Nitric oxide inhibits calpain-mediated proteolysis of talin in skeletal muscle cells

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Received 9 February 2000; accepted in final form 27 March 2000

Koh, Timothy J., and James G. Tidball. Nitric oxide inhibits calpain-mediated proteolysis of talin in skeletal muscle cells. Am J Physiol Cell Physiol 279: C806–C812, 2000.—We tested the hypothesis that nitric oxide can inhibit cytoskeletal breakdown in skeletal muscle cells by inhibiting calpain cleavage of talin. The nitric oxide donor sodium nitroprusside prevented many of the effects of calcium ionophore on C2C12 muscle cells, including preventing talin proteolysis and release into the cytosol and reducing loss of vinculin, cell detachment, and loss of cellular protein. These results indicate that nitric oxide inhibition of calpain protected the cells from ionophore-induced proteolysis. Calpain inhibitor I and a cell-permeable calpastatin peptide also protected the cells from proteolysis, confirming that ionophore-induced proteolysis was primarily calpain mediated. The activity of m-calpain in a casein zymogram was inhibited by sodium nitroprusside, and this inhibition was reversed by dithiothreitol. Previous incubation with the active site-targeted calpain inhibitor I prevented most of the sodium nitroprusside-induced inhibition of m-calpain activity. These data suggest that nitric oxide inhibited m-calpain activity via S-nitrosylation of the active site cysteine. The results of this study indicate that nitric oxide produced endogenously by skeletal muscle and other cell types has the potential to inhibit m-calpain activity and cytoskeletal proteolysis.

cytoskeleton; myotendinous junction; protease; vinculin

THE CALPAINS, A UBIQUITOUS family of calcium-dependent cytosolic cysteine proteases, are involved in many physiological and pathological processes. Current evidence indicates that calpain-mediated proteolysis can influence overall cellular protein degradation (15, 20), can modify the activity of various enzymes (e.g., protein kinase C, Ref. 24; phosphorylase kinase, Ref. 21; myosin light chain kinase, Ref. 22), and may play a role in platelet aggregation, myoblast fusion (38), and long-term potentiation (3). In addition, calpain overactivation may contribute to pathology in cerebral and cardiac ischemia, Alzheimer’s disease, arthritis, and cataract formation (45).

In skeletal muscle, calpains are thought to initiate cytoskeletal degradation by cleaving proteins impor-tant in linking components of the cytoskeleton together and to the cell membrane (15, 20). One such linker protein, talin, is colocalized with calpain at the cell membrane of cultured cells and is a preferred sub-strate for calpain (4). Thus calpain cleavage of talin may be involved in initiating breakdown of the muscle cytoskeleton. Despite the potential importance of calpain activity in proteolysis of the muscle cytoskeleton, its regulation is not well understood.

Possible regulators of calpain-mediated cytoskeletal proteolysis include calcium, calpastatin, and nitric oxide (NO). The best-characterized regulatory molecules of calpain are calcium and calpastatin. The two calpain isoforms that were first purified, μ- and m-calpain, require low micromolar and millimolar calcium concentra-tions for half-maximal activity in vitro, respectively (10). Calpastatin, which is expressed endogenously typically in greater concentrations than the calpains, binds to calpain in the presence of calcium and inhibits the activity of both isoforms (16). Recently, the NO donor sodium nitroprusside (SNP) was shown to inhibit m-calpain purified from skeletal muscle in a dose-dependent manner (29). The inhibition of m-calpain by SNP could be reversed by dithiothreitol (DTT), suggesting NO inhibited calpain via S-nitrosylation of cysteine(s). Thus NO represents a novel regulatory molecule of calpain that could inhibit cytoskeletal proteolysis in skeletal muscle cells.

In the present study, we tested the hypothesis that NO inhibits cytoskeletal breakdown in muscle cells by inhibiting calpain-mediated proteolysis of talin. We used the calcium ionophore A-23187 to increase the level of intracellular calcium in C2C12 myoblasts and activate calpain in these cells. We measured the proteolysis of talin in both the presence and absence of SNP and used a calpastatin peptide to confirm that talin proteolysis was calpain mediated. In addition, we used a casein zymogram to identify the potential target of NO on calpain. By blocking the active site with calpain inhibitor I during incubation with SNP, we sought to determine whether NO exerts its inhibitory effect by modifying the active site on calpain.

