Oxidative Stress Impairs Skeletal Muscle Repair in Diabetic Rats

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Alongside increased proteolysis, the inability to repair damaged skeletal muscle is a characteristic feature of uncontrolled diabetes. This study evaluates the role of oxidative stress in muscle-specific gene regulatory regions and myosin chain synthesis in streptozotocin (STZ)-induced diabetic and ZDF rats. In the gastrocnemius muscle of diabetic rats, prooxidant compounds were seen to increase while antioxidant levels fell. Myogenic regulatory factors—Myo, myogenin, and Jun D—were also reduced, and muscle enhancer factor (MEF)-1 DNA binding activity was impaired. Moreover, synthesis of muscle creatine kinase and both heavy and light chains of myosin were impaired, suggesting that oxidative stress triggers the cascade of events that leads to impaired muscle repair. Dehydroepiandrosterone has been reported to possess antioxidant properties. When it was administered to diabetic rats, in addition to an improved oxidative imbalance there was a recovery of myogenic factors, MEF-1 DNA binding activity, synthesis of muscle creatine kinase, and myosin light and heavy chains. Vitamin E administration to STZ-induced diabetic rats reverses oxidative imbalance and improves muscle gene transcription, reinforcing the suggestion that oxidative stress may play a role in diabetes-related impaired muscle repair. Diabetes 53: 1082–1088, 2004

Marked muscle atrophy is a characteristic feature of uncontrolled diabetes and results from both increased proteolysis and the inability to repair damaged skeletal muscle through protein synthesis. Loss of skeletal muscle, with enhanced protein breakdown, has been demonstrated in rats with experimental diabetes (1,2) as well as in type 1 diabetic patients (3). Conversely, although there is general agreement that insulin withdrawal affects fractional protein synthesis rates, the precise mechanism of impaired muscle repair in diabetes is not fully understood (4,5).

Muscle protein synthesis is regulated by a complex interaction between myogenic regulatory factors (MRFs), consisting of Myo D, Myf 5, myogenin, and MRF4, which specifically bind muscle gene DNA regulatory elements (6) and ultimately drive muscle repair. Gene transcription of both light and heavy chains of myosin and myosin creatine kinase (MCK) depends on DNA binding to muscle enhancer factor (MEF)-1 sites of the MRF, as homodimers, or as heterodimers with other HLH proteins as well as Jun-D (7–9). Impairment of the coordinated interaction between MRFs and the MEF-1 site can obviously compromise muscle protein synthesis.

Hyperglycemia impairs the prooxidant/antioxidant balance, increasing free radical and reducing antioxidant levels. There is now definite evidence that oxygen radicals contribute to the progression of diabetes and its complications (10), and promising strategies using antioxidant compounds to prevent oxidative damage in diabetes have been proposed (11,12). As far as skeletal muscle metabolism is concerned, it has been demonstrated that oxidative stress affects the expression of the redox-sensitive genes involved in protein synthesis (13) and that in vitro H2O2 inhibits myogenesis at the level of muscle-specific protein expression (14). Moreover, antioxidants prevent the decrease of MCK transcription in skeletal muscle cells (15). It has been shown that dehydroepiandrosterone (DHEA), a multifunctional steroid secreted by the adrenal gland and brain (16), possesses a multitargeted antioxidant effect (17,18) and prevents tissue damage induced by acute and chronic hyperglycemia (19).

This study investigates the effects of oxidative stress induced by chronic hyperglycemia on the expression of several genes involved in protein muscle synthesis in streptozotocin (STZ)-induced diabetic and Zucker diabetic fatty (ZDF) rats. Results show that in both models of diabetes, MRF content, MEF-1 binding activity, and synthesis of both myosin chains and MCK are clearly affected. Vitamin E and DHEA treatment counteracts the prooxidant responses to hyperglycemia and restores the muscle MRF content and synthesis of myosin chains, reversing the loss of muscle protein synthesis induced by diabetes.

