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Muscle wasting and dedifferentiation induced by oxidative stress in a murine model of cachexia is prevented by inhibitors of nitric oxide synthesis and antioxidants

Martina Buck and Mario Chojkier[1]

Department of Medicine, Veterans Affairs Medical Center and Center for Molecular Genetics, University of California, San Diego CA 92161, USA

[1]Corresponding author

In memory of Harold Weintraub, a leader in muscle biology

Muscle wasting is a critical feature of patients afflicted by AIDS or cancer. In a murine model of muscle wasting, tumor necrosis factor α (TNFα) induces oxidative stress and nitric oxide synthase (NOS) in skeletal muscle, leading to decreased myosin creatinine phosphokinase (MCK) expression and binding activities. The impaired MCK-E box binding activities resulted from abnormal myogenin–Jun-D complexes, and were normalized by the addition of Jun-D, dithiothreitol or Ref-1, a nuclear redox protein. Treatment of skeletal muscle cells with a phorbol ester, a superoxide-generating system, an NO donor or a Jun-D antisense oligonucleotide decreased Jun-D activity and transcription from the MCK-E box, which were prevented by antioxidants, a scavenger of reducing equivalents, a NOS inhibitor and/or overexpression of Jun-D. The decreased body weight, muscle wasting and skeletal muscle molecular abnormalities of cachexia were prevented by treatment of TNFα mice with the antioxidants D-α-tocopherol or BW755c, or the NOS inhibitor nitro-L-arginine.

Keywords: AIDS/cancer/Jun-D/myogenin/myosin creatine phosphokinase

Introduction

Cachexia is a frequent feature of patients afflicted with chronic diseases (for review, see Beutler, 1992; Tracey and Cerami, 1993), including AIDS, cancer and inflammatory disorders, such as arthritis and those affecting the intestine, kidney or lung (Voth *et al.*, 1990; Beutler, 1992; Tracey, 1992; Roubenoff *et al.*, 1994). The syndrome of cachexia is characterized by weight loss, decreased albumin synthesis, anemia, abnormal wound healing and impaired immunity (Tracey, 1992). In cachexia, muscle wasting accounts for the majority of weight loss, which may occur independently of the decreased food intake or malabsorption of nutrients (Tracey *et al.*, 1990; Spiegelman and Hotamisligil, 1993) that is sometimes associated with chronic diseases. Moreover, there is evidence to suggest that tumor necrosis factor α (TNFα), a product of monocytes and macrophages (Akira *et al.*, 1990), may mediate, perhaps in concert with other cytokines, the muscle wasting of cachexia (Beutler and Cerami, 1986; Fong *et al.*, 1989; Strassmann *et al.*, 1992; Spiegelman and Hotamisligil, 1993). Chronic increases in serum TNFα produced by tumor cells (Oliff *et al.*, 1987; Brenner *et al.*, 1990; Tracey *et al.*, 1990; Costelli *et al.*, 1993) or by expression of a TNFα transgene (Cheng *et al.*, 1992) can induce muscle wasting. Cachexia in tumor models can be ameliorated with antibodies against TNFα (Sherry *et al.*, 1989; Yoneda *et al.*, 1991; Costelli *et al.*, 1993). In addition, TNFα induces many other manifestations of cachexia (Tracey *et al.*, 1988; Brenner *et al.*, 1990; Yoneda *et al.*, 1991). However, in spite of the relevance of this issue to human diseases, the molecular mechanisms by which TNFα, or other mediators, induces muscle wasting remain to be determined (Beutler, 1992; Tracey and Cerami, 1993; Spiegelman and Hotamisligil, 1993). Therefore, in an attempt to elucidate this question, we evaluated the biological cascade leading to muscle wasting in a murine model of cachexia induced by chronically elevated serum TNFα (Oliff *et al.*, 1987; Brenner *et al.*, 1990; Tracey *et al.*, 1990).

Myosin is the most abundant functional protein of skeletal muscle and, in the presence of actin filaments, its ATPase activity is stimulated (Kirchberger, 1991). This process is essential for muscle contraction, since the energy required for this process is derived from ATP hydrolysis (Vale, 1994). In turn, the activity of myosin creatinine phosphokinase (MCK) is critical for differentiated skeletal muscle function, since it catalyzes the formation of ATP from phosphocreatine (Kirchberger, 1991). We found that in cachectic animals there is a decreased expression of both myosin and MCK. In addition, we found that myogenin–Jun-D complexes have a high binding affinity for the MCK enhancer, and that they comprise the vast majority of the MCK enhancer binding activities in nuclear extracts from normal skeletal muscle. These binding activities to the MCK-E box were disrupted in skeletal muscle of cachectic animals, due to the markedly decreased activity of Jun-D, and were normalized by the addition of recombinant Jun-D or recombinant Ref-1, a nuclear redox factor (Xanthoudakis *et al.*, 1992). TNFα induced oxidative stress and nitric oxide synthase (NOS) expression in skeletal muscle of cachectic animals, as described previously in other models (Wong *et al.*, 1989; Kilbourn *et al.*, 1990; Schulze-Osthoff *et al.*, 1993). These effects were also demonstrated in skeletal muscle cells treated with the phorbol ester 12-*o*-tetradecanoyl-phorbol-13-acetate (TPA), by the presence of malondialdehyde (MDA)–protein adducts, and the enhanced expression of NOS. Treatment of skeletal muscle cells with TPA, with xanthine–xanthine oxidase, a superoxide-generating system, or with 3-morpholinosyndnonomine (SIN-1), a NO donor (Lipton *et al.*, 1993), decreased Jun-D activity and transcription from the MCK-E box, which were prevented by antioxidants, NOS inhibitors and overexpression of Jun-D. Also, blocking Jun-D expression in skeletal muscle

cells with Jun-D antisense oligonucleotides inhibited transcription from the MCK-E box. Moreover, the decreased body weight, muscle wasting and skeletal muscle molecular abnormalities of cachexia were prevented by treatment of TNFα mice with the antioxidants D-α-tocopherol or BW755c, or the NOS inhibitor nitro-L-arginine. Our results provide insights into the molecular mechanisms responsible for the muscle wasting of cachexia and into potential therapeutic approaches for patients with chronic illnesses afflicted with this syndrome.

Results

Body weight loss of cachexia is prevented by antioxidants

Chinese hamster ovary (CHO) cells stably transfected with either a cytomegalovirus (CMV) vector expressing TNFα/*neo* (TNFα cells) or *neo* alone (CHO cells, control) (kindly provided by A.Oliff, Merk Sharp and Dohme Research Laboratories, West Point, PA; Oliff *et al.*, 1987), were used in our experiments as reported previously (Brenner *et al.*, 1990). The TNFα cells, but not the CHO cells, produced TNFα as measured by biological cytolytic or enzyme-linked immunosorbent assays (ELISA) (data not shown). Four-week-old male athymic nude mice were injected intramuscularly with either 3.5×10^6 CHO cells or 3.5×10^6 TNFα cells. The TNFα and CHO mice maintained essentially the same weight for the first 2–3 weeks after inoculation. After this period, TNFα animals began to develop symptoms of cachexia, including decreased weight (Figure 1A) and muscle wasting (Figure 1B). Other typical features of cachexia present in these animals include decreased albumin gene expression (Brenner *et al.*, 1990), anemia and abnormal wound healing (M.Buck *et al.*, unpublished observations). The decreased weight was not accompanied by changes in the percentage of total body water of cachectic animals when compared with control animals (74 ± 1% versus 73 ± 2%; P = NS), indicating that dehydration was not a confounding variable. Moreover, the amount of food intake, determined by using metabolic cages, was not decreased in TNFα mice immediately before the onset of weight loss (data not shown), in agreement with the report that anorexia is not a feature of this animal model (Tracey *et al.*, 1990). Because TNFα may initiate a cascade leading to oxidative stress (Meier *et al.*, 1989; Wong *et al.*, 1989; Henkel *et al.*, 1993; Schulze-Osthoff *et al.*, 1993), we assessed whether aldehyde products of lipid peroxidation, such as MDA (Schauenstein *et al.*, 1977; Chaudhary *et al.*, 1994), were present in tissues of cachectic mice. Using specific antibodies against MDA–lysine adducts (Houglum *et al.*, 1990), we found a marked increase in MDA–protein adducts in skeletal muscle of TNFα mice, while skeletal muscle of control mice had negligible levels (Figure 2). Because of this evidence, supporting an oxidative stress pathway in skeletal muscle of cachectic animals, and because aldehydes may form adducts with proteins critical for differentiated function, we treated these animals with antioxidants in an attempt to prevent muscle wasting. Interestingly, when TNFα animals received a diet supplemented with D-α-tocopherol (8 IU/g of foodstuff), a lipophilic antioxidant, the increased oxidative stress in skeletal muscle was abolished, judging by the absence of

