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Endotoxin and interferon- γ inhibit translation in skeletal muscle cells by stimulating nitric oxide synthase activity

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Abstract

The purpose of the present study was to test the hypothesis that endogenous nitric oxide (NO) negatively affects translation in skeletal muscle cells after exposure to a combination of endotoxin (LPS) and interferon (IFN) γ . Individually LPS and IFN γ did not alter protein synthesis but in combination they inhibited protein synthesis by 80% in C2C12 myotubes. The combination of LPS and IFN γ dramatically down regulated the auto-phosphorylation of the mammalian target of rapamycin (mTOR) and its substrates S6K1 and 4EBP-1. The phosphorylation of ribosomal protein S6 was decreased whereas phosphorylation of elongation factor-2 (eEF-2) and raptor was enhanced consistent with defects in both translation initiation and elongation. Reduced S6 phosphorylation occurred 8–18 h after LPS/IFN γ and coincided with a prolonged upregulation of NOS2 mRNA and protein. NOS2 protein expression and the LPS/IFN γ –induced fall in phosphorylated S6 were prevented by the proteasome inhibitor MG132. The general NOS inhibitor L-NAME and the specific NOS2 inhibitor 1400W also prevented the LPS/IFN γ –induced decrease in protein synthesis and restored translational signaling. LPS/IFN γ down regulated the phosphorylation of multiple Akt substrates including the proline rich Akt substrate-40 (PRAS40) while enhancing the phosphorylation of raptor on an AMPK regulated site. The negative effects of LPS/IFN γ were blunted by the AMPK inhibitor compound C. The data suggest that in combination LPS and IFN γ induce a prolonged expression of NOS2 and excessive production of NO that reciprocally alters Akt and AMPK activity and consequently down regulates translation via reduced mTOR signaling.

Keywords

myotube; translation; mTOR; raptor; PRAS40; AMPK; Akt

INTRODUCTION

Skeletal muscle is a major reservoir of stored amino acids and energy substrates. During critical illness and inflammatory diseases, such as sepsis, muscle protein is targeted for degradation to generate amino acids to sustain the acute phase response and provide gluconeogenic substrates (1). In addition, protein synthesis is concomitantly decreased to maintain energy reserves in the muscle. The simultaneous loss of existing muscle protein due to enhanced proteolysis and a deficit in new protein synthesis defines the cachexia of critical illness (2–4). The presence of cachexia adversely affects morbidity and mortality in septic patients and may persist for weeks after the initiating traumatic event (5).

The decrease in muscle protein synthesis observed during sepsis has been prevented in rats by pre-treating with cytokine antagonists that block either the synthesis or receptor binding of interleukin (IL)-1 or tumor necrosis factor (TNF)- α (6, 7). Although the anti-cytokine approach ameliorated sepsis-induced mortality in rats when used prophylactically, these therapies lack efficacy in a clinical setting (8, 9). Therefore, more recent research efforts have focused on later and secondary mediators of septic shock.

Administration of endotoxin (lipopolysaccharide; LPS) to naïve rats enhances nitric oxide synthase (NOS) activity that is preferentially expressed in fast twitch (gastrocnemius) but not slow twitch skeletal muscle (soleus) similar to the fiber-type specific decrease in muscle protein synthesis that occurs in septic rats (10–12). In addition, LPS induces defects in muscle function and contractility (11). The origin of LPS-induced defects in contractility is unknown but may be related to enhanced nitrosylation of contractile and/or signaling proteins (13, 14). This possible mechanism is supported by data from mice with a targeted gene deletion of S-nitrosoglutathione (GSNO) reductase, the major GSNO metabolizing enzyme in eukaryotes. GSNO reductase null mice exhibit greater muscle damage and mortality when challenged with endotoxin than do wild-type littermates (15).

Activation of NOS2 can lead to NO levels 1000-fold greater than those generated by the constitutive isoforms and it is accepted that a pathological increase in NOS2 is responsible for hypotension and cardiac dysfunction associated with septic shock (16). Yet, non-selective NOS inhibitors increased mortality in a large phase III clinical trial of patients with septic shock suggesting that we do not fully understand the pathophysiology of NO during sepsis (17). We have examined the local expression of NOS enzymes in skeletal muscle and used NOS inhibitors in C2C12 myotubes, not as a treatment modality, but rather to determine the local role of NOS2 on translation initiation in this tissue.

To better define the role of LPS and cytokines in muscle wasting and to define a mechanism by which this occurs we examined whether the combination of LPS and IFN γ altered protein synthesis and mTOR signaling in C2C12 myotubes. We observed a marked 80% decrease in protein synthesis in response to LPS and IFN γ that was associated with reduced signaling via translation initiation and elongation factors, and enhanced expression of NOS2.

MATERIALS AND METHODS

Cell culture

The C2C12 mouse myoblast cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and used for all studies. Cells were grown in 100 mm Petri dishes (Greiner Bio-One, Frickenhausen, Germany) and cultured in minimal essential media (MEM) containing 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μ g/ml) and amphotericin B (25 μ g/ml) (all from Mediatech, Herndon, VA). Cells were grown to near confluence and sub-cultured to 6-well plates for all experiments. Cells were differentiated to myotubes in 10% bovine calf serum (BCS) and fresh serum-containing media was added to the cells prior to each experiment. In most experiments cells were differentiated in 10% BCS for ≥ 7 days to obtain cultures composed of greater than 90% myotubes. Experiments were performed with ultra-pure *Escherichia coli* LPS 011:B4 (Invivogen, San Diego, CA), mouse IFN γ (Biosource, Camarillo, TX), mouse TNF α , and IL-1 α (Peprotech, Rocky Hill, NJ). The concentration of LPS, cytokines and MG132 used in these experiments was based on dose-response curves generated in these and previous studies (18–20). Additional reagents were purchased from Calbiochem (San Diego, CA) including the following NOS inhibitors: N-nitro-L-arginine methyl ester (L-NAME), N5-(1-iminoethyl) L-ornithine (L-NIO), and N-(3-Aminomethyl) benzylacetamidine (1400W) and used at concentrations found in the literature or determined by dose-response curves.

Dexamethasone was used at a concentration previously shown to inhibit protein synthesis and S6 phosphorylation (21). Dulbecco's phosphate buffered saline (DPBS), glucose, and 100X MEM amino acids were obtained from Sigma Aldrich (St. Louis, MO).

