Pentoxifylline inhibits Ca\textsuperscript{2+}-dependent and ATP proteasome-dependent proteolysis in skeletal muscle from acutely diabetic rats

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Baviera AM, Zanon NM, Navegantes LC, Migliorini RH, Kettelhut IC. Pentoxifylline inhibits Ca\textsuperscript{2+}-dependent and ATP proteasome-dependent proteolysis in skeletal muscle from acutely diabetic rats. Am J Physiol Endocrinol Metab 292: E702–E708, 2007. First published October 31, 2006; doi:10.1152/ajpendo.00147.2006.—Previous studies from this laboratory have shown that catecholamines exert an inhibitory effect on muscle protein degradation through a pathway involving the cAMP cascade. The present work investigated the systemic effect of pentoxifylline (PTX; cAMP-phosphodiesterase inhibitor) treatment on the rate of overall proteolysis, the activity of proteolytic systems, and the process of protein synthesis in extensor digitorum longus muscles from normal and acutely diabetic rats. The direct in vitro effect of this drug on the rates of muscle protein degradation was also investigated. Muscles from diabetic rats treated with PTX showed an increase (22%) in the cAMP content and reduction in total rates of protein breakdown and in activity of Ca\textsuperscript{2+}-dependent (47%) and ATP proteasome-dependent (23%) proteolytic pathways. The high content of m-calpain observed in muscles from diabetic rats was abolished by PTX treatment. The addition of PTX (10\textsuperscript{-3} M) to the incubation medium increased the cAMP content in muscles from normal (22%) and diabetic (51%) rats and induced a reduction in the rates of overall proteolysis that was accompanied by decreased activity of the Ca\textsuperscript{2+}-dependent and ATP proteasome-dependent proteolytic systems, in both groups. The in vitro addition of H-89, an inhibitor of protein kinase A (PKA), completely blocked the effect of PTX on the reduction of proteolysis in muscles from normal and diabetic rats. The present data suggest that PTX exerts a direct inhibitory effect on protein degradative systems in muscles from acutely diabetic rats, probably involving the participation of cAMP intracellular pathways and activation of PKA, independently of tumor necrosis factor-\alpha inhibition.

adenosine 3',5'-cyclic monophosphate-phosphodiesterase inhibitors; muscle atrophy; streptozotocin-diabetic rats; adenosine 3',5'-cyclic monophosphate-dependent pathway; xanthine derivatives

MAINTENANCE OF SKELETAL MUSCLE mass is often viewed as the net result of the balance between two separate processes, protein synthesis and protein degradation; these two opposite processes are believed to hold the key to the understanding of the mechanisms involved in the regulation of skeletal muscle mass. The protein turnover in skeletal muscle is regulated by numerous nutritional, hormonal, and neural factors that culminate in metabolic adjustments to the most diverse situations. Recently, much progress has been made on the control of muscle protein breakdown and the role of the different proteolytic systems (the lysosomal, the Ca\textsuperscript{2+} dependent, the ATP-ubiquitin proteasome dependent, and the residual processes) in several physiological and pathological states.

Among the factors that regulate skeletal muscle protein metabolism, the catecholamines have an important role. Although the actions of catecholamines are usually associated with catabolic processes, promoting the breakdown of both glycogen and fat for use as fuel, recent reports show that catecholamines exert an antiproteolytic effect on muscle protein metabolism. Numerous studies show that administration of \(\beta\text{-adrenergic agonists} \) markedly stimulates skeletal muscle hypertrophy (14, 25), retards atrophy in denervated muscles (27), and reduces muscle wasting in tumor-bearing rats (6).