RESEARCH DESIGN AND METHODS

Male Wistar rats (Harlan-Italy, Udine, Italy) weighing 200–220 g were cared for in compliance with the Italian Ministry of Health Guidelines (no. 86/609/EEC) and with the Principles of Laboratory Animal Care (NIH no. 85-23, revised 1985). They were provided with Picciioni pellets diet (no. 48, Gessate Milanese, Milan, Italy) and water ad libitum. Hyperglycemia was induced through a single injection of STZ (50 mg/kg) in the tail vein. Three days later, glycemia was measured with c-toluidine reagent (Sigma kit, catalog no. 035) on blood collected from the heart (1 ml). Only rats with blood glucose levels >18 mmol/l entered the experimental protocols; normoglycemic rats were used as controls.
controls. On the fourth day postinjection, hyperglycemic and control rats began DHEA treatment or vitamin E. DHEA and vitamin E (α-tocopherol) were given for 21 days, at 4 mg · day−1 · rat−1 and 400 mg/kg body wt, respectively. Crystalline DHEA and vitamin E oil were dissolved in one volume of 90% ethanol, mixed with nine volumes of mineral oil, and given daily by gastric intubation. Controls received vehicle alone. After 21 days control rats and hyperglycemic rats, with or without DHEA, were anesthetized with ether and killed by decapitation after aortic exsanguination. Blood was collected and the plasma isolated. Glycemia was evaluated as described above. The gastrocnemius muscle was isolated, weighed, and homogenized to obtain different extracts.

Beside STZ-induced diabetic rats, a genetic model of type 2 diabetes was studied. Seven-week-old ZDF (GMI, Italy) rats were treated with DHEA (4 mg · day−1 · rat−1) or vehicle alone. After 21 days, ZDF and ZDF-DHEA-treated rats were killed, and tissue extracts were isolated.

### Tissue extracts

The cytosol fraction was isolated from 20% (wt/vol) homogenates in 1.15% KCl (wt/vol) by centrifugation at 15,000g for 18 min. The supernatants were then centrifuged at 105,000g for 40 min. Protein content was determined by the Bradford assay (20). The nuclear extract was prepared by the method of Meldrum et al. (21). Briefly, 200–300 mg of gastrocnemius muscle tissue were homogenized at 10% (wt/vol) in a buffer containing 20 mmol/l HEPES, pH 7.9, 1 mmol/l MgCl₂, 0.5 mmol/l EDTA, 1% NP-40, 1 mmol/l EGTA, 1 mmol/l diethiothreitol (DTT), 0.5 mmol/l phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml aprotinin, and 2.5 μg/ml leupeptin. Homogenates were centrifuged at 1,000g for 5 min at 4°C. Supernatants were removed, and the pelleted nuclei were resuspended in 600–800 μl of extraction buffer containing 20 mmol/l HEPES, pH 7.9, 1.5 mmol/l MgCl₂, 300 mmol/l NaCl, 0.2 mmol/l EDTA, 20% glycerol, 1 mmol/l EGTA, 1 mmol/l DTT, 0.5 mmol/l PMSF, 5 μg/ml aprotinin, and 2.5 μg/ml leupeptin. The suspensions were incubated on ice for 30 min for high-salt extraction followed by centrifugation at 15,000g for 20 min at 4°C. The resulting supernatants containing DNA binding proteins were carefully removed, protein content was determined using the Bradford assay, and samples were stored at −80°C until use. Total RNA was extracted from muscle tissue using Trizol Reagent (Invitrogen, Groningen, the Netherlands) based on the method developed by Chomczynski and Sacchi (22).

### Biochemical determination on cytosol fraction

The oxidative state was determined by monitoring the generation of hydrogen peroxide (H₂O₂) as described by Zuccarato et al. (23).

