Nutritional Consequences of Critical Illness Myopathies

Apoptosis of Skeletal Muscle on Steroid-Induced Myopathy in Rats¹,²

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ABSTRACT Apoptotic cell death of differentiated skeletal muscle has been reported in an experimental steroid-induced myopathy of rats. To investigate the underlying molecular changes in the apoptosis of skeletal muscle, in situ end labeling (ISEL), Fas expression, and Western blot analysis for apoptosis-related proteins in the soleus muscle of triamcinolone acetonide (TA)-induced myopathy of rats were studied. Proteins for Western blot analysis included Fas-associated death domain (FADD) and caspase 8 for extrinsic pathway, as well as Bcl-2, Bcl-XL, Bax, Bad, Bid, Akt, p-Akt, and caspase 9 for intrinsic pathway. ISEL-positive myonuclei in TA-treated rats were 1.8 ± 1.2%, whereas Fas-positive muscle fibers were 4.5 ± 2.0%. One-fourth of Fas-positive muscle fibers had ISEL-positive myonuclei. Levels of FADD, Bax, Bad, and Bid were substantially increased in the TA-induced myopathy group, whereas Bcl-2, Bcl-XL, Akt, p-Akt, and caspase 9 did not change between control and myopathy groups. Caspase 8 activity increased in the myopathy group. These findings indicate that apoptosis of skeletal muscle in TA-induced myopathy may be triggered by Fas-Fas ligand signals and promoted mainly by overexpression of the pro-apoptotic molecules of FADD and caspase 8 involving the extrinsic pathway. The apoptotic process presented in this study supports a direct, nongenomic effect of a glucocorticoid on cellular membranes leading to cellular apoptosis rather than genomic effector mechanism of steroid hormone mediated by cytosolic steroid receptors. J. Nutr. 135: 1806S–1808S, 2005.

KEY WORDS: • apoptosis • immunocytochemistry • myopathy • steroid • Western blot

Glucocorticoids are the most potent immunosuppressive and anti-inflammatory drugs and have been widely used in the treatment of autoimmune diseases. Glucocorticoid-associated adverse effects commonly develop and include hypertension (88%), Cushingoid features (66%), adrenal suppression (56%), myopathy (50%), osteopenia (46%), growth retardation (39%), obesity and hypercholesterolemia (30%), and cataracts (14%) (1). Several clinical studies have reported the occurrence of steroid-induced myopathy in intensive care unit patients after administration of high doses of glucocorticoids with or without neuromuscular junction blocking agents (2–4). Iatrogenic steroids, especially the 9α-fluorinated ones like triamcinolone, betamethasone, or dexamethasone, can cause dose-dependent muscle wasting and weakness within weeks (5). This can be ameliorated by limitation of the steroid dose, alternate-day use, attention to exercise, and a high-protein diet. Electromyography shows myopathic features, whereas muscle biopsy reveals myopathy with selective loss of thick myosin filaments (rhabdomyolysis), necrosis, and atrophy of type II fibers (6,7).

In 3 previous studies (8–10), we investigated the development of steroid-induced myopathy of the extensor digitorum longus (EDL)⁴ and soleus in adult female rats treated with triamcinolone acetonide (TA, 5 mg·kg⁻¹·d⁻¹ for 9 d). Rats treated with TA showed substantial loss of body and muscle weight. In the TA-treated group, cross-sectional areas of type II fibers of both EDL and soleus decreased in comparison with the controls. Necrotic changes occurred only in type II fibers of the soleus. Recovery from the weight loss with type II fiber atrophy was more pronounced in the exercise group (treadmill; speed 20 m/min, duration 30 min/d, 3 d/wk for 2 wk) than in the sedentary group, but not significantly different. The steroid-induced myopathy was partly ameliorated by the exercise (8).