RNA isolation and ribonuclease protection assay (RPA)

Total RNA, DNA and protein were extracted from C2C12 cells in a mixture of phenol and guanidine thiocyanate (TRI Reagent, Molecular Research Center, Cincinnati, OH) using the manufacturer's protocol. RNA was separated from protein and DNA by the addition of bromochloropropane and precipitation in isopropanol. After a 75% ethanol wash and resuspension in formamide, RNA samples were quantified by spectrophotometry. Ten micrograms of RNA was used for each assay. Riboprobes were synthesized from a custom multi-probe mouse template set containing a probe for NOS2, GAPDH, and L32 mRNA detection. The labeled riboprobe was hybridized with RNA overnight using an RPA kit and the manufacturer's protocol (Pharminigen). Protected RNAs were separated using a 5% acrylamide gel (19:1 acrylamide/bisacrylamide). Gels were transferred to blotting paper and dried under vacuum on a gel dryer. Dried gels were exposed to a phosphorimager screen (Molecular Dynamics, Sunnyvale, CA) and the resulting data were quantified using ImageQuant™ software and normalized to the mouse ribosomal protein L32 mRNA signal in each lane.

Western blot analysis

Cell extracts were electrophoresed on denaturing polyacrylamide gels and electrophoretically transferred to polyvinylidene fluoride (PVDF) with a semi-dry blotter (Bio-Rad Laboratories, Melville, NY). The resulting blots were blocked with 5% non-fat dry milk for 1.5 h and incubated with the following antibodies from Cell Signaling Technology (Beverly, MA): phosphorylated (p)S6 serine (S)235/236 (cat # 2211), pS6 S240/244 (cat# 4838), pS6K1 threonine(T)389 and pT421/S424 (cat # 9234 and 9204), pmTOR S2448 and T2481 (cat # 2971 and 2974), pEIF-4EBP1 T37/46 (cat # 9459), pAkt substrates (cat# 9618), pRaptor S792 (cat # 2083) and pEF-2 T56 (cat # 2321). The NOS2 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, cat # SC-650). Antibodies to mouse PRAS40 (cat # NON0684) and pT246 PRAS40 (cat # PS1011) were from InVivoGen (Carlsbad, CA) and EMD Biosciences (La Jolla, CA), respectively. Unbound primary antibody was removed by washing with Tris-buffered saline containing 0.05% Tween-20 and blots were incubated with anti-rabbit or anti-mouse immunoglobulin conjugated with horseradish peroxidase. Blots were briefly incubated with the components of an enhanced chemiluminescent detection system (Amersham, Buckinghamshire, England). Dried blots were used to expose x-ray film for 1–30 minutes to achieve a signal within the linear range. Each film was then scanned with a Microtek Scanmaker 4 scanner (Microtek, Cerritos, CA) equipped with a transparency tray to generate a digital image that was then analyzed and quantified with Scion Image 3b software (Scion Corporation, Frederick MD).

Protein synthesis

C2C12 myotubes were cultured as described above and stimulated with LPS and IFN γ , either separately or in combination for 10 h. Cells were then labeled with 2 μ Ci/well of [3 H]-phenylalanine (132 Ci/mmol, Amersham, Arlington Heights, IL) in the presence of a 6000-fold excess of unlabelled phenylalanine for 4 h as previously described (18). Cells were washed twice with MEM, isolated, and cell protein was precipitated overnight at 4 C in a final concentration of 10% trichloroacetic acid (TCA). TCA pellets were washed three additional times with 10% TCA, solubilized in 2 N sodium hydroxide and counted in Scintsafe™ liquid scintillation cocktail. Under these conditions protein synthesis measurements were linear with the amount of tracer added to the cells, the number of cells per well, and the incubation time. Although the absolute incorporation of [3 H]-phenylalanine

into protein was decreased by unlabelled phenylalanine the relative changes in protein synthesis were unaltered by adding additional cold phenylalanine to the cells up to a 24,000-fold excess.

Cell viability and nitrite assays

Cell viability was measured with the Cell Titer-Blue assay as suggested by the manufacturer (Promega, Madison, WI). This assay measures the ability of viable cells to reduce the indicator dye resazurin to resorufin which is highly fluorescent. Resazurin was added to myotubes and was quantitatively and linearly converted to resorufin over time. Myotubes were therefore grown in the absence or presence of LPS/IFN γ overnight and the ability of the cells to convert one half of the added resazurin to resorufin over the last 60 minutes of culture was determined. The relative fluorescence of resorufin at 590 nm was measured in a spectrofluorometer in bottom read mode when the cells were excited at 560 nm (Molecular Devices, Spectramax Gemini EM, Sunnyvale, CA).

Nitrite and nitrate accumulation in conditioned media was measured with an Assay Designs nitric oxide assay kit in which nitrate was converted to nitrite with nitrate reductase as described by the manufacturer (Assay Designs, Ann Arbor, MI). Absorbance at 540 nm was determined in a microplate reader (Bio-Tek Instruments, Winooski, VT).

Statistics

Values are means $\pm$ SEM. Unless otherwise noted, each experimental condition was tested in triplicate and each experiment was repeated at least twice. Data were analyzed by analysis of variance followed by Student-Newman-Keuls test. Statistical significance was set at $P < 0.05$.

RESULTS

Combination of LPS and IFN γ blunts phosphorylation of mTOR substrates and decreases protein synthesis

Growth factors and nutrients signal through mTOR to increase cell size. mTOR in turn phosphorylates S6K1 and 4EBP1 to directly alter protein translation. We determined whether LPS or cytokines could alter the phosphorylation of these mTOR substrates by treating C2C12 myotubes with either LPS alone, cytokines (TNF α and IL-1 α) alone, or the combination of LPS and IFN γ in the presence of 10% BCS. LPS, TNF α , and IL-1 α alone had no effect on the T389 phosphorylation of S6K1 (Figure 1, panel A). In contrast, the phosphorylation of T389 was completely abolished when myocytes were treated with a combination of LPS/IFN γ or IL-1 + TNF α + IFN γ . Phosphorylation of the T389 site closely correlates with S6 kinase activity and is consistent with the observed 95% decrease in the phosphorylation of its substrate ribosomal protein S6 in cells treated with the combination of LPS and IFN γ . The migration of S6K1 on SDS-PAGE gels was compressed by the combination of LPS and IFN γ but the total amount of S6K1 and S6 was unchanged (Figure 1). The synthetic glucocorticoid dexamethasone has previously been shown to inhibit S6K1 activity and T389 phosphorylation (22). However, in the current study, in the presence of 10% BCS, dexamethasone did not alter the phosphorylation of either S6K1 (T389) or S6 (S235/236). Phosphorylation of the T421/S424 site on S6K1 relieves pseudosubstrate inhibition and stimulates S6K1 activity. Phosphorylation of these residues was also decreased by the combination of LPS and IFN γ , whereas individually LPS and IFN γ were without effect on the pseudosubstrate phosphorylation sites (Figure 1, Panel B). The eIF-4E binding protein 4EBP-1 was also phosphorylated on T37/46 in the presence of 10% BCS and this primes the protein for subsequent phosphorylation, release of translation initiation factor 4E, and formation of the eIF4F complex with the 40S ribosome and eIF3. Together