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MATERIALS AND METHODS

Zymography calpain assay. Purified m-calpain (Sigma) was solubilized in 50 mM Tris·HCl (pH 7.4), 1 mM DTT, and 1 mM EGTA. Samples (0.5 unit of m-calpain in 30 µl) were incubated with varying concentrations of the NO donor SNP (Sigma) and DTT for 10 min on ice. Some samples were also preincubated with 100 µM calpain inhibitor I (Calbiochem) for 10 min on ice before incubation with SNP. Samples were then subjected to casein zymography essentially as described (35). Briefly, samples were mixed with 1 µl 50% glycerol and 1 µl 0.4% bromophenol blue and then separated in a nondenaturing casein (0.2%)-polyacrylamide (10%) gel. The gel was placed in calpain activation buffer (in mM: 20 Tris·HCl, pH 7.4, 5 CaCl2, 1 DTT) at room temperature for 24 h with changes of buffer at 30 min and 1 h. Finally, the gel was stained with Coomassie blue.

Solution calpain assay. Calpain activity was measured essentially as described (17). Briefly, 0.25 unit m-calpain was solubilized in 0.4 ml 50 mM Tris·HCl, pH 7.4, containing 1 mM DTT, 5 mM CaCl2 or EDTA, and 0.5% α-casein. Samples were preincubated with varying concentrations of calpain inhibitor I for 15 min on ice. All samples were then incubated at room temperature for 30 min, and proteolysis was stopped by addition of 0.3 ml 10% trichloroacetic acid. Samples were placed on ice for 15 min, centrifuged for 15 min at 12,000 g, and the A290 of the supernatant was measured. Calpain activity was defined as the Ca2+-dependent increase in A290.

Cell culture. C2C12 myoblasts were plated on 60-mm plastic tissue culture dishes coated with 2% gelatin. Cells were grown in 5 ml of DMEM with 10% fetal bovine serum supplemented with 1% penicillin and streptomycin at 37°C and 5% CO2. The culture medium was changed every other day until the cells reached confluence.

Ionophore, NO donor, and cell fractionation. Confluent C2C12 myoblasts were plated with prewarmed (37°C) Tyrode solution (in mM: 135 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 glucose, 10 mM HEPES, pH 7.25), and then cells were incubated in 5 ml Tyrode solution with 10 µg/ml calcium ionophore (A-23187, Sigma) for 60 min at 37°C to activate calpain. Some cells were also treated with the NO donor SNP; these cells were preincubated with 2 mM SNP in Tyrode solution for 10 min and then incubated in Tyrode solution with 10 µg/ml ionophore and 2 mM SNP. Triton X-100 soluble and insoluble fractions were then prepared as described by Rodríguez Ferández et al. (36). Briefly, cells were rinsed with PBS and then incubated in 1 ml of extraction buffer (in mM: 50 N-morpholino-ethane sulfonic acid, pH 6.1, 2.5 EGTA, 5 M gCl2, and 0.5% Triton X-100) at room temperature for 2 min with gentle swirling every 30 s. The Triton X-100 soluble fraction was collected, and the insoluble fraction was scraped into 1 ml of SDS-PAGE reducing sample buffer (80 mM Tris·HCl, pH 6.8, 0.1 M DTT, 70 mM SDS, 1 mM glycerol, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 0.3 mM aprotinin). Concentrated reducing sample buffer was added to the soluble fraction, all samples were boiled for 3 min, and then protein concentrations were determined using a filter paper dye-binding assay (30).

Ionophore, NO donor, and calpain inhibitors. Confluent cells were rinsed with prewarmed (37°C) Tyrode solution, and then cells were incubated in 5 ml Tyrode solution with 50 µg/ml calcium ionophore (A-23187, Sigma) for 90 min at 37°C with or without 2 mM SNP. Other cells were treated with 50 µM calpastatin peptide inhibitor or 20 µM calpain inhibitor I (Calbiochem); these cells were preincubated with inhibitor in DMEM for 2 h and then treated with ionophore.

RESULTS

After the experimental treatment, 100 µl of media was taken for detached cell counting, and the adherent cells were scraped with a rubber policeman into the remaining media and detached cells. The cells and media were centrifuged at 500 g for 10 min at 4°C and the resulting pellet was washed with phosphate-buffered saline and centrifuged again at 500 g for 10 min at 4°C. The cell pellet was then resuspended in reducing sample buffer, cells were homogenized with a dounce homogenizer, boiled for 3 min, and then protein concentrations were determined using a filter paper dye-binding assay (30).