Moreover, to investigate whether apoptosis may contribute to the steroid-induced myopathy, rats treated with TA for 9 d were killed to detect apoptosis in situ end labeling (ISEL) and electron microscopy in soleus muscle (9), as well as DNA electrophoresis (10). Immunohistochemical stainings of Fas antigen and p53 protein were performed to examine whether apoptosis-related proteins were present in the myopathy. Muscle-fiber necrosis and apoptotic myonuclei appeared in soleus muscle following administration of TA, whereas control mus-

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⁴ Abbreviations used: EDL, extensor digitorum longus; FADD, Fas-associated death domain; ISEL in situ end labeling; TA, triamcinolone acetonide.
MECHANISM OF SKELETAL MUSCLE APOPTOSIS

Development of steroid-induced myopathy and tissue sampling. Female Sprague-Dawley rats (n = 20) weighing 180 to 210 g were maintained under standard conditions. Rats were divided into two groups. Each group was given a daily intraperitoneal injection of either physiologic saline (n = 5) or TA (Dongkwang) at a dose of 5 mg/kg body wt for 9 d (n = 15). At d 10 after completion of the injections, the soleus muscles from both legs were taken under ether anesthesia. A 10-mm-long section was taken from the midbelly of the muscles, and Fas antigen might be partly related to apoptotic muscle death in steroid-induced myopathy.

In the present study we investigated a mechanism with underlying signals of skeletal muscle apoptosis in steroid-induced myopathy of rats.

MATERIALS AND METHODS

Development of steroid-induced myopathy and tissue sampling. Female Sprague-Dawley rats (n = 20) weighing 180 to 210 g were maintained under standard conditions. Rats were divided into two groups. Each group was given a daily intraperitoneal injection of either physiologic saline (n = 5) or TA (Dongkwang) at a dose of 5 mg/kg body wt for 9 d (n = 15). At d 10 after completion of the injections, the soleus muscles from both legs were taken under ether anesthesia. A 10-mm-long section was taken from the midbelly of the soleus and quick-frozen in liquid nitrogen (∼196°C). The tissues were used for Western blot analysis.

Western blot analysis. Muscles frozen in liquid nitrogen were homogenized in a lysis buffer solution containing 100 mmol/L Tris-HCl (pH 7.4), 10 mmol/L EDTA, 50 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L leupeptin, and 20% glycerol. The homogenates were centrifuged at room temperature for 5 min at 5000 × g, and the supernatants were collected. The protein concentration in each sample was measured with a spectrophotometer using a detergent-compatible protein assay kit (Bio-Rad). An equal amount of protein from each sample was mixed with loading buffer (300 μg/lane), analyzed on 12% SDS-PAGE under reducing conditions, and then transferred onto Immobilon-P membranes (Millipore). After transfer, the Immobilon-P membranes were blocked in buffer containing 5% dry milk in PBS–0.05% Tween 20 and then incubated overnight at 4°C with primary antibodies (Table 1). Immunodetection was performed by means of peroxidase-conjugated goat antimouse, antirat, and antirabbit antibodies (SantaCruz Biotech) matched to sources of primary antibodies and visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech). Each blot was scanned into a computer, and images were stored in JPG format.

RESULTS

Compared with the control group of adult female rats, Western blotting showed that the levels of Bcl-2, Bcl-XL, and Akt did not change in the steroid-induced myopathy group. Phosphorylated Akt was mildly reduced in the steroid-induced myopathy group. Levels of Bax, Bad, and Bid were substantially higher in the steroid-induced myopathy group. Caspase 8 activity increased substantially in the steroid-induced myopathy group, whereas caspase 9 activity increased slightly. The data obtained from Western blot analysis are presented in Table 2.

DISCUSSION

In the previous experimental studies, steroid-induced myopathies were developed by daily intraperitoneal injection of

![FIGURE 1](image-url) ISEL for apoptotic cells shows a positive reaction in the soleus muscle of TA-induced myopathy (A). Fas antigen is expressed in an apoptotic cell (B).

