-treated denervated muscles than in the corresponding innervated muscles ($P < 0.001$). In both groups of denervated muscles the clathrin levels were significantly lower on day 16 than on days 4 and 8 ($P < 0.001$). There was, however, no significant difference in the clathrin levels of the two groups of denervated muscles on each of the test days, *$P < 0.001$ versus the values for denervated muscles from saline- or chloroquine-treated rats on day 16.

the saline- and chloroquine-treated rats on each test day were similar to those described elsewhere.[5] Autophagic vacuoles accumulated markedly in the chloroquine-treated muscles after denervation, in particular on days 8 and 16. In contrast, they were very rare in the contralateral, innervated chloroquine-treated muscles, as well as in the innervated and denervated muscles of the saline-treated rats.[5]

The muscle fibers in the innervated muscles from the saline- and chloroquine-treated rats had no, or only a weak, immunohistochemical reaction for clathrin on all the test days. The denervated muscles of both groups had strongly positive immunoreactivity for clathrin in the sarcoplasm on all the test days.

Immunoblotting detected an approximately 180-kDa band, mainly in the muscle extracts from the denervated muscles of the saline- and chloroquine-treated rats (Fig. 1a). Scanning densitometry showed a significant increase in the intensity of the clathrin-immunoreactive band in the denervated muscles after both saline and chloroquine treatment on all the test days ($P < 0.001$) when compared to the corresponding innervated muscles. Furthermore, the clathrin level in the denervated muscles was significantly lower on day 16 than on days 4 and 8 ($P < 0.001$). No significant differences, however, were found between the denervated muscles of both groups on each test day (Fig. 1b).

DISCUSSION

Denervation of skeletal muscle is induced by an increase in endocytotic activity as well as by an increase in exocytotic activity that primarily involves transport from the endoplasmic reticulum/Golgi apparatus.[7,12,14] High endocytotic activity mainly is associated with the coated pits/endosomes, whereas elevated exocytotic activity involves primary lysosomes derived from the endoplasmic reticulum/Golgi apparatus.[9,12,14] Clathrin is a well-known constituent of the coat of such vesicles as the coated pits/endosomes and primary lysosomes.[11]

We found specific up-regulation of clathrin in denervated muscles of chloroquine- as well as saline-treated rats as compared with the innervated control muscles of both groups. Clathrin levels, however, did not differ significantly in denervated muscles with and without chloroquine treatment and, whereas autophagic vacuoles were frequent in the former muscles, they were very rare in the latter. These findings suggest that the up-regulation of clathrin in both groups of denervated muscles reflects an increase in the number of clathrin-coated vesicles (i.e., coated pits/endosomes and primary lysosomes), which increase is induced by the increased endocytotic and exocytotic activities of these muscles. Consistent with this hypothesis, immunohistochemistry showed a strong positive reaction for clathrin in the sarcoplasm of denervated muscles with and without chloroquine treatment, but not in the corresponding contralateral innervated muscles. Denervation leads to an increase in endocytosis that persists for at least 12 days after nerves are crushed, then there is a slow decrease, and return to levels similar to those in normal innervated muscles by 21 days.[8] This time course for endocytotic activity levels is comparable to that for clathrin levels in denervated muscles with and without chloroquine treatment.

Chloroquine has been suggested to inhibit the normal trafficking of clathrin in muscle cells.[2] In our study, however, we found no differences in the up-regulation of clathrin in the denervated muscles of saline- and chloroquine-treated rats. This suggests that chloroquine does not affect the cell membrane, which provides the endocytotic pathway and mediates the transport of newly synthesized lysosomal enzymes from the exocytotic pathway via the trans-Golgi network. We speculate that autophagic vacuoles accumulate in chloroquine-treated muscles after denervation because of a decrease in the fusion rate of autophagic vacuoles and autolysosomes. Alternatively, the degradation rate of segregated cellular components within the autolysosomes may decrease, or chloroquine treatment may slow turnover of autophagic vacuoles, as described previously.[3,5,6]

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