Effects of Corticosterone on Ca\(^{2+}\) Uptake and Myofibrillar Disassembly in Primary Muscle Cell Culture

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This study was done to examine the effects of cortisolone, a glucocorticoid, on Ca\(^{2+}\) uptake, proteolysis, and Ca\(^{2+}\) channels in primary cultures of chick muscle cells, to clarify the mechanism of glucocorticoid action on muscle proteolysis. Chick muscle cells were incubated for 24 h in a medium containing cortisolone (30 ng/ml) when the cells were confluent (6 days). To examine the contribution of Ca\(^{2+}\) channels, nifedipine, a Ca\(^{2+}\) channels antagonist, was used. Ca\(^{2+}\) uptake measured with \(^{45}\)CaCl\(_2\) was increased three-fold by cortisolone, with a peak at 12 h after the treatment started. The growth of the cells estimated from the protein content and creatine kinase activity was not affected by cortisolone. Proteolysis, evaluated with \(^{3}H\)tyrosine as a label of the protein and N\(^{\text{-}}\)methylhistidine release, was unchanged by cortisolone. However, the amount of easily releasable myofilament as a measure of myofibrillar disassembly in the muscle cells was increased by cortisolone, and prevented by nifedipine. These results show that cortisolone increases Ca\(^{2+}\) uptake and starts myofibrillar protein breakdown.

Key words: corticosterone; calcium uptake; proteolysis; muscle cell

Glucocorticoids are essential for life and normal growth. On the other hand, excessive glucocorticoid hormones from endogenous or exogenous sources cause wasting of skeletal muscle. Glucocorticoids stimulate skeletal muscle proteolysis in vivo, but the mechanism is unclear. The skeletal muscle contains multiple pathways for protein breakdown including cathepsin, calpain, and proteasome. Calpain seems to be important in the initial events of skeletal muscle proteolysis caused by glucocorticoids. Glucocorticoids can activate both calpain and proteasome, but intact myofibrillar proteins are not processed by proteasome. In rats, cortisolone (CTC), a major glucocorticoid in rats and chickens, increases calpain, a Ca\(^{2+}\)-dependent protease, and skeletal muscle proteolysis. For these reasons, we speculated that glucocorticoid activates calpain, which degrades \(\alpha\)-connectin and Z-disks, which may in turn trigger skeletal muscle proteolysis.

The physiological mobilization of free intracellular calcium or Ca\(^{2+}\) signaling is needed for all cells to function, and is involved in numerous biological processes such as neurotransmission, long-term potentiation, cell secretion, muscle contraction, and cell death. Cytosolic free Ca\(^{2+}\) may be important in the regulation of calpain activity. Ca\(^{2+}\) could be mobilized from intracellular Ca\(^{2+}\) stores or from extracellular fluid to activate calpains. Glucocorticoids potentiate Ca\(^{2+}\) influx and accelerate the release of Ca\(^{2+}\) from intracellular stores in cultured hepatocytes and vascular smooth muscle cells. Glucocorticoids increase Ca\(^{2+}\) entry through the Ca\(^{2+}\) channels, and the subsequent rise in cytosolic Ca\(^{2+}\) concentration causes an increase in calpain activity followed by the start of myofibrillar disassembly. Indeed, in rats, CTC increases calpain activity and accelerates skeletal muscle proteolysis, and these effects are decreased in vivo by nifedipine (NIF), an L-type voltage-dependent Ca\(^{2+}\) channel antagonist (Ohtsuka et al., unpublished).

Z-band disruption and myofilaments release in skeletal muscle occurs in certain catabolic conditions, including starvation and treatment with glucocorticoids. The release of myofilaments from the myofibrils reflects calpain activity. Experiments were done in this study to clarify the effects in cultured chick muscle cells of CTC on Ca\(^{2+}\) uptake and muscle proteolysis using the amount of easily releasable myofilaments, N\(^{\text{-}}\)methylhistidine (MeHis) release, and breakdown of \(^{3}H\)tyrosine-labelled protein as the indices. The effects of NIF on the easily releasable myofilaments in the muscle cells treated with CTC was examined also. Cultured cells
have advantages in the investigation of hormonal actions because it is difficult in studies in vivo to eliminate the effects of other hormones.