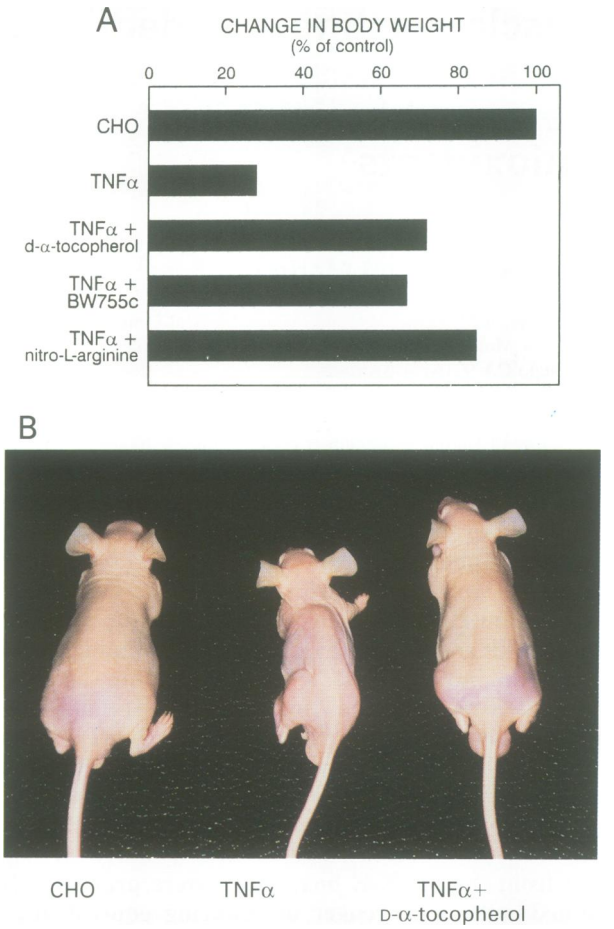

Fig. 1. Muscle wasting of cachexia is prevented by antioxidants and nitro-L-arginine. (**A**) Change in body weight. Athymic nude male mice were injected intramuscularly with either CHO cells (CHO, control group; n = 6) or TNFα-secreting cells as described in Materials and methods. Some of the TNFα mice received no treatment (TNFα group; n = 6), a diet supplemented with D-α-tocopherol (n = 6) or were treated with either BW755c (n =4) or nitro-L-arginine (n = 6) as described in Materials and methods. Values are percentages of change in body weight during the 30 day experiment, with the value for CHO animals set at 100. $P<0.05$ for all treatments compared with TNFα mice. (**B**) Representative examples of CHO, TNFα and TNFα + D-α-tocopherol groups. TNFα mice treated with either BW755c or nitro-L-arginine had an appearance similar to the TNFα + D-α-tocopherol mouse shown.

MDA–protein adducts (Figure 2), a sensitive indicator of lipid peroxidation (Houglum *et al.*, 1990). More importantly, D-α-tocopherol markedly prevented the weight loss and the muscle wasting characteristic of these animals (Figure 1A and B). As expected, the non-lipophilic antioxidant, 3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline hydrochloride (BW755c) (kindly provided by S.Moncada, Burrough Wellcome Research Laboratories, Kent, UK), did not prevent the generation of MDA–protein adducts (data not shown), since it should not affect the peroxidation of polyunsaturated fatty acids in membranes. However, BW755c (10 mg/kg twice a day orally) prevented the weight loss and muscle wasting of cachexia (Figure 1A), indicating that the oxidative stress cascade initiated by TNFα can be blocked at different levels. A similar decrease in body weight induced by the TNFα cells in 5-week-old athymic mice was also prevented by the antioxidants (data not shown). On the other hand,

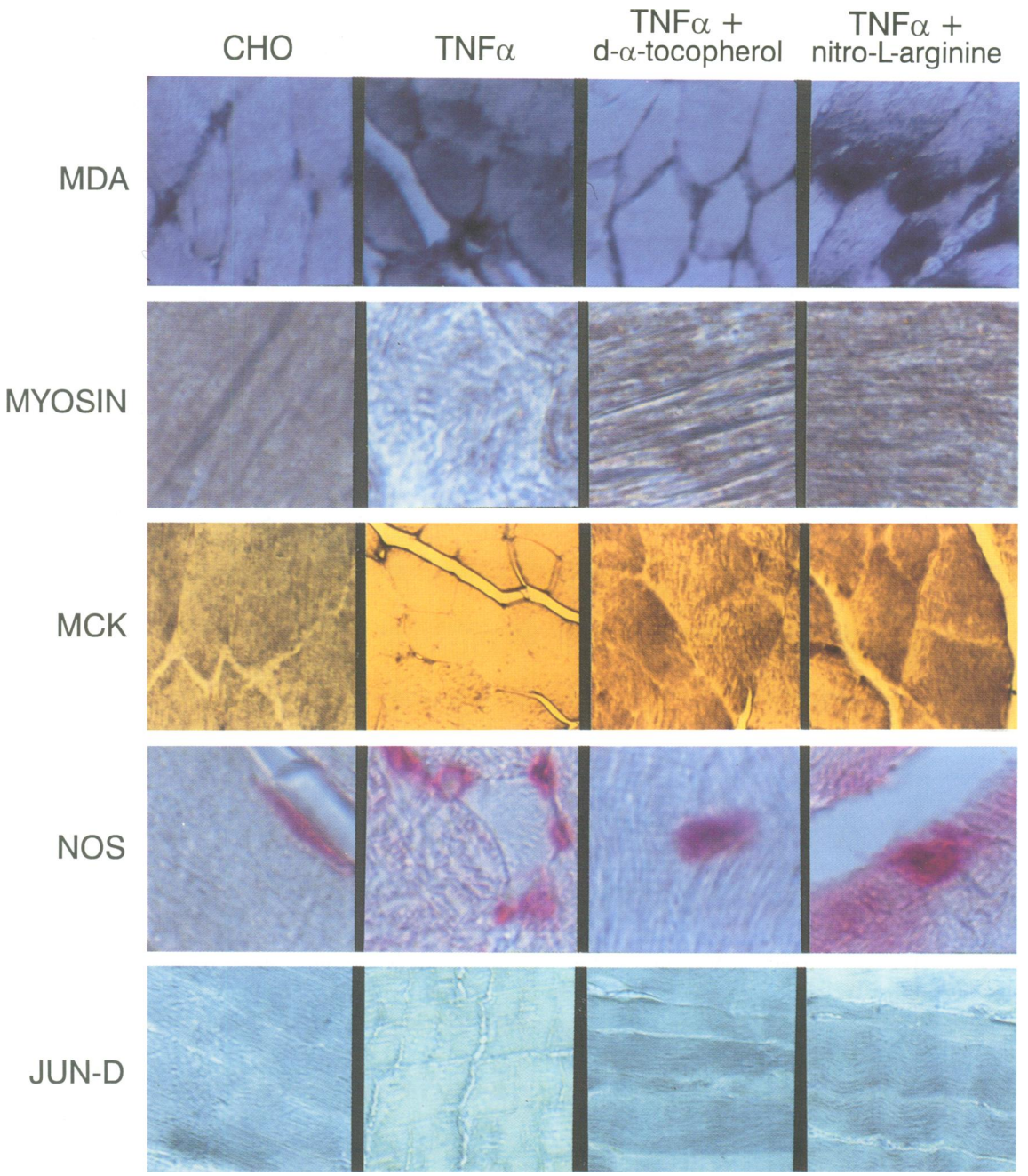

Fig. 2. Oxidative stress induces dedifferentiation and NOS expression in the skeletal muscle of cachectic mice. The experimental groups are as described in Figure 1. Immunohistochemistry for MDA–protein adducts, myosin, MCK, NOS and Jun-D were performed as described in Materials and methods. Negligible staining was observed in all immunohistochemistries when the first antibody was omitted.

indomethacin (0.5 mg/kg per day orally), an inhibitor of cycloxygenase, an enzyme activated by TNFα (Dinarello *et al.*, 1986), had no beneficial effect when given to the TNFα animals (data not shown).

The consistent effect of antioxidants on the prevention of weight loss in cachectic animals was also noticeable in the adipose tissue. Fat deposits were depleted in cachectic animals, and this effect was prevented when these animals were treated with D-α-tocopherol or BW755c (data not shown).