Western analysis. Samples containing equal amounts of protein were separated on 8% SDS-PAGE gels (25). Gels were electrophoretically transferred (BioRad) to nitrocellulose membranes at 1 A for 4 h while immersed in transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine) and chilled on ice. Membranes were blocked overnight at 4°C in blocking buffer (50 mM Tris·HCl, pH 7.6, 150 mM NaCl, 0.1% NaN3, 0.2% gelatin, 0.05% Tween 20, 3% nonfat dry milk). The blots were then overlaid for 3 h at room temperature with one of the following primary antibodies diluted in antibody buffer (50 mM Tris·HCl, pH 7.6, 150 mM NaCl, 0.1% NaN3, 0.05% Tween 20, 3% bovine serum albumin): 1) monoclonal mouse anti-talin (Sigma) diluted 1:80, 2) monoclonal mouse antivinculin (Sigma) diluted 1:200, or 3) a polyclonal rabbit antibody raised against the NH2-terminal propeptide of the large subunit of m-calpain (pro-m-calpain, a kind gift from Dr. Dorothy Croall; Ref. 11) diluted 1:750. Blots were washed 6 × 10 min with wash buffer (50 mM Tris·HCl, pH 7.6, 150 mM NaCl, 0.1% NaN3, 0.05% Tween 20, 0.2% gelatin, 0.3% nonfat dry milk), then overlaid for 1 h at room temperature with secondary antibodies conjugated with alkaline phosphatase (anti-rabbit IgG or anti-rabbit IgG, Sigma) diluted 1:3,000. Blots were washed 6 × 10 min with wash buffer, then developed with nitroblue tetrazolium and bromochloroindolyl phosphate. Relative protein concentrations were determined using an imaging system densitometer (Alpha Innotec). Uniformity of protein loading and efficiency of transfer were assessed by staining membranes with Ponceau S after transfer.

Statistical analysis. Comparisons between treatments were made using t-tests for nonnormalized values and using the nonparametric Mann-Whitney U test for normalized values. For all tests, the 0.05 level was taken to indicate statistical significance.
cytosol. SNP pretreatment prevented the decrease in the intact 235-kDa talin and the increase of the 190-kDa fragment in both the insoluble and soluble fractions.

**Ionophore, NO donor, and calpain inhibitors.** Confluent C2C12 myoblasts were also exposed to a longer treatment with higher concentration of ionophore (50 vs. 10 µg/ml, 90 vs. 60 min). For these cells, calpain activity was measured as the ratio of concentrations of the 190- and the 235-kDa bands in Western blots (talin proteolytic ratio). Ionophore treatment significantly increased the talin proteolytic ratio (Figs. 1 and 2), suggesting that calpain activity was increased. Ionophore treatment also significantly decreased vinculin concentration (Figs. 1 and 2), suggesting that calpain may have cleaved vinculin as well as talin (14), although no proteolytic fragments of vinculin were detected. SNP pretreatment of ionophore-treated cells significantly reduced both the increase in the talin proteolytic ratio and the loss of vinculin (Figs. 1 and 2), suggesting that NO inhibited the ionophore-induced increase in calpain activity. Neither ionophore nor ionophore + SNP treatment significantly altered the concentration of pro-m-calpain from control values (Fig. 2), suggesting that autolysis of the large subunit was not affected by these treatments.

Ionophore treatment of C2C12 myoblasts significantly increased cell detachment from the gelatin-coated dishes, as measured by the number of detached cells in the media (Fig. 3). Ionophore treatment also induced loss of total cellular protein as measured by the filter paper dye-binding assay (Fig. 3). These results suggest that calpain-mediated proteolysis, perhaps of the focal contact proteins talin and vinculin, induced cell detachment and loss of cellular protein.

SNP pretreatment significantly reduced both cell detachment and loss of total cell protein, suggesting that NO inhibition of calpain protected cells from detachment and loss of protein.

Some ionophore-treated cells were preincubated with the cell-permeable calpain inhibitors calpain inhibitor I and the 27-mer calpastatin peptide to verify that the measured talin proteolysis was mediated by calpain. Both calpain inhibitor I and the calpastatin peptide completely prevented the ionophore-induced increase in the talin proteolytic ratio (Fig. 4), indicating that ionophore-induced talin proteolysis was indeed calpain mediated.