LPS/IFN γ decreased the phosphorylation of the T37/46 site by 90% whereas dexamethasone was without effect.

Lastly, we determined whether LPS/IFN γ alters protein synthesis in the C2C12 cells as measured by the incorporation of [3 H] phenylalanine into protein. While neither LPS nor IFN γ alone altered protein synthesis the combination decreased protein synthesis by 80 % (Figure 1, Panel C).

LPS/IFN γ dose- and time-dependently decreases the phosphorylation of ribosomal protein S6

LPS alone did not alter the phosphorylation of rpS6. However in the presence of a constant amount of LPS (1 μ g/ml) IFN γ dose-dependently inhibited S6 phosphorylation. A decrease in pS6 was observed with a dose as low as 0.33 ng/ml of IFN γ and complete inhibition occurred at concentrations $\geq$ 1 ng/ml (Figure 2, Panel A). Similarly, in the presence of a constant amount of IFN γ (3 ng/ml), LPS dose-dependently inhibited S6 phosphorylation. A decrease in pS6 was observed with an LPS concentration as low as 10 ng/ml and complete inhibition occurred with 100 ng/ml LPS (Figure 2, Panel B). LPS and IFN γ did not reduce pS6 immediately but required at least 8 h when IFN γ was given as a pretreatment (Figure 2, Panel C).

LPS/IFN γ decreases mTOR and S6 phosphorylation coincident with increased NOS2 expression

LPS/IFN γ decreased phosphorylation of S6 on S235 and mTOR on both its autophosphorylation site (S2481) and an Akt/S6K1 consensus phosphorylation site (S2448) (Figure 3). The decrease in pS6 and pmTOR occurred independent of changes in the total amount of the two proteins. In contrast to the changes in mTOR and S6 phosphorylation, LPS/IFN γ dramatically increased the expression of NOS2 protein (Figure 2, panel C, and Figure 3). Moreover, the increase in NOS2 protein temporally preceded the reduction in pS6 (Figure 2, Panel C). Because we have previously shown that NOS2 expression is dependent upon the activation of nuclear factor kappa B (NF κ B) and inhibited when the proteasome/NF κ B inhibitor MG132 is used to prevent the degradation of the inhibitor of NF κ B (I κ B) we also asked whether MG132 could prevent the LPS/IFN γ -induced reduction in mTOR activity (19). NOS2 protein abundance and the drop in the phosphorylation of both S6 and mTOR were completely prevented by MG132 (Figure 3) suggesting that NOS2 up regulation may be required for the negative effects of LPS/IFN γ on mTOR signaling.

Although LPS/IFN γ dramatically up regulated NOS2 protein expression in C2C12 myotubes it had no effect on the total amount of NOS1 or NOS3 in these cells. In addition, LPS/IFN γ did not alter the phosphorylation of NOS3 on serine 1177 or threonine 495 two sites that regulate NOS3 activity. Finally, addition of cycloheximide to the cells blocked the expression of NOS2 and prevented the LPS/IFN γ -induced drop in pS6. These findings suggest that NOS-1 and -3 do not play a role in mTOR signaling but at this point we can not unequivocally rule out an additional role for these NOS proteins in the response of C2C12 cells to LPS/IFN γ .

LPS/IFN γ prolongs NOS2 mRNA expression

We have previously reported that LPS alone increases NOS2 mRNA expression in C2C12 cells but that the response is transient (19). Therefore, in the current study we compared the increase in NOS2 mRNA in cells treated with the combination of LPS/IFN γ to the response produced by each agent alone. LPS alone increased NOS2 mRNA 25-fold after 2 h but this response was significantly smaller than the 100- and 400-fold increase in NOS2 mRNA produced by the combination of LPS/IFN γ at 2 and 6 h, respectively (Figure 4, Panel A). In

combination, LPS/IFN γ also sustained the increased expression of NOS2 mRNA and protein for up to 18 h, whereas the response to LPS alone had waned by this time point (Figure 4, Panels A-C). Total S6 levels were unchanged.

NOS inhibitors prevent LPS/IFN γ –induced changes in mTOR, eEF2, and S6 phosphorylation

To determine whether secreted factors, such as cytokines, are necessary for the observed changes in the phosphorylation of S6, C2C12 cells were cultured with LPS/IFN γ for 5 h and then switched to either serum-free media or fresh media containing 10% BCS in the absence of immune activators. As illustrated in Figure 5, the LPS/IFN γ -induced drop in pS6 was not prevented when the media was changed to either serum-free or serum-containing media suggesting that autocrine secreted factors do not regulate S6 phosphorylation under these conditions. Secondly, because fresh media did not restore S6 phosphorylation it is unlikely that these changes are due to the depletion of amino acids, such as arginine, or serum growth factors such as insulin-like growth factor-I (IGF-I) because these would be replenished upon the addition of fresh media containing serum.

LPS/IFN γ also increased the T56 phosphorylation of eukaryotic elongation factor (eEF)-2 (Figure 5). This protein catalyzes the translocation of peptidyl-tRNA from the A site to the P site on the ribosome and its phosphorylation on T56 inhibits translational elongation. Removal of LPS/IFN γ after the initial 5 h enhanced eEF2 phosphorylation by a further 50% (Figure 5). In addition, incubation of myocytes with TNF binding protein, which neutralizes endogenous TNF α , also failed to prevent the LPS/IFN γ -induced decrease in S6 phosphorylation.