Antioxidants do not affect TNFα production

In order to characterize the mechanisms involved in the prevention of cachexia, we analyzed the level at which the antioxidants block the TNFα induction of weight loss and muscle wasting. Several lines of evidence support the notion that an oxidative pathway, targeted by both D-α-tocopherol and BW755c, responsible for the weight loss and muscle wasting in cachectic animals is a TNFα receptor and/or post-receptor event. First, we studied whether the antioxidants D-α-tocopherol and BW755c could affect the synthesis or secretion of TNFα. TNFα cells were incubated in the presence or absence of either D-α-tocopherol (50 μM) or BW755c (10 μM) for up to 72 h. No cellular toxicity was observed, in agreement with a previous report using these compounds in cultured human fibroblasts (Houglum *et al.*, 1991), and the concentration and biological activity of the secreted TNFα was

Table I. Antioxidants or nitro-L-arginine do not affect TNFα production by TNFα cells

	TNFα concentration (ng/ml)
Cells	
TNFα	70 ± 1×10^{-3}
TNFα + D-α-tocopherol	70 ± 1×10^{-3}
TNFα + BW755	60 ± 1×10^{-3}
Mice	
TNFα	3.4 ± 1.2
TNFα + D-α-tocopherol	3.0 ± 1.2
TNFα + BW755	5.0 ± 0.5
TNFα + nitro-L-arginine	2.8 ± 1.3

TNFα-secreting cells and TNFα mice were treated with D-α-tocopherol, BW755c or nitro-L-arginine, and the TNFα concentration was determined in cell media or in mouse sera by ELISA, as described in Materials and methods. P = NS for any treatment.

Table II. Antioxidants or nitro-L-arginine do not prevent anemia or impaired collagen gene expression in TNFα mice

Animals	Hematocrit (%)	Skin collagen α1(I) mRNA (% of control)
CHO	43 ± 1	100
TNFα	16 ± 1	34 ± 10
TNFα + D-α-tocopherol	18 ± 4	49 ± 15
TNFα + BW755	19 ± 3	n.t.
TNFα + nitro-L-arginine	17 ± 3	35 ± 11

Animals were treated as described in Materials and methods. Thirty days after inoculation of CHO or TNFα cells, the blood hematocrit and collagen α1(I) mRNA in the skin were determined as described in Materials and methods. $P<0.05$ for all experimental groups compared with the CHO group.

essentially identical in cell cultures treated or untreated with antioxidants (Table I). In addition, there was no discernable difference in the size of the tumors excised from TNFα mice treated or untreated with antioxidants (data not shown). More importantly, the levels of serum TNFα, although variable, were similar in TNFα mice treated with either D-α-tocopherol or BW755c (Table I). Because of the potential influence of the variable serum levels of TNFα on the biological parameters, samples from animals inoculated with TNFα cells were matched for comparable serum TNFα levels.

Finally, a more definitive proof of a similar biological activity of TNFα in TNFα mice treated or untreated with antioxidants is the presence of unaltered end-organ biological effects. Indeed, we found that two important and early components of the cachexia syndrome, anemia and decreased collagen α1(I) gene expression in the skin (Table II), which contributes to the impaired wound healing (M.Buck *et al.*, in preparation), remained abnormal in the TNFα mice treated with D-α-tocopherol or BW755c. These observations indicate that anemia and impaired skin collagen gene expression (and presumably wound healing) are affected by cellular pathways initiated by TNFα in cachectic animals, which are distinct from the one leading to body weight loss and muscle wasting. On the other hand, we found that decreased albumin synthesis in the TNFα mice, which occurs independently of weight loss, and results from decreased albumin gene transcription (Brenner *et al.*, 1990), can also be prevented by antioxidants (M.Buck *et al.*, unpublished observations).

Expression of myosin and MCK is decreased in cachectic mice

Because myosin is a major skeletal muscle structural protein (Kirchberger, 1991), we studied its expression in cachexia. Myosin expression was decreased markedly in skeletal muscle of TNFα mice when compared with control. In addition, and in contrast to the normal muscular fibril arrangement, the structure of skeletal muscle from cachectic animals was quite irregular, consisting of multiple smaller fibers (Figure 2). Interestingly, treatment with D-α-tocopherol (Figure 2) or BW755c (data not shown) prevented the depletion of myosin and markedly improved the structure of skeletal muscle from TNFα mice.

Given that MCK is a critical modulator of skeletal muscle structure and function (Kirchberger, 1991), we tested whether its expression was affected in cachectic animals. Expression of MCK protein was decreased dramatically in the muscles of cachectic animals when compared with control animals (Figure 2), but was normalized when TNFα mice were treated with D-α-tocopherol (Figure 2) or BW755c.

Because increased muscle proteolysis is induced by TNFα (Flores *et al.*, 1989; Costelli *et al.*, 1993) and may also be an important mechanism in muscle wasting during fasting (Wing and Goldberg, 1993), we analyzed whether decreased expression of the MCK gene occurred in cachectic animals. The steady-state level of MCK mRNA, as detected by *in situ* hybridization with a specific riboprobe, was inhibited markedly in the skeletal muscle of TNFα animals (Figure 3), but this change was prevented by D-α-tocopherol (Figure 3) and BW755c. No hybridization signal was detected when samples were treated with RNase, indicating that the signal was specific. These results suggest that decreased synthesis of MCK contributes to muscle dedifferentiation in cachexia.

MCK enhancer binding activities of skeletal muscle nuclei are decreased in cachectic animals

The E box, a conserved regulatory DNA region, is critical for the expression of most skeletal muscle-specific genes (Lassar *et al.*, 1989; Brennan and Olson, 1990; Weintraub *et al.*, 1990). Therefore, its binding and transcriptional activities have been used as indicators of muscle-specific differentiation (Li *et al.*, 1992b; Tapscott *et al.*, 1993; Kaushal *et al.*, 1994; Skapek *et al.*, 1995). We analyzed the binding affinity of the MCK-E box for skeletal muscle nuclear proteins from the various experimental groups. Nuclear extracts were obtained in 1% citric acid in the presence of antioxidants and protease and phosphatase inhibitors, as reported previously (Descombes *et al.*, 1990; Trautwein *et al.*, 1993; Buck *et al.*, 1994). Nuclear extracts from cachectic animals displayed a substantial decrease in DNA binding affinity for the MCK-E box (Figure 4A, lane 3) when compared with those from control animals (lane 2), and this abnormality was almost completely corrected in TNFα mice treated with either D-α-tocopherol or BW755c (lanes 4 and 5).

To determine whether the presence of an inhibitory factor, or the absence of an activity required for the normal

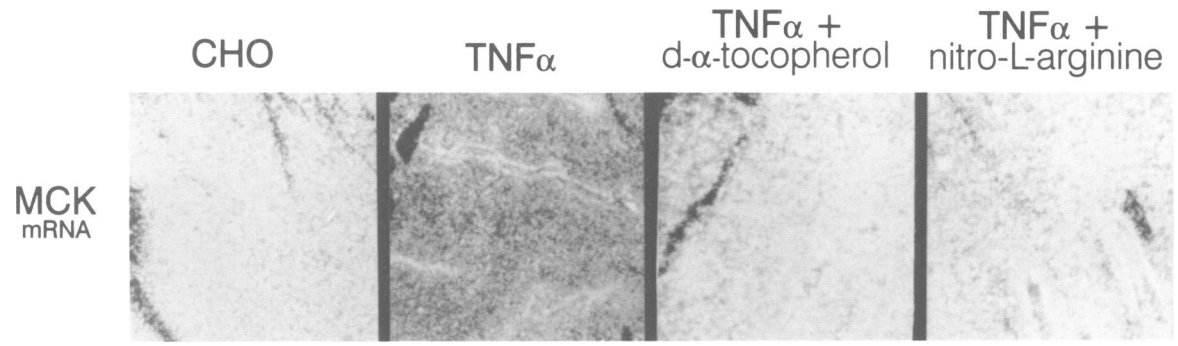

Fig. 3. Inhibition of MCK expression in skeletal muscle of cachectic mice. Representative examples of MCK mRNA detection by *in situ* hybridization with a specific riboprobe. The decrease in MCK mRNA in skeletal muscle from TNFα mice was prevented by D-α-tocopherol and nitro-L-arginine treatments. No hybridization signal was detected when samples were pre-treated with RNase.

interaction of nuclear extracts with the MCK-E box, is responsible for the abnormal DNA binding of TNFα nuclear extracts, we performed gel retardation assays mixing nuclear extracts from control and TNFα animals. The addition of nuclear extracts from TNFα animals did not inhibit the binding of control nuclear proteins to the MCK-E box (Figure 4B, lane 2), indicating that the lack of an activity, which contributes significantly towards this DNA–protein interaction, rather than the presence of an inhibitory factor, is responsible for the abnormalities observed in the nuclei of skeletal muscle from TNFα mice (Figure 4A).

In order to understand the mechanisms responsible for the deficient MCK binding activities of skeletal muscle from TNFα mice, we characterized the proteins responsible for the binding to the MCK-E box, in nuclear extracts from control animals. Incubation of nuclear extracts from control animals with a monoclonal antibody (mAb) against the critical muscle-specific transcriptional activator, myogenin (Sassoon *et al.*, 1989; Hasty *et al.*, 1993; Nabeshima *et al.*, 1993), disrupted the normal protein–DNA complex (Figure 4C, lane 2). The specificity of the protein–DNA complex was ascertained by competition with excess unlabeled MCK-E box oligonucleotide (lane 4). Because of these results, we examined whether or not the expression of myogenin was altered in TNFα mice. The immunoblot of nuclear extracts showed that myogenin was normally expressed in the skeletal muscle of cachectic animals (Figure 5A, lane 2), compared with control (lane 1).