Navegantes et al. (17) demonstrated that guanethidine-induced adrenergic blockade increases the rate of total protein degradation in rat soleus muscles after 2 days of treatment, suggesting that the sympathetic nervous system exerts an acute inhibitory control of skeletal muscle proteolysis. This activation of overall proteolysis was accompanied by an increased participation of the Ca\textsuperscript{2+}-dependent proteolytic pathway (17). The reduction in the activity of this proteolytic system induced by catecholamines, clenbuterol (selective \(\beta\text{-adrenergic agonist} \), CL-316,243 (selective \(\beta\text{-adrenergic agonist} \), and dibutyryl-cAMP in vitro suggests that catecholamines inhibit the activity of the Ca\textsuperscript{2+}-dependent proteolysis by binding to \(\beta\text{-or } \beta\text{-adrenoceptors and activating intracellular pathways involving the cAMP cascade (16, 19).\n
Drugs that induce an increase in the intracellular concentrations of cAMP, as the cAMP-phosphodiesterase inhibitors, have been employed in the treatment of many human respiratory and circulatory diseases (1, 12), and they also are used to prevent muscular atrophy in several experimental situations (8, 9). Pentoxifylline (PTX), a xanthine derivative and a nonselective cAMP phosphodiesterase inhibitor that has been used in the treatment of human clinical disorders, also induces reduction of skeletal muscle wasting. In fact, the PTX treatment induced a clear reduction in the raise of muscle protein degradation in tumor-bearing rats (3) and during sepsis (2).

The purpose of the present work is to investigate the effect of PTX on the skeletal muscle protein metabolism of rats in a classical situation of muscular atrophy, experimental diabetes. In addition to the effect of PTX treatment on the rate of protein synthesis, overall proteolysis, and activity of the proteolytic pathways (lysosomal, Ca\textsuperscript{2+}-dependent, and ATP-ubiquitin-proteasome-dependent systems), we have examined the content of m-calpain and calpastatin in extensor digitorum longus (EDL).
muscles from normal and acutely diabetic rats. The direct in vitro effect of PTX on the rate of protein degradation was investigated in the presence or not of N-(2-[p-bromocinnamylamino]-ethyl)-5-isooquinolinesulfonamide-2 HCl (H-89), an inhibitor of protein kinase A (PKA). The concentration of cAMP in muscles from PTX-treated rats in vivo and in vitro and levels of plasma tumor necrosis factor (TNF)-α are also reported.

MATERIALS AND METHODS

Animals

Because the incubation procedure requires intact muscles sufficiently thin to allow an adequate diffusion of metabolites and oxygen, young rats were used in all experiments. Male Wistar rats were housed in a room with a 12:12-h light-dark cycle and were given free access to water and a normal laboratory chow diet for at least 2 days before the beginning of the experiments. Rats of similar body weight (70–80 g) were used in all experiments, which were performed at 8:00 AM. To induce diabetes, streptozotocin (STZ, 135 mg/kg body wt) dissolved in 0.01 M citrate buffer, pH 4.5, was injected, under ether anesthesia, in the jugular vein (20). Control rats received citrate buffer. Diabetic and control rats (3 days after STZ or vehicle injection) were killed by cervical dislocation for muscle excision, and blood was collected to determine the concentration of plasma glucose, by the method of glucose oxidase. Care and treatment of experimental rats received prior institutional approval by the Ethics Committee of São Paulo State University.

PTX Treatment and In Vitro Studies

Rates of protein synthesis, overall proteolysis, and the activity of proteolytic pathways were investigated in muscles after 4 days of PTX treatment. Control and diabetic rats (1 day before and 3 days after citrate buffer or STZ administration) were injected intraperitoneally with saline or PTX (100 mg/kg body wt) dissolved in 0.9% NaCl. To investigate the in vitro effect of PTX on the rate of overall proteolysis and activity of the different proteolytic pathways, muscles from normal or diabetic rats were incubated in the presence of 10⁻³ M PTX. Contralateral muscles were used as controls. In a separate group of experiments, the rate of overall proteolysis was investigated in muscles from normal and diabetic rats incubated in the presence of 10⁻³ M PTX and 50 μM H-89, a specific PKA inhibitor.

Determination of cAMP Levels in Muscles

The intracellular levels of cAMP were measured in muscles from normal and diabetic rats treated with PTX in vivo and in vitro by using a method based on a competitive enzyme immunoassay system. Muscles were rapidly dissected and homogenized in 6% TCA. After extraction of lipid content with diethyl ether, the aqueous phase was lyophilized and resuspended in the assay buffer.

