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Expression of Endothelial Nitric Oxide Synthase in Human Peritoneal Tissue: Regulation by *Escherichia Coli* Lipopolysaccharide

MARÍA M. ARRIERO,* JUAN A. RODRÍGUEZ-FEO,* ÁNGEL CELDRÁN,* LOURDES SÁNCHEZ DE MIGUEL,* FERNANDO GONZÁLEZ-FERNÁNDEZ,* JOSÉ FORTES,† ANA REYERO,* OCTAVIO FRIEYRO,* JUAN C. DE LA PINTA,* ANGELES FRANCO,* CARLOS PASTOR,* SANTOS CASADO,* and ANTONIO LÓPEZ-FARRÉ*

**Cardiovascular Research and Hypertension Laboratory and †Department of Pathology, Fundación Jiménez Díaz, Madrid, Spain.*

Abstract. Changes in the expression of endothelial nitric oxide synthase (eNOS) in the peritoneum could be involved in the peritoneal dysfunction associated with peritoneal inflammation. Demonstrated recently in bovine endothelial cells was the existence of cytosolic proteins that bind to the 3′-untranslated region (3′-UTR) of eNOS mRNA and could be implicated in eNOS mRNA stabilization. The present work demonstrates that eNOS protein is expressed in human endothelial and mesothelial peritoneal cells. *Escherichia coli* lipopolysaccharide shortened the half-life of eNOS message, reducing eNOS protein expression in peritoneal mesothelial and endothelial cells. Moreover, under basal conditions, human peritoneal samples expressed cytosolic proteins that bind to the 3′-UTR of eNOS mRNA. The cytosolic proteins that directly bind to 3′-UTR were identified as a 60-kD protein. After incubation of human peritoneal samples with lipopolysaccharide, the binding activity of the cytosolic 60-kD protein increased in a time-dependent manner. Studies are now necessary to determine the involvement of this 60-kD protein in the regulation of eNOS expression in peritoneal cells and particularly its involvement in the peritoneal dysfunction associated with inflammatory reactions.

Nitric oxide (NO) is a gas that is generated in the endothelium through the metabolic conversion of L-arginine to L-citrulline by the endothelial nitric oxide synthase (eNOS) (1). Loss of endothelium-derived NO results in vascular abnormalities, including vasoconstriction, smooth muscle proliferation, activation of blood elements, and an increased extracellular matrix synthesis (2–5). Although initially eNOS protein was defined as a constitutive enzyme, it was later demonstrated that eNOS protein expression could be upregulated by pathophysiologic stimuli such as hypoxia, chronic exercise, and the growth state (6–8). Conversely, cytokines downregulate the expression of eNOS by destabilizing eNOS mRNA (9,10).

Regulation of mRNA stability has emerged as an important control mechanism for the regulation of mRNA levels. Although the mechanism for regulating the cellular stability of different genes has unique features, it seems that in each case, specific RNA sequences are required for the recognition of protein factors (11,12). Some of these sequences have been identified within the 3′-untranslated region (3′-UTR) of mRNA. The 3′-UTR interacts with transacting factors (regulatory proteins), which may affect the half-life of the mRNA. In this regard, we recently obtained new evidence that bovine aortic endothelial cells contain cytosolic proteins that form complexes with *in vitro* transcribed 3′-UTR eNOS mRNA (13). These cytosolic proteins seem to be involved in the stabilization of eNOS mRNA (13).

The mesothelium is a flat epithelial lining of the peritoneal, pleural, and pericardial cavities. Like the endothelium in the vessel wall, mesothelial cells provide a nonadhesive surface and gate the traffic of molecules and cells between the circulation and these body compartments. Moreover, mesothelial cells share a number of morphologic and functional properties with the endothelium, including the expression of several adhesive molecules and the production of cytokines, fibrinolytic agents, and vasodilator substances such as prostacyclin (14–16). However, whether eNOS protein is expressed in human mesothelial cells is not evident. Here we have shown that in human peritoneum, both capillary endothelial and mesothelial cells expressed endothelial-type NOS.