Because LPS/IFN γ increased NOS2 protein in the same time frame as its ability to decrease S6 phosphorylation we examined whether NOS inhibitors could block the fall in pS6. Pretreatment of myocytes with L-NAME inhibited the LPS/IFN γ -induced drop in pS6 and the rise in peEF2 (Figure 5). L-NAME also inhibited the LPS/IFN γ -induced drop in pmTOR on its autophosphorylation site and its Akt/S6K1 regulatable site. The ability of L-NAME to prevent the drop in mTOR/S6K1 signaling was manifest not only when given as a pretreatment but was also evident up to 8.5 h after the addition of LPS/IFN γ . L-NAME also significantly decreased the LPS/IFN γ -induced accumulation of nitrate/nitrite in the conditioned media of myotubes (Control: 1.19 ± 0.26 uM, LPS/IFN γ 10.6 ± 0.89 uM, LPS/IFN γ + L-NAME 2.93 ± 0.39 uM). Pretreatment of the cells with more specific NOS2 inhibitors including 1400W also inhibited the LPS/IFN γ -induced decrease in pS6 (Figure 6, Panels A and B). Both inhibitors were effective in the low micro molar range as compared to L-NAME which was capable of inhibiting LPS/IFN γ at a concentration of approximately 1 mM. The glucocorticoid dexamethasone also prevented the LPS/IFN γ -induced drop in pS6 consistent with its anti-inflammatory properties and ability to suppress NOS2 expression.

LPS/IFN γ alters the phosphorylation of Akt and AMPK substrates including the mTOR regulators PRAS40 and raptor

As mTOR activity is highly regulated by the upstream kinase Akt we next determined Akt activity by probing myocyte extracts with an antibody raised against a consensus site for Akt substrates. The antibody recognizes the motif RXRXXS/T where arginine is found in the -5 and -3 position relative to a phospho serine or threonine. LPS/IFN γ decreased the phosphorylation of a broad number of Akt substrates with molecular weights of approximately 85, 65, 52, 43 and 32 kDa (Figure 7, Panel A). The decrease in Akt substrate phosphorylation was prevented for these major substrates when C2C12 cells were pretreated with L-NAME.

We examined Akt phosphorylation to determine if upstream activation was compromised and found no difference in the level of Akt phosphorylation on the T308 site. In contrast, phosphorylation of S473 on Akt was dramatically reduced in myotubes cultured with LPS/IFN γ (Figure 7, Panel B). Tuberous sclerosis complex (TSC)-2 is phosphorylated by Akt on S939 and via a PI3 kinase-dependent pathway on T1462. We found that LPS/IFN γ decreased TSC phosphorylation on S939 but that this was associated with an equivalent fall in the total level of TSC2 such that the overall level of phosphorylation on this site and the T1462 site were unchanged or increased relative to the equal loading of total protein and GAPDH in each lane (Figure 7, Panel B).

Phosphorylation of TSC2 is thought to allow TSC1 and TSC2 to disassociate and stimulate mTOR activity. One would therefore expect diminished Akt activity to be associated with enhanced TSC1/TSC2 complex formation. Indeed transgenic expression of TSC1 stabilizes the TSC1/TSC2 complex and causes muscle wasting. Contrary to our prediction, there was no change in the amount of TSC1 bound to TSC2 when TSC2 was immunoprecipitated from LPS/IFN γ -treated C2C12 cells (Figure 7, Panel C). This suggests that LPS/IFN γ does not impact mTOR signaling at the level of tuberous sclerosis complex formation.

In contrast to the lack of an effect of LPS/IFN γ on the TSC proteins, LPS/IFN γ inhibited the phosphorylation of the Akt substrate known as proline rich Akt substrate (PRAS)-40 on T246 (Figure 7, Panel C). PRAS40 acts as an indirect inhibitor of mTOR complex 1 due to its interaction with raptor as evidenced by studies where PRAS40 is over-expressed. When PRAS40 is phosphorylated on T246 it is released from the mTOR complex permitting raptor to interact with and facilitate the phosphorylation of other mTOR substrates such as S6K1. Pretreatment of C2C12 cells with L-NAME restored PRAS40 phosphorylation on T246 consistent with its ability to re-establish Akt and mTOR signaling (Figure 7, Panel C). Similar findings were found with the NOS2 specific inhibitor 1400W which prevented the LPS/IFN γ -induced fall in S6 phosphorylation on both the S235/236 and S240/244 sites. Myotubes treated with LPS/IFN γ exhibited a 60% decrease in total PRAS40 when normalized to GAPDH. Therefore 2.5 times more extract was run to normalize for total PRAS40 in the LPS/IFN γ lanes. LPS/IFN γ still showed an additional decrease in the relative phosphorylation of PRAS40 (Figure 7, Panel C and D). 1400W prevented the LPS/IFN γ -induced fall in pPRAS40 suggesting restoration of Akt activity towards this substrate (Figure 7, Panel D).

Substrates of mTOR can bind to one of two proteins (raptor or rictor) in mTOR complex 1 and -2, respectively. Raptor therefore facilitates the selection of mTOR substrates involved in translation initiation such as S6K1 and 4EBP1 but this can be inhibited by energy stress and the 5'-AMP-activated kinase (AMPK). AMPK phosphorylates raptor on S792 and thereby limits the phosphorylation of mTOR substrates such as S6K1 (23). LPS/IFN γ increased the phosphorylation of raptor on S792 in C2C12 myotubes and this was prevented by inhibition of NOS2 activity (Figure 7, Panel D). The changes in raptor phosphorylation were independent of changes in the total amount of raptor and house keeping proteins such as GAPDH.

NOS inhibition prevents LPS/IFN γ –induced changes in myotube diameter

Cell stresses that inhibit mTOR activity are often associated with decreased cell size. We treated C2C12 myotubes with LPS/IFN γ for 15 h and stained the cells with Ponceau S to visualize cell protein. Myotubes stained red and their diameter was clearly decreased by LPS/IFN γ (Figure 8, Panel A). Although LPS/IFN γ decreased cell size it did not alter total DNA in the C2C12 myotubes consistent with the fact that myotubes derived from S6K1 null mice have a normal number of nuclei but are smaller. In addition, LPS/IFN γ did not alter the number of viable cells as measured by the release of lactate dehydrogenase (data not shown)

or the ability of myotubes to reduce the indicator dye resazurin to resorufin. When resazurin was added to myotubes it was quantitatively and linearly converted to resorufin over time and myotubes grown in the absence (100 ± 7.5 RFU) or presence of LPS/IFN γ (95 ± 9.9 RFU) had similar cell viability and/or metabolic activity as measured by this method.