Measurement of Plasma TNF-α

In a separate group of control and diabetic animals treated or not with PTX in vivo, the plasma concentrations of TNF-α were determined by a double-ligand ELISA.

Incubation Procedure to Measure the Rates of Protein Degradation

EDL muscles were rapidly dissected, with care being taken to avoid damaging the muscles. The muscles were maintained by pinning them on inert plastic supports and incubated at 37°C in Krebs-Ringer-bicarbonate buffer, pH 7.4, equilibrated with 95% O₂-5% CO₂ containing glucose (5 mM), and in the presence of cycloheximide (0.5 mM) to prevent protein synthesis and the reincorporation of tyrosine back into proteins. Tissues were preincubated for 1 h and then incubated for 2 h in fresh medium of identical composition.

The rates of overall proteolysis and of the different proteolytic systems were determined by measuring the rate of tyrosine release in the incubation medium. Because muscles cannot synthesize or degrade tyrosine, its release reflects the rate of protein breakdown. Tyrosine was assayed as previously described (24).

To measure the participation of the different proteolytic pathways, muscles were incubated as described in detail previously (17, 20). Because of a limitation of the method to investigate the contribution of the Ca²⁺-dependent proteolysis in muscles from diabetic rats (free/stretch model), the effect of PTX in vitro in this proteolytic system was determined in muscles incubated in the presence of inhibitors of lysosomal- and ATP proteasome-dependent proteolytic pathways, where the remaining proteolytic activity estimates the participation of the Ca²⁺-dependent process, since the activity of residual pathway did not change between the groups.

Measurement of Rates of Protein Synthesis

Rats were killed by cervical dislocation for muscle excision. The muscles were rapidly dissected, maintained at approximately resting length, and incubated at 37°C in Krebs-Ringer-bicarbonate buffer, pH 7.4, equilibrated with 95% O₂-5% CO₂, containing glucose (5 mM) and all 20 amino acids at concentrations similar to those of rat plasma (22). The procedure used for measurement of rates of protein synthesis in muscles from normal and diabetic rats treated with PTX was as previously described (15).

Western Blotting Analysis of Muscle Calpain and Calpastatin

After PTX treatment, EDL muscles were harvested and homogenized in 20 mM Tris-HCl and 5 mM EDTA, pH 7.5, in the presence of 1 mg/ml leupeptin and 100 mg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride. The homogenate was centrifuged at 14,000 g at 4°C for 20 min, retaining the supernatant, and protein content was determined by the method of Lowry et al. (13) using BSA as a standard. An equal volume of sample buffer (20% glycerol, 125 mM Tris-HCl, 4% SDS, 100 mM dithiothreitol, and 0.02% bromophenol blue, pH 6.8) was added to homogenate, and the mixture was boiled and subjected to SDS-PAGE on 8% acrylamide gels (10). Gels were electroblotted on nitrocellulose membranes (23), and the antibodies were used to detect the corresponding protein levels: m-calpain polyconal antibody (1:500) and calpastatin polyclonal antibody (1:1,000). Primary antibody was detected by peroxidase-conjugated secondary antibody (1:10,000) and visualized by enhanced chemiluminescence reagents. Band intensities were quantified using the LabImage Program (Version 2.7.1).

Drugs

All drugs and reagents were purchased from Sigma Chemical (St. Louis, MO), Calbiochem EMD Biosciences (La Jolla, CA), American Biosciences (Piscataway, NJ), Santa Cruz Biotechnology (Santa Cruz, CA), and Alexis Biochemicals.

Statistical Methods

Means of muscle samples from different groups of animals were analyzed using Student’s nonpaired t-test. Paired t-test was used to compare the means of muscle samples of the same group. P < 0.05 was taken as criterion of significance.