Hydroxynonenal (HNE) concentration was also determined on fresh cytosol fractions by the method of Esterbauer et al. (24). An aliquot of cytosol (0.5 ml) was added to an equal volume of acetonitrile-acetic acid (96:4, vol/vol). After centrifugation at 250g for 20 min at 4°C, the supernatant was directly injected into a high-performance liquid chromatographer (Waters Associated, Milford, MA) Symmetry C₁₈ column (5 mm, 3.0×150 mm). The mobile phase was acetonitrile–bidistilled water (42%, vol/vol). The HNE concentration was calculated by comparison with a standard solution of HNE of known concentration. The reduced-to-oxidized glutathione ratio was evaluated using the Owen’s and Belcher method (25). Tumor necrosis factor (TNF)-α was determined using an enzyme immunoassay specific for rat TNF-α (Quantikine M; R&D Systems, Minneapolis, MN), following the manufacturer’s instructions.

### Western blot analysis

Jun D, Myo D, and myogenin were detected on cytosol extracts by Laemmli’s method (26). Equal amounts of proteins (60 μg) were separated on 10% SDS-polyacrylamide gels, then blotted onto nitrocellulose membranes (Amersham, Braunschweig, Germany). The membranes were blocked with 5% (wt/vol) nonfat dry milk in 5 mmol/l Tris-HCl, pH 7.4, containing 200 mmol/l NaCl and 0.05% (vol/vol) Tween 20 (TBS-Tween), for 1 h at 25°C, incubated with rabbit polyclonal antibodies against Jun D, Myo D, and myogenin, and reacted with peroxidase-labeled anti-rabbit immunoglobulin (Bio-Rad) in TBS-Tween containing 2% (wt/vol) nonfat dry milk. Immunoreactive proteins were detected through the chemiluminescence assay (ECL; Amersham) and subsequent exposure to film for 2–10 min. Anti-β-actin antibody served as loading control. Specific bands were quantified by densitometry using analytic software (Multi-Analyt; Bio-Rad, Munich, Germany), and the net intensity of bands in each experiment was normalized for the intensity of the corresponding β-actin band before comparison between control and treated samples.

### DNA binding assay

Electrophoretic mobility shift assay was performed as described by Pahl et al. (27). MEF-1 consensus oligonucleotides (5'-GATT CCC CCC AAC ACC TGC TGC-3') were labeled with [32P]ATP using T₄ polynucleotide kinase and purified on the QIAquick Nucleotide Removal kit. For the electrophoretic mobility shift assay, 5 μg of nuclear proteins were incubated with 100,000 cpm of [32P]labeled MEF-1 oligonucleotide probe for 30 min at room temperature in binding buffer containing 55 mmol/l HEPES-KOH, pH 7.8, 0.5 mmol/l EDTA, 0.5 mmol/l DTT, 10% glycerol, 0.25 mmol/l spermidine, and 0.1 μg/μl poly dI/dC in a final volume of 40 μl. Protein-DNA complexes were resolved by electrophoresis through 5% native polyacrylamide gels containing 10% glycerol and 1× Tris-glycine buffer. Gels were dried under vacuum and exposed for 8–12 h to Amersham Hyperfilms (Amersham) at −80°C with intensifying screens. Specificity of binding was assessed by competition with a 50-fold molar excess of unlabelled oligonucleotides. MEF-1 specific bands were quantified by densitometry using analytic software (Multi-Analyt; Bio-Rad).

### RT-PCR analysis

Total RNA was reverse transcribed at 42°C for 40 min, using AMV reverse transcriptase (Finnzymes, Espoo, Finland) in the presence of oligo(dT) primer (Invitrogen). The PCR system contained 5 μl of extracted RNA, 1.5 μl of 10 × PCR buffer, 1 μl of 0.2 mmol/l dNTP (Finnzymes), 1.25 units TopDNA polymerase (Finnzymes), and 50 pmol of sense and antisense primers in a total volume of 50 μl. Amplification was carried out as follows. For muscle kinase (MCK) and myosin light chains (MLC 1, MLC 3): 1×3 min at 94°C; 20×30s at 94°C, 30 s at 58°C, and 30 s at 72°C; and 1×7 min at 72°C. For myosin heavy chains (MHC I, MHC IIa, MHC IIb, MHC IIx) and cyclophilin (CYC); 1×3 min at 94°C; 25×1 min at 94°C, 1 min at 50°C, and 1 min at 72°C; and 1×7 min at 72°C.