Materials and Methods

Primary cell culture. Myoblasts were isolated from the thigh muscle of 13-day-old chick embryos. The muscle tissue was digested with Dispase (2,000 U/ml, Godo Shusei Co., Ltd., Tokyo, Japan) for 10 min at 37°C. The cell suspension was passed through a stainless steel net (200 mesh) and centrifuged at 100 × g for 3 min. The supernatant was aspirated and the cell pellet was dispersed into 3 ml of a basal growth medium (M-199 containing 15% calf serum and 2.5% chicken embryo extract). The cell suspension was transferred to an uncoated 35-mm culture dish to allow fibroblast attachment. After 40 min, unattached cells were transferred to a new dish. This adhesion method was repeated a total of 3 times to eliminate fibroblasts, and then the cells were counted and plated on gelatin-coated 6-well plates at the density of 2.5 × 10⁵ cells/well. The cells were grown at 37°C in a 5% CO₂-enriched atmosphere of humidified air. The medium was replaced every 2 days. After being cultured for 6 days, the cells became confluent and some 90% of the cells formed myotubes.

Ca²⁺ uptake measurement. On day 6, the medium was replaced by a medium containing CTC (30 ng/ml) and ⁴⁰CaCl₂ (2 μCi/ml). CTC was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and ⁴⁰CaCl₂ was from Amersham Pharmacia Biotech UK Ltd. CTC was dissolved in ethanol before addition to the medium (final ethanol concentration in culture medium was 0.1%). The medium of the control group did not have CTC. Six wells were used for each treatment. After 5 and 30 min, and at 1, 2, 6, 12, and 24 h, the culture medium was discarded and the muscle cells were removed and quickly washed three times with ice-cold 25 mM Tris (pH 7.5) containing 140 mM NaCl and 1 mM LaCl₃. Then cells were weighed and digested in 2 ml of 1 M NaOH for the measurement of ⁴⁰Ca. Radioactivity was measured with ACSII (Amersham, Arlington Heights, IL) as the scintillation fluid and a liquid-scintillation counter (Tri-Carb 2100TR, Packard, Meriden, USA). Proteins were measured by Lowry’s method with bovine serum albumin as the standard.

Measurement of growth and myofibrillar proteolysis. On day 6, the medium was replaced by a medium containing CTC (30 ng/ml), and 24 h after the replacement, the medium and cells were collected. After the medium was collected, the cell monolayer was washed three times with ice-cold PBS, and the cells were scraped off with a rubber policeman with 3 ml of Tris buffer (pH 6.8). The cells were then homogenized with a homogenizer and centrifuged at 1250 × g for 10 min at 4°C. The supernatant was quickly frozen and stored at −80°C until analysis. Protein in the recovered cells was measured by Lowry’s method as mentioned above.

Creatine kinase (EC 2.7.3.2) activity of the cells was assayed by the method of Rosalki and MeHis in the medium was assayed by the method of Hayashi et al. as a measure of skeletal myofibrillar protein breakdown.

Measurement of protein degradation. The rate of degradation of long-lived proteins was measured by the method of Gulve and Dice. In brief, the long-lived proteins were radiolabeled for 2 days with [³H]tyrosine (1 μCi/ml) after preculture for 4 days. [³H]Tyrosine was purchased from American Radiolabeled Chemicals, Inc (St. Louis, MO). After radiolabeling, cells were rinsed and placed in a nonradioactive chase medium (2 mM tyrosine) for 2 h to allow degradation of the short-lived proteins. The cells were rinsed and then put into nonradioactive experimental medium. The medium and cells were collected at 24 h. The medium was added 20% trichloroacetic acid and the supernatant was recovered. The cells were dissolved in 0.5 M NaOH and the radioactivities in the supernatant and solubilized cell solution were measured with a scintillation counter.