![TABLE 1](image-url)

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Clone</th>
<th>Dilution</th>
<th>Catalog No.</th>
<th>Source</th>
</tr>
</thead>
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<td>Bad (C-20)</td>
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<td>Caspase 9</td>
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<td>#9276</td>
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<td>Actin</td>
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![TABLE 2](image-url)

<table>
<thead>
<tr>
<th>Apoptosis-related proteins</th>
<th>Control</th>
<th>TA myopathy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase 8</td>
<td>+</td>
<td>++++</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bcl-XL</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Akt</td>
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<td>+</td>
</tr>
<tr>
<td>P-Akt</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bax</td>
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<td>+++</td>
</tr>
<tr>
<td>Bad</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Bid</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Caspase 9</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

+++: Semi-quantitative changes of protein levels by Western blot analysis. (±: mildly decreased, +: protein level in control group, +++: mildly increased, ++++: moderately increased, ++++: greatly increased).
Akt protein levels did not change; however, phosphorylated Akt prolonged treatment with glucocorticosteroids.

Apoptosis is the endpoint of an energy-dependent cascade of molecular events, initiated by physiologic or harmful stimuli and consisting of 4 separable but overlapping components: signaling pathways, control and integration, common-execution phase, and removal of dead cells (11–14). Basal Akt was a positive transmembrane signal determinant of apoptosis in previous studies (9,10). The Fas molecule, synonymously referred to as APO-1, contains a cytoplasmic “death domain” shared with the type I tumor necrosis factor receptor (15). To date, apoptosis induced by Fas antigen has been extensively investigated in the lymphoid systems (16,17). Immunohistochemical studies have demonstrated that Fas antigen is expressed on muscle fibers from patients with various muscle wasting diseases, but not in normal muscle cells (18,19).

The control and integration stage is performed by specific proteins that connect death signals to the execution program. There are commonly 2 broad schemes for this stage, which are not mutually exclusive. One involves the direct transmission of signals by specific adapter proteins to the execution mechanism, as described for the Fas-Fas ligand model and target cell killing by cytotoxic T lymphocytes (15,20,21). Fas-Fas ligand interacts with 3 death domains, consisting of FADD, RAIDD, and Daxx (22,23). Initiator caspase molecules, caspases 2 and 8, potentiate the process (24). The second scheme involves members of the Bcl-2 family of proteins, which play major and ubiquitous roles in apoptotic regulation largely by regulating mitochondrial function (25,26).

In the present study, the levels of proapoptotic proteins (caspase 8, Bax, Bad, and Bid) were higher in the steroid-induced myopathy group than in the control group, whereas antiapoptotic proteins (Bcl-2, Bcl-XL) did not change between the 2 groups. Akt protein levels did not change; however, phosphorylated Akt levels decreased slightly. These elevated apoptotic signals result in mitochondrial permeability changes (27) with cytochrome c release from the mitochondria into the cytosol (28,29). Other proteins, such as p53 protein and viral protease inhibitor proteins, are also involved in apoptotic regulation (30,31). However, p53 protein was not involved in steroid-induced myopathy in the previous study (9). The execution phase is the final pathway of the actual apoptotic death program and is accomplished largely by the caspase family of proteases, consisting of caspase 9 and caspases 3, 6, 7 (29,32).

Apoptosis is a regulated form of cell death that involves the activation of a family of proteases known as caspases. Caspases are proteolytic enzymes that cleave specific target proteins, leading to the degradation of mitochondrial DNA and the release of pro-apoptotic molecules. This process is mediated by the activation of initiator caspases, such as caspase 1, 2, and 8, which then activate execution caspase 3. The expression and activation of these caspases are critical in the development of steroid-induced myopathy.

CONCLUSION

TA can induce myopathy, and apoptosis of skeletal muscle develops. Apoptosis may be triggered by Fas-Fas ligand signals, and initiator caspase 8, Bax, Bad, and Bid promote apoptosis. The apoptotic process presented in this study might be different from the classic apoptotic process through the glucocorticoid-receptor complex and then DNA binding in the nucleus with subsequent altered gene expression.

LITERATURE CITED