PCR products were subjected to electrophoresis on 1.5% agarose gel in the presence of ethidium bromide. Gels were photographed and analyzed with the PS program Kodak Science 1D Image System. The net intensity of bands in each experiment was normalized for the intensity of the corresponding CYC band before comparison between control and treated samples.

### Statistical analysis

All results are presented as means ± SD. Differences between means were analyzed for significance using one-way analysis of variance with the Bonferroni post hoc test (28).

### RESULTS

Three-week-old STZ-induced diabetic rats showed lower body weights and lower gastrocnemius muscle weights than the control group (P < 0.001) (Table 1). Normoglycemic rats treated with DHEA or vitamin E did not differ in either parameters from the control group treated with vehicle alone. Although STZ-induced diabetic rats treated

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**TABLE 1**

| Control (n = 10) | 6.04 ± 0.64 | 258.2 ± 14.92 | 1.71 ± 0.09 |
| Vitamin E (n = 8) | 6.08 ± 0.59 | 280.9 ± 10.62 | 1.68 ± 0.01 |
| DHEA (n = 8) | 6.21 ± 0.63 | 289.4 ± 11.80 | 1.78 ± 0.08 |
| STZ (n = 10) | 20.91 ± 2.39* | 234.0 ± 13.45* | 1.21 ± 0.06* |
| STZ + vitamin E (n = 10) | 20.73 ± 2.25* | 254.8 ± 5.48* | 1.54 ± 0.17* |
| STZ + DHEA (n = 10) | 21.52 ± 2.81* | 267.5 ± 23.57† | 1.55 ± 0.17† |
| ZDF (n = 5) | 23.41 ± 3.09 | 343.3 ± 12.51 | 1.27 ± 0.09 |
| ZDF + DHEA (n = 7) | 25.5 ± 2.35 | 346.6 ± 20.63 | 1.50 ± 0.08† |

Data are means ± SE. *P < 0.001 vs. C; †P < 0.05 vs. STZ; ‡P < 0.001 vs. ZDF.

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**DIABETES, VOL. 53, APRIL 2004 1083**
with DHEA or vitamin E showed plasma glucose levels similar to those found in STZ-induced diabetic rats (20 mmol/l), body and gastrocnemius muscle weights were significantly higher than those of untreated diabetic rats (\(P < 0.05\)). Body weight of ZDF rats treated with DHEA did not differ from those of untreated ZDF rats, while the weight of the gastrocnemius muscle was significantly lower in untreated ZDF rats than in ZDF rats treated with DHEA (\(P < 0.001\)).

**Oxidative balance.** Hyperglycemia induces oxidative stress in tissues as demonstrated by the increased \(H_2O_2\) and HNE levels in the muscle cytosol of STZ diabetic rats (Fig. 1). DHEA treatment (4 mg/day for 3 weeks) or vitamin E treatment (400 mg/kg for 3 weeks) significantly decreased \(H_2O_2\) levels (\(P < 0.001\)) compared with untreated STZ-induced diabetic rats. Increased \(H_2O_2\) levels can cause harmful effects as well as increasing oxidative damage, evaluated in terms of end products of lipid peroxidation. HNE concentrations markedly increased in STZ-induced diabetic versus control rats (\(P < 0.001\)). The concentration of this aldhehyde decreased by \(60\%\) when DHEA or vitamin E was administered to diabetic rats. Alongside increased levels of \(H_2O_2\) and HNE, we found an increased level of TNF-\(\alpha\), a redox-sensitive cytokine, in the gastrocnemius muscle cytosol of STZ-induced diabetic rats. Again, DHEA treatment decreased the TNF-\(\alpha\) level (by \(30\%\) vs. STZ, \(P < 0.05\)). In ZDF rats, oxidative stress in the gastrocnemius muscle cytosol was less evident: \(H_2O_2\) and HNE levels were \(15\%\) higher than in ZDF rats treated with DHEA. However, we found an imbalance in nonenzymatic cell antioxidant defenses in both models. The reduced-to-oxidized glutathione ratio, widely accepted as specific indicator of oxidative stress, significantly decreased in both STZ-induced diabetic and ZDF rats and improved when DHEA or vitamin E were administered (Table 2).