Because phosphorylation of myogenin on Thr87, within the DNA binding domain, such as that induced by basic fibroblast growth factor, has an inhibitory effect on the binding to, and the transcription from, the MCK-E box (Li *et al.*, 1992a), we treated nuclear extracts from TNFα mice with phosphatase in an attempt potentially to dephosphorylate putative phosphoproteins and eventually reconstitute their activity, as described previously (Trautwein *et al.*, 1994). However, no changes were observed after the phosphatase treatment (data not shown), suggesting that phosphorylation of myogenin (or of another critical MCK binding protein) is not the mechanism responsible for the decreased binding affinity of skeletal muscle proteins from the TNFα mice for the MCK-E box. Furthermore, phosphatase treatment improved the binding of liver nuclear proteins from the same animals to the albumin promoter D-site (M.Buck *et al.*, in preparation), indicating the reliability of the method.

Jun-D binding to MCK enhancer is inhibited in skeletal muscle of cachectic animals

Because TNFα, like phorbol esters, can induce c-Jun expression and activity (Brenner *et al.*, 1989), and because c-Jun interacts physically with Myo-D (Bengal *et al.*, 1992) and may repress the transcription activity of myogenin (Li *et al.*, 1992b), we assessed c-Jun binding activity in nuclear extracts of skeletal muscle. However, AP-1 binding activities were not increased in skeletal muscle nuclear extract from cachectic animals (data not shown). Moreover, AP-1 binding activities of normal skeletal muscle are not related to c-Jun or Jun-B, since antibodies against these factors did not affect the DNA–protein complex. In addition, the migration pattern of the complex did not correspond to that of c-Jun or c-Jun–Fos dimers. The AP-1 binding activities of normal skeletal muscle were disrupted almost completely with Jun-D antibodies (data not shown), in agreement with the effect of AP-1 oligonucleotides on the Jun-D–myogenin–MCK-E box complex (see below).

Given that Jun-D may act synergistically with myogenin to enhance transcription from MCK-E box chimeric reporter genes in C2C12 muscle cells (Li *et al.*, 1992b), and that Jun-D is present constitutively in myoblasts (Li *et al.*, 1990), we tested the potential role of Jun-D in the MCK-E box binding activities of skeletal muscle nuclear extracts from control animals. First, we found that an AP-1 oligonucleotide competed for the binding of the nuclear proteins (Figure 4C, lane 5), suggesting that a protein(s) that binds to the AP-1 site also forms a complex with myogenin and the MCK-E box. Specific antibodies against either c-Jun (Figure 4C, lane 3) or Jun-B (data not shown), two AP-1 binding proteins (Nakabeppu *et al.*, 1988; Ryseck and Bravo, 1991), did not affect the protein–DNA complex, suggesting that neither c-Jun nor Jun-B participates in the myogenin–MCK-E box complex. However, antibodies against Jun-D, another AP-1 binding protein (Nakabeppu *et al.*, 1988; Ryseck and Bravo, 1991), markedly disrupted the binding to the MCK-E box oligonucleotide of skeletal muscle nuclear proteins from the CHO–TNFα mix (Figure 4B, lane 4), and from TNFα mice treated with D-α-tocopherol (Figure 4D, lanes 3 and 4), indicating that Jun-D interacts with myogenin and/or the MCK-E box complex. Treatment of TNFα mice with D-α-tocopherol or BW755c normalized the affinity of nuclear proteins for the MCK-E box (Figure 4A, lanes 4 and 5). This included the participation of both myogenin

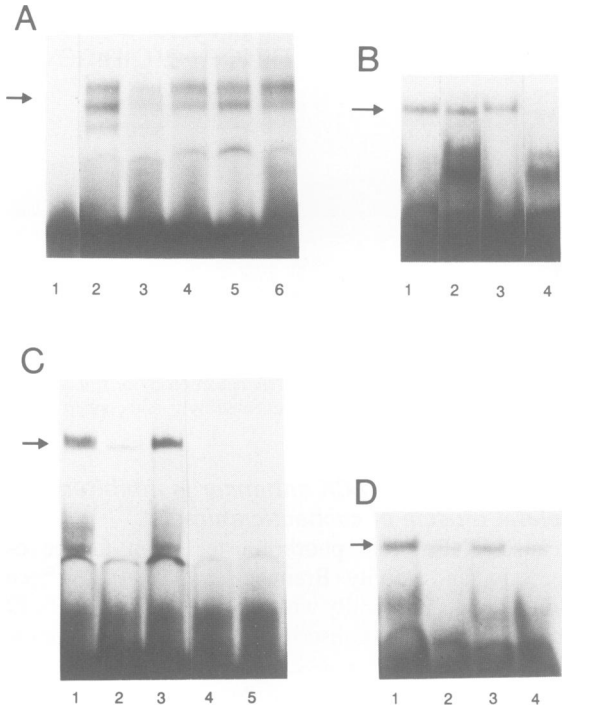

Fig. 4. MCK-E box binding activities are decreased in skeletal muscle from cachectic animals. Mobility shift analysis of skeletal muscle nuclear extracts. Equal amounts of nuclear protein were incubated with ^{32}P-labeled MCK-E box oligonucleotide (1 ng). The DNA–protein complexes were resolved by electrophoresis on a 6% non-denaturing polyacrylamide gel. The position of the bound DNA is indicated by arrows. Some samples were incubated with specific antibodies or unlabeled oligonucleotide as indicated by + signs. The lanes are shown below in parenthesis. (**A**) Representative samples of CHO (2); TNFα (3); TNFα/D-α-tocopherol (4); TNFα/BW755c (5); and TNFα/nitro-L-arginine (6). In lane 1, the probe was processed without nuclear extracts. (**B**) Representative samples of CHO (1); CHO/TNFα mix (2); CHO/TNFα mix + myogenin antibodies (3); and CHO/TNFα mix + myogenin and Jun-D antibodies (4). (**C**) Representative samples of CHO (1); CHO + myogenin antibodies (2); CHO + c-Jun antibodies (3); CHO + MCK-E box oligonucleotide (4); and CHO + AP-1 oligonucleotide (5). (**D**) Representative samples of TNFα/D-α-tocopherol without additions (1); or with the addition of myogenin antibodies (2); Jun-D antibodies (3); or myogenin and Jun-D antibodies (4).

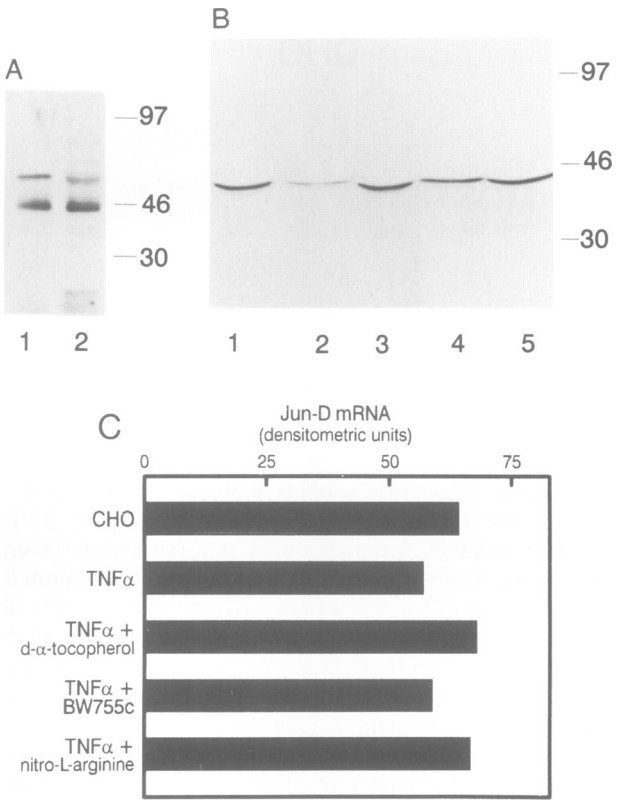

Fig. 5. Expression of myogenin and Jun-D in skeletal muscle of cachectic mice. (**A**) Western blot was performed as described in Materials and methods using mAbs against myogenin in extracts of skeletal muscle from CHO (lane 1) and TNFα (lane 2) mice. The mobility of molecular weight standards is shown. (**B**) Western blot using antibodies against Jun-D (amino acids 327–341) in extracts of skeletal muscle from CHO (lane 1); TNFα (lane 2); TNFα/D-α-tocopherol (lane 3); TNFα/BW755c (lane 4); and TNFα/nitro-L-arginine (lane 5) mice. (**C**) Analysis of Jun-D mRNA by dot-blot hybridization with a specific riboprobe as described in Materials and methods for samples shown in Figure 1A. The amount of RNA was determined by 18S RNA; P = NS for any experimental condition.

and Jun-D in the DNA–protein complex, to levels comparable with that of control animals, judging by the disruption of the complex with antibodies against each or both of these transcription factors (Figure 4D, lanes 2–4).