Measurement of easily releasable myofilaments. Easily releasable myofilaments were measured as described by Williams et al. The myofilament proteins were first isolated by soaking of the muscles at 4°C in a low-salt buffer (LSB; 0.1 M KCl, 2 mM MgCl₂, 2 mM EGTA, 0.5 mM dithiothreitol, and 10 mM Tris-maleate, pH 7.0) containing 1% Triton X-100, for 90 min. After being soaked for 90 min, the muscle cells were homogenized at 4°C in the same solution with a Polytron homogenizer (Brinman, Westbury, N.Y.). The homogenate was centrifuged at 1500 × g for 10 min and the pellet was suspended with a Pasteur pipette in 10 ml of LSB containing 1% Triton X-100, filtered through two layers of gauze cloth, and recentrifuged. The resulting myofilament pellet was washed once in LSB containing 1% Triton X-100 and three times in LSB. The myofilaments were extracted from the myofilament fraction by repeated pipetting (10 passages through a Pasteur pipette) in 1.5 ml of LSB containing 5 mM ATP. The suspension was layered over 0.75 ml of LSB containing 20% glycerol in a conical tube and centrifuged at 1500 × g for 10 min. The supernatant, including the layer-containing glycerol, was collected with a Pasteur pipette and was centrifuged through 0.5 ml of LSB containing 20% glycerol. The final supernatant contained the released myofilaments and the pellet of the residual myofibril-
Fig. 1. Effects of CTC on Ca\textsuperscript{2+} Uptake in Cultured Chick Muscle Cells.
Cells were incubated for 24 h in a medium containing \textsuperscript{45}CaCl\textsubscript{2} (2 \mu Ci/ml). Symbols: ○, control medium; ●, medium containing CTC (30 ng/ml). Values are means ± SD. (n = 6). *P < 0.05 and **P < 0.01 vs control.

Fig. 2. Effects of CTC on Protein Content (A) and CK Activity (B) of Cultured Chick Muscle Cells.
Cells were incubated for 24 h in a control medium or a medium containing CTC. Open bars, control medium; closed bars, medium containing CTC (30 ng/ml). Values are means ± SD. (n = 6).

Fig. 3. Effects of CTC on MeHis Release (A) and Long-lived Protein Degradation Measured with [\textsuperscript{3}H]Tyrosine (B) in Cultured Chick Muscle Cells.
Cells were incubated for 24 h in a control medium or a medium containing CTC. Open bars, control medium; closed bars, medium containing CTC (30 ng/ml). Value are means ± SD. (n = 6).

Fig. 4. Effects of CTC on Easily Releasable Myofilaments.
Cells were incubated for 24 h in a control medium or a medium containing CTC. Open bar, control medium; closed bar, medium containing CTC (30 ng/ml). Values are means ± SD. (n = 6). *P < 0.05 vs control.

Results
The uptake of Ca\textsuperscript{2+} was increased by CTC, with a peak seen at 12 h (Fig. 1). The protein content and CK activity, used as measures of growth and differentiation of the muscle cells, were not affected by CTC (Fig. 2). MeHis release and the rate of breakdown of long-lived protein in skeletal muscle were not affected by CTC (Fig. 3). The amount of easily releasable myofilaments in muscle cells was increased by CTC (Fig. 4).

The increment of easily releasable myofilaments due to CTC treatment was reduced by NIF (Fig. 5). Ca\textsuperscript{2+} channels may have a role in the effect of CTC on myofibrillar disassembly. There was no difference between the control and NIF groups in the amount of easily releasable myofilaments.

Discussion
Glucocorticoids stimulate muscle protein breakdown\textsuperscript{38,29} and inhibit muscle protein synthesis\textsuperscript{4,30}.
retarding growth of rats. The skeletal muscle contains multiple pathways for protein breakdown, including the lysosomal, calcium-dependent, and cytosolic ATP-ubiquitin-dependent proteolytic systems. Among the proteolytic pathways and proteinases, calpain and its activator Ca\(^{2+}\) are thought to play important roles in the initiation of glucocorticoid-induced muscle proteolysis. Calpain, a Ca\(^{2+}\)-activated neutral protease, seems to mobilize the Z-disks of muscle cells, resulting in muscle protein degradation. The calcium concentration in skeletal muscle was increased by glucocorticoid treatment and then calpain activity was increased in an experiment in vivo (Ohtsuka et al., unpublished). Ca\(^{2+}\) concentration in the cytosol might be a signal to start the proteolysis. We postulated that glucocorticoids increase Ca\(^{2+}\) uptake through cytoplasmic Ca\(^{2+}\) channels, and that a subsequent rise in the cytosolic Ca\(^{2+}\) concentration causes an increase in calpain activity, followed by the start of skeletal muscle proteolysis. There are many studies in vitro showing effects of Ca\(^{2+}\) on skeletal muscle protein breakdown. Baracos et al. have reported that an overload of Ca\(^{2+}\) around muscle cells using an ionophore or a medium of high Ca\(^{2+}\) concentration accelerates muscle protein degradation. However, the mechanism of the catabolic effects of glucocorticoids is not completely clarified. Glucocorticoids seem to affect the rate of Ca\(^{2+}\) uptake in cultured cells. In the liver and vascular smooth muscle cells, Ca\(^{2+}\) uptake is stimulated by corticosterone and dexamethasone, but in C2C12 and C2 muscle cells, the uptake is inhibited by dexamethasone and α-methylprednisolone. These differences in Ca\(^{2+}\) uptake seem to be due mainly to the timing of the measurement after hormonal treatment. In the experiments mentioned, the effects of glucocorticoids on Ca\(^{2+}\) uptake might show change with time as they did in this experiment.