RESULTS

In Vivo and In Vitro Effects of PTX on Intracellular Levels of cAMP in Muscles from Normal and Diabetic Rats

As shown in Fig. 1, cAMP content did not differ significantly in muscles from normal and diabetic rats treated with
vehicle. In vivo PTX treatment induced a 22% increase in cAMP levels in muscles from diabetic rats. However, cAMP content in muscles from normal rats was not significantly affected by PTX treatment. The addition of PTX to the incubation medium induced a significant increase in the cAMP levels in muscles from normal (22%) and diabetic (51%) rats (Fig. 1).

Effects of PTX Treatment on Plasma Levels of TNF-α

Plasma levels of TNF-α in diabetic rats were significantly higher (61.3 ± 8.8 pg/ml; n = 5; P < 0.05) than in controls (37.6 ± 3.9 pg/ml; n = 5). The PTX treatment decreased the plasma TNF-α concentrations (19.4 ± 4.0 pg/ml; n = 6; P < 0.05) in diabetic rats but had no effect in the control group (36.7 ± 4.5 pg/ml; n = 6).

Evaluation of Different Proteolytic Processes in Muscles from Diabetic Rats Treated with PTX In Vivo

The weight of muscles of rats 3 days after STZ injection was smaller (26.5 ± 0.89 mg; n = 7) than that of normal rats (33.0 ± 1.21 mg; n = 7). PTX treatment did not affect the mass of muscles from normal (32.7 ± 1.99 mg; n = 7) or diabetic (26.8 ± 1.42 mg; n = 7) rats. The rate of total protein degradation in EDL muscles from diabetic rats was 60% higher (P < 0.05) than in muscles from normal rats (Fig. 2). Treatment of diabetic rats with PTX induced a reduction of 17% (P < 0.05) in muscle proteolysis. Normal rats treated with PTX did not show any change in muscle protein degradation when compared with the normal rats treated with vehicle (Fig. 2).

Lyosomal proteolytic activity. The activity of the lysosomal proteolytic pathway did not differ significantly in muscles from diabetic and normal rats and was not affected by PTX treatment of these animals (data not shown).
Ca\(^{2+}\)-dependent proteolytic activity. In agreement with previous observations (20), the participation of the Ca\(^{2+}\)-dependent proteolytic system was markedly increased (175%) in muscles from acutely diabetic rats (Fig. 2). A significant 47% reduction in the participation of this process was observed in muscles from diabetic rats treated with PTX compared with rats treated with the vehicle. However, in normal rats treated with PTX, the Ca\(^{2+}\)-dependent proteolysis did not differ significantly from control muscles (Fig. 2).

ATP-dependent proteolytic activity. The activity of the ATP-dependent proteolytic system in EDL muscles from diabetic rats was 97% higher than normal controls (Fig. 2). PTX treatment induced a significant decrease (23%) in this proteolytic activity in diabetic rats. No significant effect of PTX treatment was found on the ATP-dependent proteolytic activity of muscles from normal rats (Fig. 2). No difference in the residual process was apparent in muscles from normal and diabetic rats, treated or not with PTX.

**In Vivo Effect of PTX on Calpain and Calpastatin Protein Levels in Muscles from Normal and Diabetic Rats**

The protein levels of m-calpain and calpastatin in muscles from diabetic rats were increased (115%) and decreased (31%), respectively, compared with the normal group (Fig. 3). The PTX treatment induced a 70% decrease of m-calpain content in muscles from diabetic rats but had no effect in the normal group (Fig. 3). The lower content of calpastatin in muscles from diabetic rats was not affected by PTX treatment (Fig. 3).

**In Vitro Effect of PTX on the Proteolytic Pathways in Muscles from Normal and Diabetic Rats**

The rate of overall proteolysis in EDL from diabetic rats (0.448 ± 0.021 nmol tyrosine·mg \(^{-1}\)·2 h \(^{-1}\), n = 6) was reduced by 16.5% (P < 0.05) when muscles from the same animals were incubated in the presence of PTX (0.374 ± 0.025 nmol tyrosine·mg \(^{-1}\)·2 h \(^{-1}\); n = 6; Fig. 4). PTX (10\(^{-3}\) M) added to the incubation medium of muscles from diabetic rats did not affect the activity of the lysosomal proteolytic system (data not shown) but induced a significant decrease in the activity of Ca\(^{2+}\)-dependent and ATP-dependent proteolytic processes (14 and 20%, respectively; Fig. 4).