Peritoneal inflammation has been implicated as a major cause of morphologic and functional alterations of the peritoneum (17,18). Loss of eNOS expression in mesothelial and capillary endothelial cells may contribute to altering their functional properties. Therefore, we analyzed the effect of bacterial lipopolysaccharide (LPS) on the expression of eNOS protein in

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Correspondence to Dr. Antonio López-Farré, Cardiovascular Research and Hypertension Laboratory, Fundación Jiménez Díaz, Av Reyes Católicos, 2, Madrid 28040, Spain. Phone: 91-5504821; Fax: 91-5494764; E-mail: ALopeza@fjd.es

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human peritoneal samples, determining the presence of cytosolic proteins that specifically bind to the 3′-UTR eNOS mRNA.

Materials and Methods

Tissue Samples

Peritoneum samples were obtained from hernia sacs removed during indirect surgical hernia repairs. Biopsies were obtained from six patients under sterile conditions.

All patients gave oral informed consent. The peritoneum was carefully approached from the antemesothelial side. Routine pathologic examination of the visceral peritoneum disclosed signs of inflammation linked to neoplasia extension or diffuse ischemic lesions. The samples were quickly isolated with a curved surgical clamp and cut. We tried to avoid undue manipulation, particularly extended exposition and surgical handling, which usually cause tissue disruption. Peritoneal samples were washed with isotonic saline to remove the remaining blood and cut into similar portions. The peritoneal portions were preincubated in RPMI medium containing 10% fetal calf serum, 5 mM glutamine, 2×10^{-5} U/L penicillin, and 2×10^{-5} μg/L streptomycin during 1 h. Afterward, the medium was removed and replaced by fresh RPMI medium containing 10% fetal calf serum. LPS (10 μg/ml) was added for different periods of time. Peritoneal samples were then frozen in liquid nitrogen for molecular biology determinations or embedded in paraffin wax for immunohistochemistry analysis.

Western Blot Analysis

eNOS protein was analyzed in human peritoneum by Western blotting as described previously (19). Human peritoneal samples were homogenated and lysed in Laemmli buffer containing 2-mercaptoethanol (20). Equal amounts of protein (20 μg/lane) estimated by bicinchoninic acid reagent (Pierce, Rockford, IL) were loaded. To verify that equal amounts of proteins had been loaded in the gel, we ran and stained a parallel gel with identical samples with Coomassie to compare the intensities of protein bands. Proteins were separated on denaturing sodium dodecyl sulfate (SDS)-10% polyacrylamide gels. The separated proteins were then blotted into nitrocellulose (Immobilon-P, Millipore Corp., Iberica, Madrid, Spain). Blots were blocked overnight at 4°C with 5% nonfat dry milk in Tris-buffered saline-Tween (20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween-20). Western blot analysis was performed with a monoclonal antibody against eNOS (Transduction Laboratories, Lexington, UK). Blots were incubated with the first antibody (1:2500) for 1 h at room temperature and, after extensive washing, with the second antibody (horseradish peroxidase-conjugated anti-mouse Ig antibody) at a dilution of 1:1500 for 1 h. Specific eNOS protein was detected by enhanced chemiluminescence (ECL; Amersham Iberica, Madrid, Spain). Prestained protein markers were used for molecular mass determinations.

Immunohistochemistry

eNOS expression was also detected by immunohistochemistry. Human peritoneal pieces were incubated in the presence and in the absence of LPS (10 μg/ml) for 24 h. Then they were dehydrated with sequentially increasing concentrations of ethanol followed by xylene and embedded in paraffin wax. Paraffin blocks were sectioned at 4 μm on a standard rotary microtome (Leitz 1512, MICROM GmbH, Walldorf, Germany), and the sections were recovered from a water bath on acid-alcohol–cleaned slides. Sections were stained with hematoxylin and eosin using an automated staining system.