This investigation was first designed to find whether Ca\(^{2+}\) uptake can trigger skeletal muscle proteolysis by CTC. Furthermore, this experiment was done to survey the changes with time in the Ca\(^{2+}\) uptake. We found that CTC increased Ca\(^{2+}\) uptake in a time-dependent way. Therefore, it is likely that glucocorticoids increase Ca\(^{2+}\) entry into muscle cells and increase in calpain activity followed by the start of muscle myofibrillar disassembly. It is also of interest that the increased Ca\(^{2+}\) uptake due to CTC did not last long. Several studies have shown that the proteolytic effect of CTC on the skeletal muscle peaks several days after the treatment started and gradually disappears. The reason for this effect of CTC might be explained by the transitory effect of CTC on Ca\(^{2+}\) uptake.

The regulation of plasma membrane Ca\(^{2+}\) influx is critical for the initiation and maintenance of a variety of signal transduction cascades. In nonexcitable cells, the depletion of endoplasmic reticulum Ca\(^{2+}\) stores activates plasma membrane Ca\(^{2+}\) channels, a process called capacitative Ca\(^{2+}\) entry. Depletion of sarcoplasmic reticulum Ca\(^{2+}\) stores causes a prolonged increase in cytoplasmic Ca\(^{2+}\) dependent on extracellular Ca\(^{2+}\). This induction of Ca\(^{2+}\) influx is brought about by various signals including soluble cytosolic factors.

Our previous papers have described how the administration of CTC causes muscle protein breakdown in chickens and rats. Calpain has been implicated in the degradation of myofibrillar elements. Calpain activation may trigger the dissociation of myofibrillar elements. Baracos et al. have shown that Ca\(^{2+}\) is a critical in the regulation of intracellular protein breakdown in skeletal muscle. Hong and Forsberg reported recently that the treatment of cultured myotubes with dexamethasone, a synthetic glucocorticoid, increases the concentrations of mRNA for cathepsin B and D and m-calpain but that mRNAs for the proteasome C2 subunit are not affected by the treatment. These results suggest that glucocorticoids regulate lysosomal and calcium-dependent but not proteasome-dependent protein breakdown.

Several studies have provided evidence that dexamethasone stimulates proteasome- and calpain-dependent proteolysis in cultured L6 myotubes. Dexamethasone stimulates protein degradation and the expression of mRNA that encodes the ubiquitin-proteasome system in C2C12 myotubes. This effect is inhibited by the proteasome inhibitor MG132. However, increased levels of mRNA for various enzymes do not necessarily reflect increased proteolytic activity, and there is not a necessary relationship between mRNA levels and protein breakdown.

Release of myofilaments from the myofibrils may reflect calcium-dependent calpain activity.
finding is consistent with hypothesis that CTC causes muscle protein breakdown by affecting Ca\(^{2+}\) uptake. Indeed, we showed here that the amount of easily releasable myofilaments was increased by CTC. However, MeHis release and the breakdown of long-lived protein were not affected by CTC. The findings suggest that the proteolytic system of cultured muscle cells may be incomplete. Glucocorticoid is likely to exert its full effect on myofibrillar protein degradation in the presence of other hormones such as thyroid hormone. Indeed, we have reported synergistic effects of CTC and thyroid hormone on muscle proteolysis. Concentrations of factors such as thyroid hormone in the medium may not meet the demand to activate proteolytic enzymes other than calpain.

In conclusion, this study showed that the administration of CTC causes Ca\(^{2+}\) uptake and results in myofibrillar disassembly.

References

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