To examine whether a NO donor could mimic the effect of LPS/IFN γ we treated C2C12 myotubes with sodium nitroprusside (SNP) and examined the phosphorylation of translational regulators. SNP decreased the phosphorylation of S6 similar to LPS/IFN γ (Figure 8, Panel B). In addition, SNP decreased basal protein synthesis in C2C12 cells and limited IGF-I-stimulated protein synthesis (Figure 8, Panel C).

LPS/IFN γ and nutrient deprivation decrease S6 phosphorylation by different mechanisms

Nutrient deprivation is a well characterized model in which mTOR activity and translation initiation are severely depressed. Therefore, we examined whether amino acid and glucose starvation would also decrease S6 phosphorylation via an L-NAME inhibitable mechanism. Myotubes grown in DPBS (ie. no glucose or amino acids, 4h) exhibited a dramatic reduction in S6 phosphorylation that was not reversed by L-NAME (Figure 9, Panel A). Nutrient deprivation was also associated with increased T56 phosphorylation of eEF2 consistent with other studies showing the glucose- and energy-dependent regulation of this phosphorylation site. The nutrient deprivation-induced rise in p-eEF2, like the fall in pS6, was not prevented by L-NAME (Figure 9, Panel A). As nutrient deprivation can also activate NF κ B signaling we tested whether MG132 could block the fall in mTOR signaling due to nutrient deprivation as it does for LPS/IFN γ . Pretreatment of C2C12 cells with MG132 did not prevent the nutrient deprivation-induced decrease in pS6 (Figure 9, Panel B).

In contrast to the effects of nutrient deprivation, repletion of total amino acids (TAA) to nutrient deprived cells dose-dependently increased S6 phosphorylation. Maximal stimulation of pS6 occurred at 50–100 % of the normal concentration of amino acids found in MEM (Figure 9, Panel C). Starvation of myocytes for 12 h dramatically down regulated S6 phosphorylation and this was reversible upon re-addition of a combination of amino acids and glucose (Figure 9, Panel D). In contrast, myocytes incubated with LPS/IFN γ followed by starvation decreased pS6 to undetectable levels, and addition of amino acids and glucose to the cells failed to re-stimulate S6 phosphorylation. These data suggest that LPS/IFN γ not only lowers the basal phosphorylation of S6 but that inflammation per se induces a state of nutrient resistance at the level of mTOR signaling.

Because pharmacological activators of AMPK such as 4-carboxamide-1- β -D-ribofuranoside (AICAR) are often used to mimic nutrient deprivation/energy stress we examined whether L-NAME could prevent the loss in mTOR signaling due to both LPS/IFN γ and AICAR. Both treatments inhibited S6 phosphorylation in myotubes and this was independent of a change in the total amount of S6 or myosin heavy chain (MHC) (Figure 10, Panel A). Whereas L-NAME prevented the LPS/IFN γ -induced fall in pS6, it failed to block the decrease produced by AICAR. LPS/IFN γ increased phosphorylation of raptor on its AMPK consensus site (S792) and L-NAME prevented the LPS/IFN γ -induced rise in p-raptor (Figure 10, Panel A). In contrast, AICAR increased raptor phosphorylation (S792) and this was not prevented by L-NAME. These data suggest that AICAR, by stimulating AMPK directly, alters mTOR signaling independent of the generation of NO.

To determine whether the LPS/IFN γ -induced decrease in mTOR signaling was mediated by activation of AMPK, cells were incubated with the AMPK inhibitor 6-[4-(2-piperidin-1-ylethoxy)-phenyl]-3-pyridin-4-pyrazol[1,5- α] pyrimidine (Compound C, 40 μ M). As illustrated in Figure 10 (Panel B), compound C limited the ability of LPS/IFN γ to decrease S6 and increase eEF2 phosphorylation. The altered phosphorylation of these proteins was

independent of changes in the total level of S6, eEF2, and B-tubulin. These results suggest that AMPK may mediate some of the negative effects of LPS/IFN γ on mTOR signaling in myotubes.

L-NAME inhibits the LPS/IFN γ -induced decrease in protein synthesis

C2C12 myotubes were grown in 10% BCS with either LPS alone, IFN γ alone, or LPS/IFN γ . After 10 h, the cells were pulse labeled with [3 H]-phenylalanine for 4 h to measure protein synthesis. Some cells were also treated with L-NAME to inhibit NOS activity. Vehicle, LPS, and IFN γ – treated cells all had equivalent levels of protein synthesis (Figure 11). In contrast to the above, the combination of LPS/IFN γ decreased protein synthesis by 80%. The NOS inhibitor L-NAME completely prevented the LPS/IFN γ -induced fall in protein synthesis.

DISCUSSION

Accumulating evidence from rodent studies suggests that LPS and sepsis alter translation initiation in skeletal muscle by disrupting mTOR signaling (24, 25). We have furthered these findings herein by demonstrating that a combination of LPS and IFN γ , but neither immune-modulator alone, disrupts mTOR signaling in cultured skeletal muscle cells. LPS/IFN γ decreased both the autophosphorylation of mTOR and the phosphorylation of an Akt/S6K1-regulated site on the enzyme. Depressed mTOR signaling extended to the mTOR substrates S6K1 and 4E-BP1, and these changes were comparable to those in skeletal muscle. Moreover LPS/IFN γ decreased the phosphorylation or altered the amount of several S6K1 substrates (i.e. S6, eIF4B, and PDCD4 (data not shown)) suggesting that S6K1 activity is also decreased.

Although LPS alone can induce NOS2 expression, LPS cannot independently diminish mTOR signaling or protein synthesis in myotubes. The combination of LPS and IFN γ was necessary to produce both the increase in NOS2 protein as well as the reduction in mTOR substrate phosphorylation and protein synthesis. Even though IFN γ alone does not alter NOS2 expression this cytokine dramatically enhanced the magnitude and duration of LPS-stimulated NOS2 mRNA and protein expression. LPS alone inhibits mTOR signaling in vivo but this signal is likely to be enhanced by the release of endogenous IFN γ (25, 26). In this regard, Di Marco et al have reported that IFN γ stabilizes the TNF α -induced increase in NOS2 mRNA leading to a greater loss of the muscle-specific transcription factor MyoD (27). Our analysis broadens this finding by demonstrating that IFN γ also acts synergistically with LPS to enhance the expression of NOS2 mRNA and protein. Pretreatment with IFN γ may prepare myotubes to respond to LPS with a more rapid increase in NOS2 based on the cytoplasmic localization of NOS2 RNA binding proteins such as HuR. As discussed below, the excessive production of NO is then likely to impair protein synthesis by multiple mechanisms.