**MRF levels.** To evaluate MRF content, we performed a Western blot analysis of Jun D, Myo D, and myogenin on cytosol obtained from the gastrocnemius muscle of control rats and of STZ-induced diabetic rats treated or not with DHEA (4 mg/day) or with vitamin E (400 mg/kg) for 3 weeks. Data are means ± SD of 8–10 rats per group.
50% in STZ-induced diabetic rats, in which oxidative stress is clearly present. In the muscle tissue of STZ-induced diabetic rats treated with DHEA, the content of these regulatory factors is partially restored (Fig. 2). Likewise, in ZDF rats, the content of the three regulatory proteins is higher in DHEA-treated rats than in untreated ZDF rats (Fig. 3). Figure 4 shows MRFs levels in vitamin E–treated STZ-induced diabetic rats. Vitamin E administered to STZ-induced diabetic rats protected the muscle from oxidative damage and restored MRFs to levels close to those of control rats.

**MEF-1 binding activity in nuclear extracts.** In addition to the reduced content of regulatory muscle proteins, we also found that oxidative stress compromises MEF-1 activation. MEF-1 DNA binding activity decreased by 30% in diabetic rats compared with the control group and was restored in diabetic rats treated with DHEA (Fig. 5).

**Modulation of the transcription of MEF-1–dependent genes.** RT-PCR was performed to detect MCK transcript levels in controls and in STZ-induced diabetic rats treated or not treated with DHEA or vitamin E. As shown in Fig. 6, MCK transcripts, clearly detected in control and DHEA-treated rats, were significantly reduced in STZ-induced diabetic rats, while remaining close to control levels in STZ-induced diabetic rats treated with DHEA. Figure 6B shows gene expression of the MHC. The expression of the isofrom MHC IIb gene is lower in STZ-induced diabetic rats than in the control group. When DHEA was given to STZ-induced diabetic rats, MHC expression was partially restored. Expression of MLC, isoforms 1 and 3 (Fig. 6C and D), which are the predominant MLC isoforms in the adult (29), was also lower in STZ-induced diabetic rats. Expressions of light chains were restored to near normal levels in the muscle tissue of STZ-induced diabetic rats treated with DHEA. A similar pattern was observed when vitamin E was administered to STZ-induced diabetic rats (Fig. 6E and F): both MCK and MHCIIb expressions were restored in treated rats. A slightly lower expression of MEF-1–dependent genes was observed in untreated ZDF rats than in ZDF rats treated with DHEA (data not shown).

**DISCUSSION**

Skeletal muscle repair involves two principal stages: arrest of myoblast proliferation and the subsequent expression of myotube-specific genes (30). Binding of the myogenic
regulatory factors to the MEF-1 site, a DNA motif containing the sequence CANNTG, is the key step in the expression of some skeletal muscle-specific genes. Here we show that Myo D and myogenin proteins are reduced in the muscle tissue of diabetic rats. Development of the skeletal muscle lineage (31) and differentiation of pluripotent stem cells into multinucleated myotubes (32) are regulated by the binding of homo- and heterodimers of MRFs to the MEF-1 site. In diabetic rats, not only are Myo D and myogenin proteins reduced, but MEF-1 DNA binding activity is also impaired. Since Myo D both induces its own transcription and activates the transcription of myogenin (33), its reduction compromises the formation of dimers that bind and activate the MEF-1 site and impairs the terminal differentiation of myotubes (31). Beside impairing MEF-1 binding activity due to decreased levels of myogenic-transcription factors, the reduction of Jun D level might further compromise MEF-1 binding activity. Jun D, a ubiquitous E protein, can also form heterodimers with the MRFs and exhibits high binding to E boxes (6). Impaired MEF-1 binding activity resulting from an abnormal myogenin-Jun D complex has been reported in mice.