### Table 1. Intact and large proteolytic fragment of talin in Triton X-100 insoluble and soluble fractions

<table>
<thead>
<tr>
<th></th>
<th>Insoluble</th>
<th></th>
<th>Soluble</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>235 kDa</td>
<td>190 kDa</td>
<td>235 kDa</td>
<td>190 kDa</td>
</tr>
<tr>
<td>Control</td>
<td>0.95 ± 0.03</td>
<td>0.40 ± 0.02</td>
<td>1.00 ± 0.03</td>
<td>0.51 ± 0.04</td>
</tr>
<tr>
<td>Ionophore</td>
<td>0.81 ± 0.04*</td>
<td>0.47 ± 0.02*</td>
<td>0.93 ± 0.02*</td>
<td>0.66 ± 0.04*</td>
</tr>
<tr>
<td>Ionophore + SNP</td>
<td>0.91 ± 0.07</td>
<td>0.35 ± 0.02</td>
<td>0.94 ± 0.04</td>
<td>0.47 ± 0.06</td>
</tr>
<tr>
<td>SNP</td>
<td>0.88 ± 0.04</td>
<td>0.39 ± 0.03</td>
<td>0.83 ± 0.09</td>
<td>0.43 ± 0.05</td>
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Values presented are means ± SE for n = 9 measurements. Note, values normalized to soluble 235-kDa band for each blot. SNP, sodium nitroprusside. *P < 0.05 compared with control.
Zymography. Casein zymography was used to determine the effects of the NO donor SNP on purified m-calpain activity. Clearing bands in the casein-loaded gel indicated calpain-mediated casein proteolysis and SNP was expected to inhibit calpain activity and reduce the amount of clearing. SNP showed a dose-dependent inhibition of calpain-mediated proteolysis, and the reducing agent DTT prevented this inhibition in a dose-dependent manner (Fig. 5A). In other experiments, DTT added to the zymography activation buffer reversed SNP-induced inhibition of calpain activity (data not shown). Together, the zymography data suggest that NO inhibits calpain activity by S-nitrosylation of the active site cysteine (1).

DISCUSSION

The major finding of this study was that the NO donor SNP inhibited calpain-mediated talin proteolysis and cytoskeletal breakdown in C2C12 muscle cells.
m-Calpain was found previously to be colocalized with talin in focal contacts of cultured cells, and talin was found to be a preferred substrate of m-calpain within focal contacts (4). Thus m-calpain proteolysis of talin was thought to play a role in focal contact physiology. Our data indicate that ionophore-induced talin proteolysis may release talin from the membrane into the cytosol. Because the 47-kDa domain of talin has been proposed to stabilize attachment to the membrane (32), loss of this domain after proteolysis could induce release of talin into the cytosol. The more severe treatment of ionophore-induced talin proteolysis, loss of vinculin, cell detachment, and loss of total protein. These data support the view that calpain proteolysis can induce focal contact disassembly, cytoskeletal disruption, and cell detachment. The observations that NO inhibited the ionophore-induced changes in both experiments suggest that NO inhibition of m-calpain can protect cells from cytoskeletal proteolysis. In muscle tissue, neuronal NO synthase is colocalized with talin at a focal contact analog, the myotendinous junction (7, 8, 42). Thus endogenously produced NO could play an important role in regulating calpain-mediated cytoskeletal proteolysis during remodeling at the myotendinous junction.

Our data are apparently in contrast with those of a previous investigation (43), which found only intact talin in the cytosol and only cleaved talin at the membrane. The differences between studies may be a result of the different methods used; the previous investigation measured newly synthesized talin that had been labeled with \(^{35}\)S for 2 h before cell fractionation. The short duration of labeling could account for the lack of cleaved talin in the cytosol, because the half-life of talin in the cytosol has been reported to be 14–15 h (27). However, it is difficult to reconcile the difference seen in the membrane fraction. We found mostly intact talin in the membrane, whereas the previous investigators found only cleaved talin. Proteolysis during isolation of the membrane fraction in the previous study could account for the difference in results. However, the cysteine protease inhibitor E-64 did not influence the relative amounts of intact and cleaved talin in the membrane fraction for the previous study, suggesting that proteolysis did not occur during isolation.