Translation initiation and elongation are highly dependent on anabolic signals generated from growth factors and nutrients. NO inhibits growth factor signaling at several levels. Akt (also called PKB) is a putative target for NO action. Mice lacking Akt1 exhibit muscle atrophy and conversely over expression of Akt prevents the expression of the atrophy-related ubiquitin E3 ligases atrogin-1 and MuRF-1 (28–30). Furthermore, S6K1 is a critical target of Akt in growth factor and nutrient signaling. Deletion of S6K1 results in a reduction of muscle cell volume and S6K1 deficient cells fail to respond to stimulation by a membrane targeted form of Akt suggesting Akt is necessary for activation of S6K1 (31). NO donors rapidly inactivate Akt by S-nitrosylation and mutation of cysteine 224 to serine restores Akt activity

Regardless of the exact mechanism, our data shows that LPS/IFN γ inhibits Akt activity in C2C12 myotubes based on a dramatic decrease in the phosphorylation of Akt substrates recognized to contain a consensus Akt phosphorylation site. The most noteworthy Akt substrate which exhibited decreased phosphorylation is the protein PRAS40. PRAS40 is a newly recognized inhibitor of mTOR that interacts with the mTOR complex 1 protein raptor via its TOR signaling motif (32). Phosphorylation of PRAS40 by Akt facilitates its disassociation from raptor and promotes its binding to 14-3-3 proteins that sequester the inhibitor away from mTOR. Phosphorylation of PRAS40 on T246 was dramatically down regulated by LPS/IFN γ , consistent with decreased mTOR activity and protein synthesis.

Herein we report a strong temporal association between the expression of NOS2 protein and a fall in mTOR signaling in C2C12 cells treated with LPS/IFN γ . Maximal expression of NOS2 occurred 8–18 h after addition of LPS/IFN γ to the cells and the fall in mTOR signaling coincided with this time frame. In contrast, whereas LPS alone also induced NOS2, the increase was more transient, of smaller magnitude, and was ineffective at reducing mTOR signaling. Lastly, MG132 prevented the up regulation of NOS2 and restored mTOR signaling. It seems likely that MG132 prevents the activation of NF κ B by LPS and this in turn inhibits NOS2 gene expression. We have previously demonstrated that MG132 blocks LPS-induced NOS2 expression if added 30 minutes prior to LPS but is ineffective when added 120 minutes afterwards. LPS also increased NF κ B reporter activity in C2C12 cells and this was likewise inhibited by MG132 (19).

In contrast to the proteasome inhibitor which blocked the LPS-induced increase in NOS2 protein expression when given as a pretreatment, the NOS inhibitor L-NAME prevented derangements in mTOR signaling when introduced 5–8 h after the addition of LPS/IFN γ . These data are consistent with the idea that L-NAME inhibits NOS2 activity but not NOS2 expression. We cannot exclude the possibility that L-NAME affects other NOS isoforms but we confirmed these original findings by showing that a more specific NOS2 inhibitor (1400W) also prevented the LPS/IFN γ -induced fall in pS6. Lastly, we found that LPS/IFN γ had no effect on either the amount or phosphorylation of NOS1 and NOS3 protein. Overall our data are consistent with the inducible form of NOS (NOS2) mediating the effects of LPS/IFN γ in myocytes. Yet, over expression of NOS2 and silencing of NOS2 mRNA are required in future studies to determine whether NOS2 is both necessary and sufficient for LPS/IFN γ -mediated inhibition of mTOR signaling.

Because mTOR and S6K1 are major determinants of cells size we stained myotubes with Ponceau S to detect muscle protein. Myotubes treated with LPS/IFN γ exhibited a dramatic decrease in diameter compared to control cells whereas the number of nuclei and DNA were unchanged. Although cell shrinkage can affect translation we find that the decrease in protein synthesis precedes the morphological changes in cells size. In addition although osmolarity can also alter mTOR signaling we find that the effect of addition of glucose and total amino acids to myotubes can not be mimicked by the addition of other sugars (e.g. fructose and 2-deoxyglucose) or a mixture of non-essential amino acids (33). Moreover, we find that L-NAME consistently prevents LPS/IFN γ -induced morphological changes, changes in Akt and mTOR signaling, and changes in protein synthesis.

Muscle wasting during critical illness and sepsis is often compared to the loss of muscle mass during starvation. Yet, total parenteral nutrition does not stave off the loss of muscle during sepsis due to the development of insulin resistance (34). The data suggest the mechanism of tissue wasting during starvation and sepsis is inherently different when it comes to the responsiveness to nutrients.

In a cell culture model of starvation we observed that nutrient deprivation dramatically reduced S6 phosphorylation in C2C12 myotubes and the magnitude of the response was comparable to that produced by LPS/IFN γ . Yet, unlike LPS/IFN γ , L-NAME failed to prevent the drop in pS6 due to nutrient deprivation and AICAR suggesting these responses are NOS-independent. Starved myocytes responded to being replenished with amino acids with a 3-fold increase in S6 phosphorylation. In contrast repletion of amino acids and glucose to myocytes that had been pretreated with LPS/IFN γ failed to increase pS6. These results demonstrate a strong LPS/IFN γ -induced nutrient resistance in myocytes and this response is consistent with the muscle leucine resistance observed in rats administered endotoxin (25).

Our data show that both nutrient deprivation and LPS/IFN γ increase eEF2 phosphorylation in myocytes. Similar to the LPS/IFN γ -induced changes in pS6 the rise in eEF2 phosphorylation is diminished by L-NAME. In contrast, L-NAME failed to antagonize the increase in eEF2 phosphorylation due to nutrient deprivation. The mechanism appears to be innately different for the two stresses in that the LPS/IFN γ -induced rise in peEF2 is NOS-dependent whereas the starvation-induced increment in peEF2 is NOS-independent. It is also noteworthy that eEF2 activity may not only be modified by phosphorylation but that this elongation factor is also a substrate for S-nitrosylation (35, 36).