An additional, more rapidly migrating protein–DNA complex was formed when nuclear extracts from TNFα mice were added to control nuclear extracts (Figure 4B, lane 2). The migration of this complex corresponded to the migration of the DNA–recombinant myogenin complex (data not shown), and it was disrupted by antibodies against myogenin (lane 3), indicating that myogenin, in excess over Jun-D activity, formed this additional complex. This complex reappeared when Jun-D antibodies were further added to the reaction (lane 4), also suggesting an interaction between myogenin, Jun-D and the MCK-E box.

If the inability of skeletal muscle extracts from cachectic animals to bind to the MCK-E box is due mainly to the decreased Jun-D activity, as suggested by the above experiments, the normal protein–DNA complex should be reconstituted with recombinant Jun-D. Therefore, bacterially expressed recombinant Jun-D was purified by affinity chromatography, and added to skeletal muscle nuclear extracts from cachectic animals, in amounts comparable with that present in skeletal nuclei from control animals as determined by immunoblots. The addition of Jun-D normalized the nuclear extract–MCK-E box complex (Figure 6A, lane 2), which now included not only Jun-D (lane 4), but also myogenin, as indicated by the disruption of the shift induced by mAbs against myogenin (lane 3). Collectively, these data indicate that Jun-D is indispensable for the MCK binding activities of control skeletal muscle nuclei, and that Jun-D is sufficient to normalize the decreased MCK binding activities of skeletal muscle from cachectic animals. Jun-D interacts not only functionally with myogenin (Li *et al.*, 1992b), but also physically, given the reincorporation of myogenin to the MCK–protein complex of skeletal muscle from cachectic mice in the presence of Jun-D.

In agreement with the gel retardation analysis, immunohistochemistry (Figure 2) and Western blots (Figure 5B) for Jun-D, using specific antibodies that do not react with Jun-B or c-Jun, demonstrated a decrease in the expression of this transcription factor in the skeletal muscle of TNFα mice when compared with control animals, and restoration

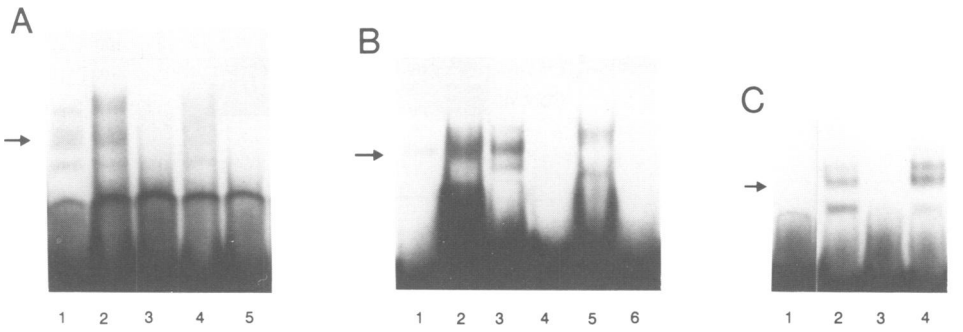

Fig. 6. Jun-D and Ref-1 normalize the MCK-E box binding activities of skeletal muscle from cachectic animals. Mobility shift analysis of skeletal muscle nuclear extracts. Samples were incubated with ^{32}P-labeled MCK-E box oligonucleotide, and processed as described in Figure 4. The position of the bound DNA is indicated by arrows. Some samples were incubated with specific antibodies, recombinant (r) protein or DTT as indicated by the + signs. (**A**) Representative samples of TNFα without additions (lane 1); or with addition of r Jun-D (lane 2); r Jun-D and myogenin antibodies (lane 3); r Jun-D and Jun-D antibodies (lane 4); and r Jun-D, myogenin and Jun-D antibodies (lane 5). (**B**) Representative samples of TNFα without additions (lane 1); or with the addition of DTT (lane 2); r Ref-1 (lane 3); DTT, myogenin and Jun-D antibodies (lane 4); r Jun-D (lane 5); and oxidized r Jun-D (lane 6). (**C**) Representative samples of human skeletal muscle cells untreated (lane 2); cultured with either Jun-D antisense oligonucleotide (lane 3); or Jun-D sense oligonucleotide (lane 4). In lane 1, the probe was processed without nuclear extracts.

of its expression in TNFα mice treated with D-α-tocopherol or BW755c (Figures 2 and 5B). To ascertain the mechanisms by which Jun-D protein expression is down-regulated in the skeletal muscle of cachectic animals, we analyzed the steady-state levels of Jun-D mRNA. No differences in Jun-D mRNA were found between control and experimental groups (Figure 5C), indicating a translational or post-translational regulation of Jun-D (see below). The modulation of the AP-1 proteins (Jun/Fos) involving reduction–oxidation (Abate *et al.*, 1990), through a highly conserved cysteine domain (KCR) in the DNA binding region of AP-1 and related factors (Xanthoudakis *et al.*, 1992), is mediated by the nuclear redox factor Ref-1 (Xanthoudakis and Curran, 1992). Therefore, we determined whether Ref-1 would normalize the binding of skeletal muscle nuclear extracts from TNFα mice to the MCK-E box. The addition of either purified recombinant Ref-1 or dithiothreitol (DTT) as described (Xanthoudakis *et al.*, 1992) (Figure 6B, lanes 2 and 3) normalized the DNA–protein complex, suggesting that the critical mechanism involves oxidation of a transcription factor, presumably the KCR domain of Jun-D. Moreover, the normalization of this MCK-E box nuclear protein complex with DTT was abolished by incubation with antibodies against myogenin and Jun-D (Figure 6B, lane 4). In addition, the normalization of the DNA–protein complex occurred with unoxidized recombinant Jun-D (Figure 6B, lane 5), but not with oxidized Jun-D (Figure 6B, lane 6).

Jun-D expression modulates MCK binding and transcription activities in skeletal muscle cells

Human skeletal muscle cells cultured in a defined medium without serum exhibited a differentiated phenotype, and nuclei from these cells had a high binding affinity for the MCK-E box (Figure 6C, lane 2). As expected, treatment of normal skeletal muscle cells with a Jun-D antisense oligonucleotide but not with a sense oligonucleotide blocked the expression of Jun-D (data not shown). This specific inhibition of Jun-D expression with a Jun-D antisense oligonucleotide was sufficient to disrupt the binding of nuclear proteins to the MCK-E box (Figure 6C, lane 3), emphasizing the critical role of Jun-D in skeletal muscle differentiated functions. The Jun-D sense oligonucleotide had no effect on the MCK-E box–nuclear extract complex (lane 4).

Because mouse skeletal muscle C2C12 cells under specific culture conditions also exhibit a differentiated phenotype (Brennan and Olson, 1990; Li *et al.*, 1992a) but, unlike human skeletal muscle cells (unpublished observations), they are transfected readily (Li *et al.*, 1992a), we performed additional experiments with these cells. C2C12 cells were refractory to treatment with recombinant TNFα, as reported previously for isolated murine skeletal muscle (Moldawer *et al.*, 1987), suggesting that the effects of TNFα on skeletal muscle of cachectic mice may require induction of a cascade which is not available in cultured skeletal cells. Given that many of the effects of TNFα, including oxidative stress, could be mimicked by phorbol esters (Brenner *et al.*, 1989; Schreck *et al.*, 1991; Henkel *et al.*, 1993; Traenckner *et al.*, 1994), we treated C2C12 cells with TPA. In these cells, TPA (60 ng/ml) induced oxidative stress judging by the presence of MDA–protein adducts (Figure 7A), mimicking the findings in skeletal muscle of TNFα mice. Moreover, treatment of C2C12 cells with either TPA or with xanthine (4 μM)–xanthine oxidase (1 mU/ml), a superoxide-generating system (McWhirter and Hille, 1991), inhibited transcription from a chimeric reporter gene containing a multimerized MCK-E box element (4RTK-CAT) (Weintraub *et al.*, 1990; Brennan and Olson, 1990; Tapscott *et al.*, 1993) (Figure 7B). However, Jun-D expression was unchanged in cells treated with TPA (Figure 7C), or in any of the experimental conditions depicted in Figure 7B, suggesting that a post-translational modification of Jun-D induced by oxidative stress was a likely mechanism. Therefore, we tested whether Ref-1 would rescue the impaired MCK-E box–nuclear protein complex from skeletal muscle cells treated with TPA. Similar to the finding in the skeletal muscles from cachectic mice, both Ref-1 and DTT (data not shown) normalized the MCK-E box binding activities of nuclear extracts, indicating that an oxidative mechanism may be responsible for the observed abnormality. In addition, we used BW755c, an antioxidant, and methylene blue, a scavenger of reducing equivalents which are needed for the formation and stability of aldehyde–protein adducts (Brenner and