Different from the in vivo treatment, PTX added to the incubation medium reduced by 25% (P < 0.05) the rate of overall proteolysis (Fig. 4) and induced a decrease in the activities of Ca\(^{2+}\)-dependent (11%) and ATP-dependent (13%) degradative systems (Fig. 4) in muscles from normal rats. No change was observed in lysosomal and residual proteolytic activities in muscles from these animals.

To investigate the participation of PKA in the intracellular action of PTX, muscles from normal and diabetic rats were incubated in the presence of PTX and H-89, a specific inhibitor of PKA. The rate of total proteolysis in EDL from normal and diabetic rats was 14 and 23% decreased, respectively (P < 0.05), in the presence of PTX; however, the addition of H-89 completely blocked the antiproteolytic effect of PTX on these muscles (Fig. 5).

**Evaluation of the Rates of Protein Synthesis in Muscles from Normal and Diabetic Rats Treated with PTX In Vivo**

The data in Fig. 6 show the rates of protein synthesis in EDL muscles from normal and diabetic rats 4 days after PTX treatment. Rates of protein synthesis in muscles from diabetic rats (7 rats, 0.146 ± 0.010 nmol Tyrr·mg \(^{-1}\)·2 h \(^{-1}\) were 51% lower than in normal rats (8 rats, 0.295 ± 0.017). PTX did not affect the rates of protein synthesis in muscles from normal and diabetic animals.

**DISCUSSION**

The present data show that PTX treatment for 4 days induces a decrease in the rate of total protein degradation in EDL muscles from diabetic animals (3 days after STZ) through a clear reduction in the activity of the Ca\(^{2+}\)-dependent and ATP proteasome-dependent proteolytic systems (Fig. 2), two degradative processes well known to be activated in the acute phase of experimental diabetes (20). On the other hand, PTX did not modify the reduced rate of muscle protein synthesis observed in the same animals (Fig. 6). The fact that PTX treatment prevented the plasma TNF-\(\alpha\) increase in diabetic animals suggests that this drug may reduce protein catabolism indirectly by suppressing high levels of TNF-\(\alpha\) in diabetic rats. This is in agreement with previous studies showing that the antiproteolytic effect of PTX and other xanthine derivatives is due to the inhibition of TNF-\(\alpha\) synthesis, which is frequently associated with skeletal muscle-wasting conditions (5, 7, 11) in experimental models that exhibit increased production of cytokines, like sepsis or cancer. Nevertheless, an alternative...
hypothesis is that PTX reduces muscle protein catabolism directly by increasing cAMP intracellular levels in skeletal muscle. Hinkle et al. (8) recently showed that inhibition of muscle phosphodiesterase activity prevents muscle mass and function loss in two disuse atrophy models (casting and denervation) without a cytokine-mediated mechanism. In previous studies, Navegantes et al. (18) have shown that addition of nonhydrolyzable cAMP analogs and activation of the Gs (GTP-binding protein stimulatory)-coupled β-adrenergic receptor in vitro inhibit proteolysis in skeletal muscle from normal rats. It would thus appear that the raise in the cAMP intracellular levels is one of the regulatory mechanism(s) to prevent excessive breakdown of protein in skeletal muscle. In agreement with these previous findings, the present study shows that the increase in intracellular cAMP concentration induced by PTX treatment or addition of this drug to the incubation medium

Fig. 4. Rates of total protein degradation and Ca^{2+}-dependent and ATP-dependent proteolytic activities in EDL muscles from normal and diabetic rats incubated in the presence of PTX. Values are means ± SE of 7–9 muscles. The same experiment was repeated 2 times with similar results. *P < 0.05, diabetic vs. normal (•) and normal or diabetic + PTX in vitro vs. normal or diabetic (#).

Fig. 5. Rates of protein degradation in EDL muscles from normal and diabetic rats incubated in the presence of PTX and H-89. Values are means ± SE of 7–9 muscles. The same experiment was repeated 2 times with similar results. *P < 0.05, PTX in vitro vs. control (•) and PTX + H-89 in vitro vs. PTX in vitro (#).