Many of the effects of LPS/IFN γ on mTOR signaling were replicated by the addition of an NO donor to C2C12 myotubes. SNP strongly inhibited the phosphorylation of ribosomal protein S6. In addition, SNP inhibited the basal level of protein synthesis and IGF-I-stimulated protein synthesis. These data suggest that NO mediates the negative effects of LPS/IFN γ treatment on both mTOR signaling and protein synthesis. Lee et al have shown that NO donors induce apoptosis in myoblasts and we find that although myotubes are more resistant to NO than myoblasts, that long-term treatment (>24 h) with LPS/IFN γ also induces continued expression of NOS2 and eventually cell death in myotubes (37). Therefore we were careful to perform our experiments on viable myotubes as evidenced by a lack of lactate dehydrogenase release from LPS/IFN γ treated cells and the fact that LPS/IFN γ failed to alter the metabolic activity and viability of myotubes as measured by the conversion of resazurin to resofurin.

Some of the negative effects of LPS/IFN γ on mTOR signaling are mediated by AMPK since they are reversed by the AMPK inhibitor compound C. Compound C also blocked the increase in eEF2 phosphorylation due LPS/IFN γ . One hypothesis is that the generation of NO, due to LPS/IFN γ , alters the activity of cytochrome C oxidase resulting in a decrease in the cellular level of ATP. AMPK may sense the NO-induced change in energy charge and signal through the TSC proteins to inhibit mTOR. The mechanism does not appear to be operational in our model because formation of the TSC1/TSC2 complex was not increased in myocytes incubated with LPS/IFN γ . This result suggests that AMPK affects other components of mTOR signaling.

NOS2 is also activated during sepsis in vivo where it negatively affects skeletal muscle mitochondrial function (19, 38). Nitric oxide-induced changes in the energy charge of myotubes may provoke an AMPK-dependent phosphorylation of raptor on S792. Phosphorylation of this site increases the association of raptor with 14-3-3 proteins and diminishes its ability to associate with mTOR substrates such as S6K1. Neutralization of raptor in this way may have the same effect as deletion of the raptor gene which decreases mTOR signaling, reduces muscle mass, and results in muscle dystrophy (39). The increase in S792 phosphorylation on raptor in LPS/IFN γ treated myotubes is consistent with an AMPK-dependent decrease in mTOR signaling due to LPS/IFN γ .

Although our data do not unequivocally demonstrate a direct causative link between the changes in mTOR signaling, S6K1, S6 phosphorylation and translation we have clearly demonstrated that the changes occur in the same time frame and that both the alterations in signaling and protein synthesis are reversible by the addition of inhibitors of NOS2 expression, L-NAME and more specific NOS2 inhibitors such as 1400W. In addition, the very dramatic decrease in protein synthesis is suggestive of a decrease in both translation initiation and elongation consistent with diminished mTOR signaling and increased phosphorylation of eEF2. Therefore, our study provides a molecular explanation for reduced mTOR signaling and protein translation in skeletal muscle cells exposed to LPS/IFN γ .

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Figure 1. The combination of LPS and IFN γ decreases phosphorylation of mTOR substrates and protein synthesis in C2C12 myocytes

A, C2C12 myotubes were treated with LPS (1 μ g/ml), IFN γ (3 ng/ml), TNF α (40 ng/ml), IL-1 α (40 ng/ml), dexamethasone (1 μ Mole/L), or a combination of LPS and IFN γ . Cell extracts were isolated after 12 h and run on SDS-PAGE gels with proteins transferred to PVDF membranes which were probed for pT389 S6K1, total S6K1, pS235/236 S6, or total S6 protein. B, Extracts were run and transferred as described above and probed for pT421 S6K1, total S6K1, pT37/46 4EBP-1, and total 4EBP-1. C, C2C12 myotubes were cultured as described above and stimulated with LPS, IFN γ , or the combination of the two for 10 h. Cells were then labeled with [3 H]-phenylalanine (Phe) for 4 h and the amount of radioactively labeled protein was determined as described in the Methods. Values are means $\pm$ SEM and are expressed as a percentage of the incorporation in control cells isolated at the same time point. Bars with different letters are statistically different ($P < 0.05$).

Figure 2. LPS/IFN γ decrease S6 phosphorylation dose-dependently in C2C12 myocytes

A, C2C12 myotubes were treated with LPS (1 μ g/ml) alone or in combination with an increasing amount of IFN γ . Cell extracts were isolated after 12 h and run on SDS-PAGE gels with proteins transferred to PVDF membranes and probed for pS235/236 S6 and total S6 protein. B, C2C12 cells were cultured with a constant concentration of IFN γ (3 ng/ml) alone or in combination with an increasing amount of LPS. C, C2C12 myotubes were pretreated with IFN γ overnight followed by LPS. Cell extracts were isolated 1 to 12 h after the addition of LPS and analyzed for pS235/236 S6, total S6, or NOS2 as described above. Panels are representative blots of two or more studies.

Figure 3. LPS/IFN γ -induced decrease in mTOR signaling is coincident with the expression of NOS2

C2C12 cells were treated with the proteasome inhibitor MG132 alone (20 μ M), the combination of LPS and IFN γ or pretreated with MG132 for 15 min to block the effect of LPS/IFN γ . Cell extracts were analyzed for pS235/236 S6 and total S6 protein as described above as well as two sites (S2448 and S2481) on mTOR, and NOS2. Protein expression blots are representative of two or more studies.

Figure 4. LPS/IFN γ sustains NOS2 mRNA expression in C2C12 myocytes

A, C2C12 myotubes were treated with either LPS or IFN γ alone or the combination for 2, 6, or 10 h. RNA was isolated in TriReagentTM and NOS2 mRNA content was determined from triplicate wells by ribonuclease protection assay. All data were normalized to L32 mRNA. Values are means $\pm$ SEM and are expressed as a fold increase compared to control cells isolated at the same time point. Errors bars are relatively small and are therefore not visible at this scale. B, An image of NOS2, L32, and GAPDH mRNA from the RPA at the 10 h time point is shown for comparison. C, Myotubes were cultured as above with a combination of LPS and IFN γ , isolated after 2, 6, 10, and 18 h and probed for NOS2 and pS6 protein. Total S6 levels were unchanged.