Our data also contrast with those of Michetti et al. (29), who found that NO inhibits ionophore-induced autolysis of an m-calpain-like isoform in situ in human neutrophils. We did not detect any change in autolysis of the large subunit of m-calpain in C\(_2\)C\(_1\)2 muscle cells with ionophore stimulation or SNP treatment, using an antibody specific for the propeptide of the large subunit of m-calpain (11). This finding is counter to the premise that autolysis occurs before proteolysis (10). However, recent studies have shown that substrate proteolysis can occur without large subunit autolysis of m-calpain (13, 23, 37). Thus autolysis may not be required for m-calpain to cleave talin.

Our finding that NO inhibits calpain by S-nitrosylation of the active site cysteine is consistent with previous reports of NO inhibition of cysteine proteases. NO has been reported to inhibit cathepsin B (40) and papain (44) via S-nitrosylation of the active site cysteine. Recently, caspase proteases have been found to be inhibited by S-nitrosylation of the active site cysteine and to be S-nitrosylated or denitrosylated by physiologically relevant stimuli (12, 28). Thus NO may be a common regulatory molecule of cysteine proteases in vivo. NO has also been reported to alter the activity of a number of nonprotease enzymes via S-nitrosylation of cysteine (e.g., glyceraldehyde-3-phosphate dehydrogenase, Ref. 31; aldolase, Ref. 33; alcohol dehydrogenase, Ref. 34). Thus S-nitrosylation of cysteine could represent a common posttranslational modification and regulatory mechanism for many enzymes with widely varying functions.

Oxygen free radicals have been reported to inhibit proteolytic activity of \(\mu\)-calpain (18, 19). For purified \(\mu\)-calpain, treatment with hydrogen peroxide was found to inhibit activity at all calcium concentrations, and this inhibition could be reversed with DTT (18). For neuroblastoma cells, treatment with the oxidants doxorubicin (which produces superoxide radicals) or 2-mercaptopyridine-\(N\)-oxide (which produces hydroxyl radicals) decreased ionophore-stimulated proteolysis of an exogenous cell-permeable fluorescent calpain substrate and the endogenous calpain substrates tau and spectrin (19). These oxidants also decreased the rate but not extent of \(\mu\)-calpain autolysis. Because the effects of oxidation on purified m-calpain activity were not studied, it is not clear whether oxidation of m-calpain contributed to the decrease in proteolysis of calpain substrates in situ. In short, oxygen free radicals as well as NO may represent novel regulatory molecules of calpain.

The finding that NO inhibits calpain-mediated cytoskeletal proteolysis in situ has many potential in vivo implications. In skeletal muscle, colocalization of NO synthase and talin at the myotendinous junction (8, 42) suggests that NO inhibition of calpain could be an important component in the regulatory system of myotendinous junction remodeling. Mechanical loading and activation of muscle, which increase the production of NO from the neuronal isoform of NO synthase in muscle cells (2, 41), could be expected to stabilize cytoskeletal attachments to the cell membrane via NO inhibition of calpain-mediated proteolysis. However, the quantity of NO that is generated in response to increases in muscle activation or loading that is available for calpain inhibition will also vary with the concentration and distribution of NO-scavenging molecules, which have not been measured in the present investigation. Thus an additional but unexplored mechanism through which modulation of NO activity can influence cytoskeletal protein stability would be through the regulation of the production and distribution of NO scavengers in vivo.

The use of calcium ionophore to increase cytosolic calcium concentrations in our experiments is likely to have induced an increase in intracellular calcium that is similar to that caused by massive mechanically or chemically induced injury in vivo. The ability of NO to
protect cells from ionophore-induced, calpain-mediated proteolysis suggests that NO produced endogenously could play a similar role in vivo. Previous findings showing that calpain proteolysis may play a role in injury or disease of skeletal muscle (5, 39), cardiac muscle (46), and brain (26) suggest that NO-mediated inhibition of calpain may provide a therapeutic approach to protect these tissues from injury or disease. This speculation has received support by recent observations that NO can protect skeletal (9) and cardiac (6) muscle from injury after ischemia-reperfusion; it is possible that the mechanism of protection in these studies may include NO inhibition of calpain.

This investigation was supported by National Aeronautic and Space Administration Grant NAG2-1193 and National Institutes of Health Grants AR-40343 and AR-08479.

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