Fig. 6. Rates of protein synthesis in EDL muscles from normal and diabetic rats treated or not with PTX. Values are means ± SE of 7–9 muscles. The same experiment was repeated 2 times with similar results. *P < 0.05, diabetic vs. normal.
(Fig. 1) may be responsible, at least in part, for the reduction of muscle protein degradation in acutely diabetic rats, independent of its effect on TNF-α inhibition.

The present data show that the rate of overall proteolysis in muscles from normal rats was not affected by in vivo PTX treatment. In contrast, PTX in vitro reduced the rates of overall proteolysis in muscle from both normal and diabetic rats. It seems reasonable to speculate that, when normal animals are treated with PTX, the consequent raise in the cAMP intracellular content is countered by a rapid mechanism triggered by insulin stimulating the activity of the cAMP phosphodiesterases and preventing the PTX-induced reduction of proteolysis. This hypothesis is supported by the increased levels of cAMP observed only in muscles from PTX-treated diabetic rats but not in PTX-treated normal rats (Fig. 1). Thus the addition of PTX to the incubation medium, without insulin, raises the cAMP content and decreases proteolysis in skeletal muscle from normal rats (Fig. 1). The antiproteolytic effect of PTX in vitro was inhibited by H-89, a PKA inhibitor, further supporting the idea that activation of the cAMP messenger system via a PKA-dependent pathway is one of the regulatory mechanisms(s) to prevent excessive skeletal muscle protein breakdown.

The data of present work indicate that the high activity of the two proteolytic systems (Ca\(^{2+}\)-dependent and ATP proteasome dependent) decreases in muscles from diabetic rats treated in vivo (Fig. 2) and in muscle from normal and diabetic rats incubated in the presence of PTX in vitro (Fig. 4). In agreement with these results, we have previously found that the activity of the Ca\(^{2+}\)-dependent system is reduced in normal rat muscles incubated in the presence of 10\(^{-3}\) M dibutyryl-cAMP or isobutyl methylxanthine, a nonselective phosphodiesterase inhibitor (19). We show in the present work that m-calpain content is downregulated in muscles from diabetic rats treated with PTX in vitro, suggesting that the PTX-induced decrease in Ca\(^{2+}\)-dependent proteolysis is the result of inhibited calpain activity. Although calpastatin levels were not altered by PTX, the possibility that calpastatin activity was increased by treatment cannot be ruled out. Previous reports found that treatment of rats with PTX reduces the increased expression of different components of ATP proteasome-dependent proteolysis in cancer (3) and septic rats (4). More recently, it has been demonstrated that β2-adrenergic agonist treatment attenuates muscle atrophy induced by hindlimb unweighting through inhibitory effects on the ubiquitin-proteasome pathway (26). Since β2-agonists activate PKA in rat skeletal muscle (21), it could be speculated that proteasome and/or the process of protein ubiquitination is regulated by this kinase. The inhibition of ATP-dependent proteolytic activity and ubiquitin ligase gene expression (Navegantes LC, Wing S, Gomes MD, Baviera AM, Migliorini RH, Kettelhut IC, unpublished observations) by isobutyl methylxanthine or clenbuterol observed in skeletal muscles from normal rats in vitro is consistent with this hypothesis.

In summary, the present work shows that PTX, a nonselective phosphodiesterase inhibitor, decreases in vivo the activity of Ca\(^{2+}\)-dependent and ATP-dependent proteolysis in EDL muscles from diabetic rats. The inhibitory effect of PTX in vitro on these proteolytic systems suggests the participation of intracellular pathways involving the cAMP and activation of PKA. Although the suppression of high levels of plasma TNF-α in diabetic rats might have contributed to the antiproteolytic effect of PTX observed in vivo, this is the first report to show a direct inhibitory effect of this drug on protein degradative systems in fast-twitch rat skeletal muscle. This finding raises the possibility of using this drug to treat acute skeletal muscle atrophy in different muscle-wasting conditions.

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