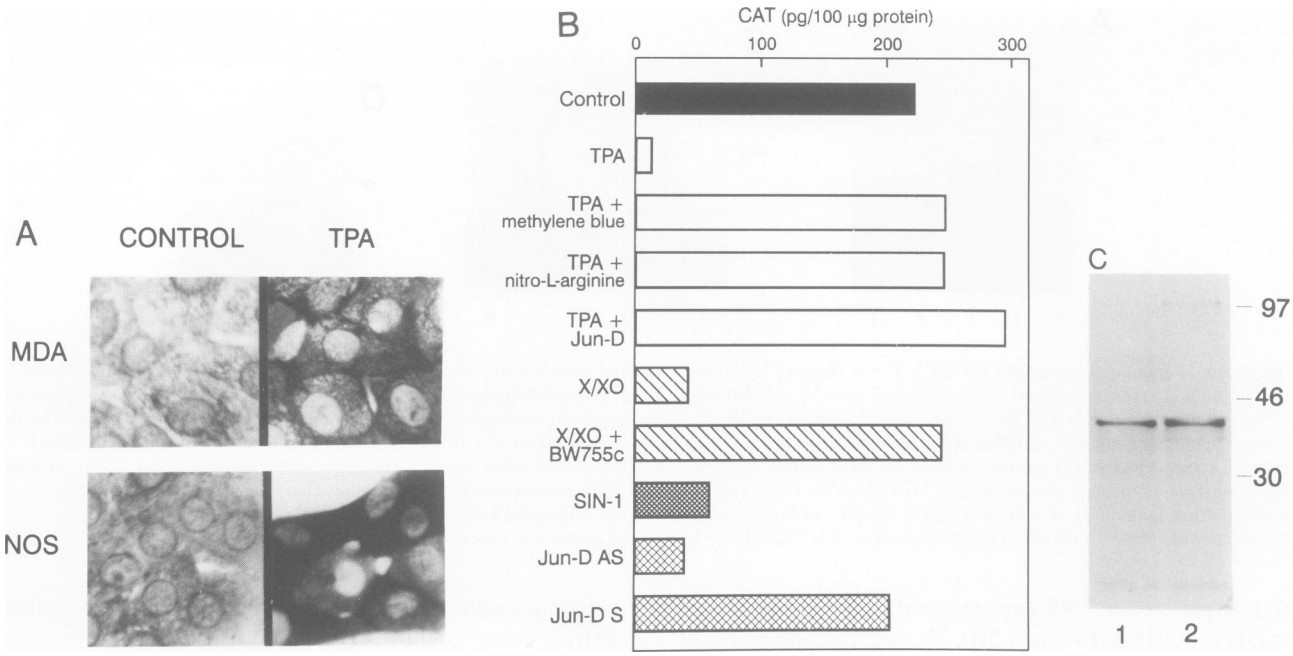

Fig. 7. Oxidative stress inhibits transcription from the MCK-E box in mouse skeletal muscle cells. Mouse skeletal muscle C2C12 cells were cultured and treated as described in Materials and methods. (**A**) Immunohistochemistry for MDA–protein adducts and NOS was performed for control and TPA-treated C2C12 cells. (**B**) C2C12 cells were transfected with 4RTK-CAT alone or with RSV-Jun-D and treated with TPA; methylene blue; nitro-L-arginine; xanthine (X)–xanthine oxidase (XO); BW755c; SIN-1; Jun-D antisense oligonucleotide (AS); and Jun-D sense oligonucleotide (S), as indicated. The cells were collected 48 h later and CAT activity was determined. The results represent averages of three independent experiments. $P<0.05$ for TPA, X/XO, SIN-1 and Jun-D antisense oligonucleotide. (**C**) Western blot was performed using antibodies against Jun-D for control (lane 1) and TPA-treated (lane 2) C2C12 cells.

Chojkier, 1987; Chojkier *et al.*, 1989), to assess whether the inhibition of MCK-E box transcription activities by oxidative stress utilizes these pathways. As depicted in Figure 7B, BW755c (100 μM) and methylene blue (10 μM) normalized the decreased transcription from 4RTK-CAT, induced by TPA or xanthine–xanthine oxidase. As expected, treatment of C2C12 skeletal muscle cells with a Jun-D antisense oligonucleotide but not with a sense oligonucleotide was sufficient to disrupt the transcription from 4RTK-CAT (Figure 7B), emphasizing the critical role of Jun-D in skeletal muscle differentiated functions. Moreover, overexpression of Jun-D in TPA-treated cells was sufficient to normalize the dramatic inhibition of transcription from 4RTK-CAT (Figure 7B).

NOS activity mediates effects of oxidative stress on muscle dedifferentiation and muscle wasting

Because oxidative stress pathways are known to interact with NO to modulate cytoprotective or cytotoxic effects (Lipton *et al.*, 1993; Stalmer, 1994), and NO itself plays a role in muscle function (Kobzik *et al.*, 1994), we analyzed this pathway with regard to muscle dedifferentiation and muscle wasting. In mouse skeletal muscle C2C12 cells, the decrease in MCK-E box binding activities induced by oxidative stress was associated with a higher expression of NOS (Figure 7A). Therefore, we investigated whether N^5-[nitroamidino]-L-2,5-diaminopentanoic acid (nitro-L-arginine) (500 μM), a potent inhibitor of NOS (Kobzik *et al.*, 1994), would prevent the effects of TPA in these cells. As shown in Figure 7B, the inhibitor of NOS normalized the decreased transcription from 4RTK-CAT. Additional support for a role for NO was obtained by treating the skeletal muscle cells with SIN-1, a source of NO (Lipton *et al.*, 1993). SIN-1 (0.6 μM) treatment, similarly to oxidative stress, markedly decreased 4RTK-CAT transcription in skeletal muscle cells (Figure 7B).

Because of the strong suggestion, derived from the experiments with C2C12 cells, that the NO pathway may mediate (or act synergistically with) the effects of oxidative stress on skeletal muscle, we studied these interactions in cachectic animals. As shown in Figure 2, the expression of NOS was negligible in the skeletal muscle of control animals, but was increased substantially in TNFα mice. Interestingly, this increased expression of NOS was prevented in TNFα mice treated with the antioxidants, D-α-tocopherol (Figure 2) or BW755c. These results strongly suggest that oxidative stress induces NOS expression in the skeletal muscle of TNFα mice, in agreement with the enhanced NOS expression observed in skeletal muscle cells treated with TPA (Figure 7A).

In subsequent experiments, we treated TNFα mice with the NOS inhibitor, nitro-L-arginine, and analyzed its effects on muscle wasting and dedifferentiation. Addition of nitro-L-arginine to the drinking water (50 μg/ml) prevented the weight loss and muscle wasting of the TNFα animals to a considerable extent (Figure 1A). Moreover, treatment with the NOS inhibitor also prevented the muscle abnormalities characteristic of the TNFα mice including: (i) decrease in myosin expression (Figure 2); (ii) decrease in MCK expression (Figures 2 and 3); (iii) decrease in MCK-E box binding affinity (Figure 4A, lane 6) and (iv) decreased expression of Jun-D (Figures 2 and 5B, lane 5). As expected, nitro-L-arginine did not affect the induction of either MDA–protein adducts (Figure 2) or NOS (Figure 2), upstream components of the cascade leading to the synthesis of NO, in the skeletal muscle of

TNFα mice. Similarly to the observations made in TNFα mice treated with antioxidants (see above), nitro-L-arginine treatment did not affect the tumor size, the production of TNFα, or some end-organ biological effects of TNFα, such as anemia or inhibition of collagen gene α1(I) expression in the skin (Table II).

Discussion

Because the animal model of cachexia developed by Oliff and co-workers (Oliff *et al.*, 1987) closely recapitulates the main features observed in patients with this syndrome, i.e. muscle wasting, decreased albumin synthesis, anemia and impaired wound healing (Oliff *et al.*, 1987; Brenner *et al.*, 1990; Tracey *et al.*, 1990; M.Buck *et al.*, unpublished observations), it provides a valuable system to analyze the molecular mechanisms responsible for these abnormalities. At the onset of weight loss, TNFα serum levels were only moderately increased (100–300 pg/ml) as described previously (Brenner *et al.*, 1990). At the time of sacrifice, the TNFα mice had high serum TNFα levels; however, similar values have been found in patients with trauma or infectious, parasitic and neoplastic diseases (Scuderi *et al.*, 1986; Waage *et al.*, 1987; Grau *et al.*, 1989; Goodman *et al.*, 1990). Although a hypercatabolic state has been suggested as a possible explanation for the weight loss in cachexia (Starnes *et al.*, 1988; Costelli *et al.*, 1993; Roubenoff *et al.*, 1994), the physiological and molecular disturbances leading to this syndrome have remained elusive (Beutler and Cerami, 1986; Spiegelman and Hotamisligil, 1993; Tracey and Cerami, 1993).