Figure 5. L-NAME inhibits LPS/IFN γ -induced changes in mTOR signaling

A, C2C12 myotubes were treated with a combination of LPS and IFN γ for 5 h and switched to either fresh SFMEM or MEM containing 10% BCS to remove immune activators. Subsets of these cells also had the NOS inhibitor L-NAME (2 mM) added at the 5 h time point. Cell extracts were isolated after 12 h and run on SDS-PAGE gels with proteins transferred to PVDF membranes. Membranes were probed for pS235/236 S6, total S6 protein, peEF2 T56, or total eEF2. Protein expression blots are representative of two or more studies.

Figure 6. Multiple NOS inhibitors block the LPS/IFN γ -induced fall in S6 phosphorylation
 A, C2C12 myotubes were treated with LPS and IFN γ or pretreated with various NOS inhibitors, 1400W (Panel A) or L-NIO (Panel B), to block the decrease in pS6. Cell extracts were isolated after 12 h and run on SDS-PAGE gels with proteins transferred to PVDF membranes. Membranes were probed for pS235/236 S6 and total S6 protein. Protein expression blots are representative of two or more studies.

Figure 7. LPS/IFN γ inhibits Akt signaling and phosphorylation of PRAS40

A, C2C12 myotubes were treated with LPS/IFN γ or LPS/IFN γ and L-NAME (2 mM) and isolated after 14 h. Cell extracts were probed for Akt substrates phosphorylated on serine (S) or threonine (T) within the context of the amino acids RXXRXXS/T where arginine is found in the -5 and -3 position. LPS/IFN γ decreased the phosphorylation of multiple proteins containing this phospho-motif relative to control extracts and L-NAME restored the phosphorylation of these proteins. B, Extracts from myotubes treated with LPS/IFN γ were also probed for pAkt serine 473, total Akt, pTSC2 S939 and T1462, total TSC2 and GAPDH. C, Myotubes were treated as described above and cell extracts isolated and immunoprecipitated with a TSC2 antibody. Immunoprecipitates were probed for either TSC1 or TSC2. Additional whole cell extracts were probed for total PRAS40 and PRAS40 phosphorylated on T246 as well as GAPDH. D, Some myotubes were also treated with a more specific inhibitor of NOS2 (1400W, 25 μ M) to prevent LPS/IFN γ induced changes in mTOR signaling. 1400W prevented the LPS/IFN γ -induced fall in pS6 on both the S235/236 and S240/244 sites and the drop in PRAS40 phosphorylation on the T246 site. Conversely, LPS/IFN γ increased the phosphorylation of raptor on an AMPK consensus site (S792) and this was also blocked by 1400W. Note that in panels C and D, 2.5 $\times$ as much extract was run in lanes 4–6 for the gels probed for total and phosphorylated PRAS40 to normalize the total amount of PRAS40 to that found in the other lanes compared to GAPDH.

Figure 8. LPS/IFN γ decreases myotube diameter

A, C2C12 myotubes were treated with LPS/IFN γ , L-NAME, or LPS/IFN γ and L-NAME. Cells were stained with Ponceau S for 3–4 minutes, rinsed twice with TBS and stored in TBS containing 20% glycerol. Phase contrast pictures of the stained myotubes were taken on an inverted microscope. B, Additional cells were treated with an increasing dose of sodium nitroprusside (SNP) and cell extracts isolated and probed for pS6 (S235/236) and total S6. C, Further cells were grown in 24-well plates, allowed to differentiate, and treated with IGF-I, SNP (100 μ M), or the combination in serum free media. Protein synthesis was measured by labeling with [3 H]-phenylalanine over a 4 h period and TCA precipitating labeled protein as described in the Methods. Values are means $\pm$ SEM and are expressed as a percentage of the incorporation in control cells isolated at the same time point. Bars with different letters are statistically different ($P < 0.05$).

Figure 9. Combination of LPS/IFN γ makes myotubes resistant to nutrient signaling

A, C2C12 myotubes were either kept in MEM with 10% BCS or switched to Dulbecco's phosphate buffered saline (DPBS) containing 2.2 g/L of sodium bicarbonate in the presence or absence of L-NAME (2 mM). Cell extracts were isolated after 4 h and run on SDS-PAGE gels with proteins transferred to PVDF membranes which were probed for pS235/236 S6, total S6, pT56 eEF2, or total eEF2. **B**, Some cells were switched to DPBS as described above and treated with MG132 (20 μ M) for 4h. Extracts were probed for pS6 and total S6. **C**, Some of the cells were nutrient deprived and then had MEM amino acids added back to the cells at 0–100% of the concentration of total amino acids normally found in the media. Extracts were run and transferred as described above and probed for pS235/236 S6 and total S6. Values are means $\pm$ SEM and are expressed as absorption units relative to cells grown in DPBS which was set to 1.0. The inset shows a blot for pS6 in nutrient deprived cells (DPBS) and cells re-stimulated with amino acids (AA). **D**, C2C12 myotubes were cultured as described with the exception that they were treated with LPS/IFN γ for 5 h followed by a switch to DPBS for 12 additional hours. Some of the nutrient deprived cells were re-stimulated with 1X total amino acids (TAA) and 1 mM glucose and the cell extracts isolated after 15 minutes. Extracts were run and transferred as described above and probed for pS235/236 and total S6.

Figure 10. AMPK inhibition partially blocks LPS/IFN γ -induced changes in mTOR signaling
 A, C2C12 myotubes were treated with either LPS/IFN γ or AICAR (1 mM) for 14 h in the presence or absence of L-NAME (2 mM). Cell extracts were isolated and probed for pS6, total S6, pRaptor (S792), total raptor or myosin heavy chain (MHC). B, Additional cells were treated with the AMPK inhibitor compound C (0–40 μ M) at the same time as LPS/IFN γ . Cell extracts were isolated and probed for pS6, total S6, peEF2, total eEF2 and B-tubulin.

Figure 11. L-NAME inhibits the LPS/IFN γ -induced decrease in protein synthesis in C2C12 cells
A, C2C12 myotubes were cultured as described above and stimulated with LPS, IFN γ , or the combination of LPS/IFN γ for 10 h. Cells were then labeled with [3 H]-phenylalanine for 4 h. Additional cells were pretreated with L-NAME (2 mM) prior to the addition of LPS and/or IFN γ . The amount of radioactively labeled protein was determined as described in the Methods. Values are means $\pm$ SEM and are expressed as a percentage of the incorporation in control cells isolated at the same time point. Bars with different letters are statistically different ($P < 0.05$).