FIG. 6. Expression of MCK (A), MHC IIb (B), MLC 1 (C), and MLC 3 (D) from gastrocnemius muscle tissue from control and diabetic rats, treated or not treated with DHEA (4 mg/day). Expression of MCK and of MHC IIb from gastrocnemius muscle tissue from diabetic rats treated or not treated with vitamin E (400 mg/kg) is represented in panels E and F. Semi-quantification of RT-PCR was achieved by densitometric analysis of the net intensity of bands. The relative expression of each gene as shown is normalized to the CYC signal from the same RT product. Normalized data are expressed as means ± SD of 8–10 rats per group.
treated with TNF-α; it was normalized by the addition of Jun D or Ref-1, a nuclear redox protein (15). We also show that MCK synthesis is reduced in diabetic rats, probably dependent on the loss of MEF-1 binding activity. MCK is a key enzyme critical for differentiated skeletal muscle function. The MEF-1 site has been identified in the enhancer region of MCK and is considered to be fundamental for MCK transcription (34). In skeletal muscle cells, MCK transcription is decreased by prooxidants and prevented by antioxidants (15). Moreover, the inactivation of MCK by \( \text{H}_2\text{O}_2 \) that has been reported in rabbit muscles is dependent on the MEF-1 site (35). The MEF-1 site has also been found within the promoter of MHC and MLC genes (9,36). MHC IIb, together with MHC IIA and MHC IID/X, accounts for 90% of MHC in adult muscles. MEF-1 binding is required for the expression of MHC IIb (37), and the presence of Myo D in the MEF-1 complex facilitates the high-level expression of MHC IIb (8). Here we show that MHC IIb synthesis is reduced in the gastrocnemius muscle of diabetic rats. As far as MLC is concerned, we show a lower expression of MLC 1 and MLC 3 isoforms in diabetic rats. Previous studies report that skeletal MLC, isoforms 1 and 3, are activated in the late embryonic stage and that this activation persists in the adult as predominant MLC isoforms (9). These two isoforms, 1 and 3, are called “essential” light chains because they possess a constitutive function. Thus impairment of MEF-1 binding activity compromises several steps involved in muscle protein synthesis.

We also show that in the gastrocnemius muscle of diabetic rats, prooxidant compounds are increased and the reduced-to-oxidized glutathione ratio is reduced, which suggest that the impairment of myogenic regulatory factors and myosin chains may be due to oxidative stress. It has been demonstrated that free radicals affect the expression of redox-sensitive genes involved in myogenic differentiation (38) and that \( \text{H}_2\text{O}_2 \) inhibits myogenesis in cell culture (39).

Moreover, antioxidants exert a beneficial effect in experimental models of chronic injury in diabetic animals (40,41). Here we show that, in diabetic rats treated with DHEA or vitamin E, oxidative balance is restored and muscle gene transcription improved. DHEA treatment prevents the reduction of antioxidant compounds and the increase of oxidant species induced in other tissues by chronic hyperglycemia (40). The finding that DHEA both preserves the key steps in muscle protein synthesis, which are compromised by chronic hyperglycemia, and improves the muscle repair process in a similar manner to vitamin E, the major chain-breaking antioxidant (42), strongly suggests that DHEA’s action mechanism involves its multitargeted antioxidant properties (39,43). However, we cannot exclude that, as has been reported for vitamin E (44), an additional nonantioxidant effect may be involved.

Whatever the effect of DHEA is due to DHEA itself, to its metabolites, or to a combination of both remains unclear. Some effects of DHEA might depend on estrogen. However, we found negligible variation of both 17β-estradiol and testosterone concentrations in rats treated with 4 mg DHEA (45). Nevertheless, we have shown elsewhere that DHEA, but not a variety of other steroids including androstenedione, 17β-estradiol, ADIOL, and dihydrotestosterone, protects bovine retinal capillary pericytes against glucose-induced lipid peroxidation (46). In conclusion, whatever the active molecule(s) and antioxidant and nonantioxidant mechanism(s) involved, our data clearly demonstrate that DHEA supplementation can protect the muscle tissue of STZ-induced diabetic rats from activation of a variety of target genes relevant to muscle protein synthesis and muscle repair. This protection has been confirmed in ZDF rats showing that the effect is not limited to the type 1 model of diabetes.

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