Here, we have demonstrated that the antioxidants, D-α-tocopherol and BW755c, and the NOS inhibitor nitro-L-arginine prevented the weight loss and the muscle wasting in this animal model of cachexia (Figure 1). Similarly to the findings in patients with cachexia, the weight loss in cachectic animals occurs, at least in the early stages, independently of decreased food intake and without changes in total body water content. The induction of an oxidative pathway in the skeletal muscle of TNFα animals was indicated by the presence of MDA–protein adducts, which result from the oxidation of polyunsaturated fatty acids (Houglum *et al.*, 1990; Chaudhary *et al.*, 1994; Holvoet *et al.*, 1995). These findings are in agreement with evidence that TNFα stimulates oxidative stress in many cells and tissues (Wong *et al.*, 1989; Schulze-Osthoff *et al.*, 1993). In addition, NOS expression was stimulated markedly in the skeletal muscle of TNFα mice, and this effect is prevented by treating these animals with D-α-tocopherol or BW755c, suggesting that NO may mediate the muscle wasting and dedifferentiation induced by oxidative stress (Figure 2 and see below).

In order to ascertain that the dramatic effects of D-α-tocopherol and BW755c were not the spurious result of decreased synthesis of TNFα, we analyzed the influence of antioxidants on TNFα cells. Antioxidants did not affect either TNFα cell viability or the secretion of biologically active TNFα by these cells. Moreover, the TNFα cells' tumor size as well as the serum levels of TNFα were similar in TNFα animals whether or not they were treated with D-α-tocopherol, BW755c or nitro-L-arginine. Finally, end-organ biological effects of TNFα, such as anemia and decreased collagen α1(I) gene expression, remained abnormal in TNFα animals treated with D-α-tocopherol, BW755c or nitro-L-arginine. Collectively, these results indicate that a TNFα receptor or post-receptor pathway(s) in skeletal muscle is the target of antioxidants. This pathway may include activation of other cytokines such as interleukin (IL)-1β and IL-6 (Dinarello *et al.*, 1986; Akira *et al.*, 1990; Schulze-Osthoff *et al.*, 1993), which in turn could induce muscle wasting (Flores *et al.*, 1989; Fong *et al.*, 1989; Strassmann *et al.*, 1992; Spiegelman and Hotamisligil, 1993).

In cachectic animals, the skeletal muscle fibrils are depleted of myosin, a major structural protein, and their organization is disrupted. This decrease in myosin protein content in TNFα mice was prevented by D-α-tocopherol, BW755c or nitro-L-arginine treatment, indicating that oxidative pathways and activation of NOS are critical in the development of muscle wasting in cachectic animals. Because MCK plays an important role in skeletal muscle differentiated functions (Kirchberger, 1991), it has been used as an indicator of muscle-specific gene expression (Li *et al.*, 1992b; Tapscott *et al.*, 1993; Kaushal *et al.*, 1994; Skapek *et al.*, 1995). The expression of the MCK gene was reduced markedly in the skeletal muscle of cachectic animals. In agreement with previous reports in muscle cell lines (Brennan and Olson, 1990), myogenin is a major component of the MCK-E box binding activities of nuclear extracts from normal skeletal muscle. Although, the MCK-E box binding activities were decreased substantially in nuclear extracts from cachectic skeletal muscle, the expression of myogenin was essentially unchanged. In addition, phosphatase treatment of skeletal muscle nuclear extracts from cachectic animals did not modify their binding affinity for the MCK-E box, suggesting that phosphorylation of myogenin or other nuclear factors, which could inhibit its binding to cognate DNA sequences (Li *et al.*, 1992a), was not responsible for the impaired DNA binding affinity. Addition of skeletal muscle nuclear extracts from cachectic animals to those from normal animals did not inhibit the binding of the latter to the MCK-E box. These data strongly suggested that the lack of an activator, rather than the presence of an inhibitor, is responsible for the decreased MCK binding activities in skeletal muscle from cachectic animals.

Using cognate *cis*-elements and specific antibodies for various transcription factors, we demonstrated that Jun-D, but not Jun-B or c-Jun, is a major contributor to the MCK binding activities of normal skeletal muscle. This finding allowed us to establish that Jun-D binding activity and Jun-D protein were decreased in skeletal muscle nuclear extracts from cachectic animals. The addition of recombinant Jun-D to these nuclear extracts, in concentrations similar to those found in nuclear extracts from normal skeletal muscle, normalized the MCK binding activities. The nuclear redox factor Ref-1 is sensitive to the redox state of the cell (Xanthoudakis and Curran, 1992), functions as a DNA repair enzyme (Xanthoudakis *et al.*, 1992), stimulates the DNA binding activity of several transcription factors and may itself be under post-translational regulation. In this context, we found that although Ref-1 expression is induced in the skeletal muscle of cachectic animals (unpublished observations), it is apparent that this mechanism is insufficient to protect Jun-D from the effects of oxidation. More importantly,

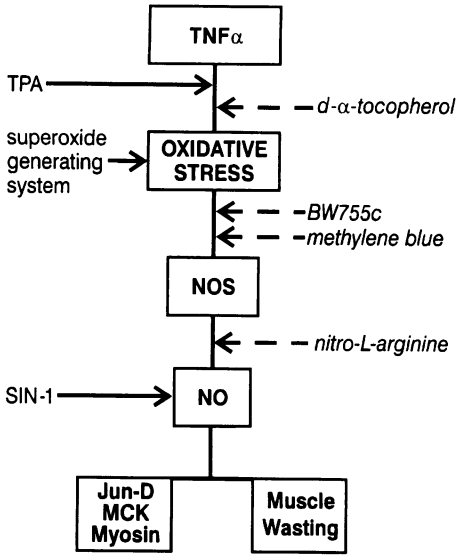

Fig. 8. Oxidative stress induces skeletal muscle dedifferentiation and wasting. Induction of an oxidative stress cascade leads to decreased Jun-D activity and decreased expression of MCK and myogenin. The muscle wasting and/or the dedifferentiation of skeletal muscle can be induced by TNFα, TPA, a superoxide-generating system (xanthine–xanthine oxidase) and SIN-1 and prevented by D-α-tocopherol, BW755c, methylene blue and nitro-L-arginine.

addition of recombinant Ref-1 or DTT normalized the MCK-E box binding activities of nuclear extracts from TNFα mice, strongly suggesting an oxidative modification of a critical binding factor, such as Jun-D. Interestingly, the Jun-D-reconstituted MCK–nuclear extract complex from skeletal muscle of cachectic animals now contained myogenin, indicating a direct or indirect physical association between Jun-D and myogenin. The precise nature of the eventual interaction between myogenin-E12 or other heterodimers (Murre *et al.*, 1989; Brennan and Olson, 1990) and Jun-D remains to be determined. Of relevance to future therapeutic approaches in the treatment of cachexia is the fact that the antioxidants D-α-tocopherol and BW755c, or the NOS inhibitor, nitro-L-arginine, normalized the following skeletal muscle abnormalities in TNFα mice: (i) myosin expression; (ii) MCK expression; (iii) MCK binding activity and (iv) Jun-D binding activity (Figure 8).

Although c-Jun, by virtue of its interactions with Myo-D and myogenin, may repress skeletal muscle differentiated functions such as MCK gene expression (Bengal *et al.*, 1992; Li *et al.*, 1992b), we found that c-Jun activity was unchanged from control levels in the nuclei of skeletal muscle from cachectic animals.

Because proteolysis has been reported to be induced in skeletal muscle by a single injection of TNFα (Flores *et al.*, 1989), and it has also been incriminated as a mechanism of muscle wasting during fasting (Wing and Goldberg, 1993), we analyzed whether protein degradation could contribute to the muscle dedifferentiation of cachexia. The synthesis and nuclear translocation of NF-κB is the result of proteolysis by the ubiquitin–proteasome pathway of the NF-κB precursor (Palombella *et al.*, 1994) and the I-κB inhibitor (Palombella *et al.*, 1994; Traenckner *et al.*, 1994), respectively, and because NF-κB activation can be induced by TNFα and oxidative stress (Henkel *et al.*, 1993; Palombella *et al.*, 1994), we assessed NF-κB activity in skeletal muscle. NF-κB binding activities were comparable in skeletal muscle nuclear extracts from control and cachectic animals (data not shown). These data exclude the participation of the NF-κB pathway in the muscle wasting of the cachectic animals. However, it was conceivable that proteolysis of a critical regulatory pathway participates in the cascade leading to muscle wasting in cachexia. In this context, degradation of Jun-D is induced by serum in fibroblasts, and it may play a role in modulating the proliferation of these cells (Pfarr *et al.*, 1994). In addition, Treir and co-workers (1994) have reported recently that another AP-1 factor, c-Jun, undergoes proteolysis via the ubiquitin system, and that Jun-D is also ubiquitinated in fibroblasts. However, we found that Jun-D protein is not degraded rapidly in skeletal muscle cells treated with TPA, a phorbol ester which mimics the effects of TNFα on cellular functions including oxidative stress (Brenner *et al.*, 1989; Henkel *et al.*, 1993), with a superoxide-generating system or with SIN-1, a donor of NO. Nonetheless, we determined that the down-regulation of Jun-D expression in the skeletal muscle from cachectic animals occurs at the translational or post-translational level, given that Jun-D mRNA was not affected.

More definitive evidence for a critical role of Jun-D in skeletal muscle differentiated function was obtained by the use of antisense Jun-D oligonucleotides and by over-expression of Jun-D. An antisense, but not a sense, Jun-D oligonucleotide blocked not only Jun-D expression but also MCK transcription activities in skeletal muscle cells. In addition, transfection of skeletal cells with a vector expressing Jun-D normalized the inhibition of MCK transcription induced by TPA. Collectively, these results indicate that Jun-D is necessary for the expression of skeletal muscle differentiated function. Addition of recombinant Jun-D or recombinant Ref-1 was sufficient to normalize the impaired MCK binding activities in skeletal muscle nuclear extracts from cachectic TNFα mice, and skeletal muscle cells, treated with components of the oxidative cascade resulting in dedifferentiation. Experiments in transgenic animals, including Jun-D 'knock-out' with and without expression of various deleted/mutated Jun-D transgenes, should clarify the role of Jun-D in muscle differentiated functions.

The weight loss and muscle wasting of cachexia are common findings in patients with AIDS, cancer and diseases characterized by chronic inflammation (Beutler and Cerami, 1986; Tracey and Cerami, 1993). At present, there is no satisfactory treatment for these complications, which contribute significantly to the morbidity and mortality of patients with cachexia (Tracey and Cerami, 1993). Our results strongly suggest that oxidative stress and NO mediate the skeletal muscle abnormalities, including the decreased affinity of Jun-D for the MCK-E box, and the muscle wasting characteristic of cachexia (Figure 8). Thus, this study provides insights into this biological enigma, as well as potential therapeutic approaches utilizing antioxidants and/or NOS inhibitors (Kobzik *et al.*, 1994), for patients with AIDS, cancer and chronic inflammatory diseases.

Materials and methods

Mouse model of cachexia

CHO cells transfected with either the human TNFα gene cloned into a mammalian expression vector (TNFα cells) or with the mammalian expression vector alone (CHO cells, control) were kindly provided by Dr A.Oliff (Merk Sharp and Dohme Research Laboratories, West Point, PA; Oliff *et al.*, 1987). Cells were grown in Dulbecco's modified essential medium supplemented with 10% fetal calf serum, penicillin (50 U/ml) and streptomycin (50 μg/ml). The TNFα cells, but not the CHO cells, produced TNFα. Four-week-old male nude mice were injected intramuscularly with either 3.5×10^6 CHO cells or 3.5×10^6 TNFα cells as we described previously (Brenner *et al.*, 1990). Nude mice were housed in a temperature- and humidity-controlled facility. Animals in the treatment groups received D-α-tocopherol (8 IU/g of foodstuff), BW755c (10 mg/kg in 100 μl of 1% sucrose, twice a day orally), nitro-L-arginine (50 μg/ml of drinking water) or indomethacin (0.5 mg/kg per day orally). The mice in all groups were weighed twice a week. Animals had free access to food and water and they were sacrificed at 30 days post-inoculation. TNFα levels in cell culture media and mouse sera were measured by a biological cytolytic assay and by an ELISA, using mAbs against human TNFα as described (Brenner *et al.*, 1990).

Immunohistochemistry and in situ hybridization

Sections from paraffin-embedded blocks were deparaffinized, passed through a graded series of alcohol, rehydrated with phosphate-buffered saline (PBS) and stained with specific IgG purified antibodies. Immunohistochemical detection of MDA–protein adducts, myosin, MCK, inducible nitric oxide synthase (iNOS) and Jun-D was performed in skeletal muscle sections as described previously (Houglum *et al.*, 1990; Buck *et al.*, 1994), using the avidin–biotin–alkaline phosphatase system (Vector Laboratories, Inc.). Cultured cells (C2C12) were treated with TPA (60 ng/ml) overnight and fixed with 50% acetone, 50% methanol at –20°C for 20 min. Antibodies against MDA–lysine epitopes (Houglum *et al.*, 1990), myosin (R & D), MCK (Dakko), Jun-D (Santa Cruz Biotechnologies) and iNOS (Transduction Laboratories) were used. For *in situ* hybridization, the hybridization, washing procedures and RNase digestion of mismatched sequences, as well as detection of the ^{35}S-labeled probe, were performed as described (Brenner *et al.*, 1990). All sections were processed with the same batches of probe and reagents. Samples were pre-treated with RNase as negative controls.

Nuclear extract preparation and gel retardation assay

Nuclei were prepared by a modification of the procedure described previously (Descombes *et al.*, 1990; Buck *et al.*, 1994). Tissue or cells were homogenized in 5% citric acid, 0.5% NP-40, 10 mM NaF, 10 mM Na pyrophosphate and 10 μM butylated hydroxytoluene with a glass Dounce homogenizer with a loose fitting pestle. The homogenized cells were placed above a cushion consisting of 2.0 M sucrose. The nuclei were precipitated by a 4000 *g* centrifugation at 4°C for 20 min, and frozen at –70°C. DNA concentrations were determined by spectrophotometric absorbance at 260 nm. Gel retardation analysis of protein–DNA complexes was performed in the presence of dIdC, with an oligonucleotide spanning the E box of the MCK gene, as described previously (Descombes *et al.*, 1990; Trautwein *et al.*, 1993; Buck *et al.*, 1994). The sense oligonucleotides were MCK-E box (5′ CCC AAC ACC TGC TGC CTG AGC C 3′) and AP-1 (5′ TGA CAT CAT GGG CTG TCG ACC ATG 3′). Expression of recombinant Jun-D protein was induced with IPTG, from the coding region of Jun-D cDNA (ATCC) cloned into a PRSET vector, and purified with a Probond Resin (Invitrogen). Recombinant Jun-D was oxidized by exposure to air at room temperature for 18 h in the presence of leupeptin and aprotinin (Descombes *et al.*, 1990), but without reducing agents or antioxidants. Purified recombinant Ref-1 (0.2 μg) (kindly provided by S.Xanthoudakis, Hoffman-La Roche) or 10 mM DTT was added to some samples as described (Xanthoudakis *et al.*, 1992).

Cell culture and transfection

Human skeletal muscle cells (Clonetics) were cultured in a defined skeletal muscle serum-free basal medium (SkBM, Clonetics). C2C12 cells (ATCC) were cultured in minimal essential medium supplemented with 10% fetal bovine serum. Cells were cultured without serum from the time of transfection. Treatments were started immediately after transfection and continued for an additional 48 h. The sequences of Jun-D oligonucleotide phosphorothioate were antisense (5′ CAG CGC GTC CTT CTT CAT 3′) and sense (5′ ATG AAG AAG GAC GCG CTG 3′). DNA transfection into skeletal muscle cells was carried out with lipofectin as recommended by the manufacturer (BRL). The total amount of transfected DNA was 2 μg. The 4RTk-CAT plasmid (1 μg) was transfected with either RSV-Jun-D (1 μg) or control RSV-β galactosidase (1 μg). Cells were harvested 48 h after transfection and CAT content determined by ELISA as described by the manufacturer (5′ Prime 3′ Prime). Detection of myogenin or Jun-D in the skeletal muscle tissue or cells was performed by Western blot following the chemiluminescence protocol (DuPont), using anti-rat myogenin mAbs (F5D) (kindly provided by W.Wright, University of Texas, Southwestern), or polyclonal antibodies against Jun-D (Santa Cruz Biotechnologies) as described (Trautwein *et al.*, 1993; Buck *et al.*, 1994). RNA was purified from muscle with Trizol following the manufacturer's protocol (BRL). Slot blots and hybridization with a riboprobe for Jun-D or 18S RNA (Ambion) was performed as described (Brenner *et al.*, 1989).

Statistical analysis

Results are expressed as the mean of at least triplicates, unless stated otherwise. Either the Student-*t* or the Fisher's exact test was used to evaluate the differences in the means between groups, with a P value of <0.05 as significant.

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