The sections were incubated further with phosphate-buffered saline containing 3% normal goat serum (ICN Biochemicals Ltd, Barcelona, Spain) and 0.1% Triton X-100 for 30 min and subsequently with a monoclonal antibody against eNOS protein (Transduction Laboratories) at the dilution of 1:20 for 1 h or monoclonal antibodies to identify mesothelial (HBME-1) and endothelial (factor VIII) cells. The sections were washed in phosphate-buffered saline containing 0.05% bovine serum albumin, then incubated with biotinylated antiserum to goat IgG (1:300 dilution, Vector Laboratories Ltd, Madrid, Spain) for 1 h and in a solution of peroxidase-linked avidin-biotin complex (ABC kit, Vector Laboratories Ltd) for 1 h as previously reported (19,21). To reveal the peroxidase activity, the nickel-enhanced diaminobenzidine procedure was used.

Northern Blot Analysis

To test the stability of mRNA, human peritoneal samples were preincubated with actinomycin D (10 μg/ml) for 1 h. Then, human peritoneal samples were incubated in the presence and in the absence of LPS (10 μg/ml) during different periods of time. Each experiment was performed four times. Total RNA was isolated according to the method of Chomazynski and Sacchi (22). Twenty micrograms of RNA were fractionated in 1.3% agarose-formaldehyde gels and transferred by capillarity to Genescreen nylon membranes (Dupont, Boston, MA). The membranes were prehybridized at 42°C for 4 h in a solution containing 50% formamide, 1% SDS, 5× SSC (1× SSC is 150 mM NaCl plus 150 mM sodium citrate), 1× Denhardt's solution, 100 μg/ml denatured herring sperm DNA, and 50 mM phosphates (pH 6.5) and hybridized for 16 to 18 h in the same solution supplemented with dextran sulfate (10% final concentration) containing 500,000 cpm of radiolabeled eNOS probe per milliliter. The cDNA probe used was the *Hin*dIII-*Bam*HI fragment of pNOS UTR-L (see below). The equal loading of RNA was confirmed by the ethidium bromide staining of 28S and 18S rRNA.

Plasmids and In Vitro *Transcription*

Oligonucleotides complementary to eNOS cDNA (GenBank accession number, BTNIOXSY) were purchased from Bio-synthesis, Inc. (Lewisville, TX). pNOS-UTR plasmids were prepared as previously reported (13). In brief, oligonucleotide 1 (5′-GGATCTAGAACGCTATCACGAGGACATT-3′) and oligonucleotide 2 (5′-AGGAAGCTTAGTAGGTCTCCTAACTTCTG-3′) were used to produce by reverse transcriptase PCR (from BAEC total RNA) a fragment covering 166 bases of the coding region and 393 bases of the 3′-UTR of eNOS cDNA (from 3485 to 4012). Amplification products were purified after agarose gel electrophoresis, subjected to restriction endonuclease digestion with *Xba*I and *Hin*dIII and ligated to pGEM4Z (Promega, Madison, WI) to create plasmid pNOS-UTR-L. To produce single-stranded RNA, plasmids were linearized with the corresponding restriction enzyme and transcribed with SP6 or T7 RNA polymerase. Radiolabeled RNA was produced according to the manufacturer's recommendations (Promega) with ^{32}P-CTP (Amersham Iberica).

Band-Shift Assays

After incubation with LPS (10 μg/ml) for different periods of time, the human peritoneal samples were frozen in liquid nitrogen. Afterward, the samples were pulverized and resuspended in hypotonic buffer (25 mM Tris-HCl [pH 7.9], 0.5 mM ethylenediaminetetraacetate, and 1 mM phenylmethylsulfonyl fluoride) followed by four freezing and thawing cycles and by centrifugation at 12,000 g at 4°C for 15 min. The supernatant was removed, supplemented with glycerol (10% final concentration), and frozen at −70°C until use. The protein content of the cytosolic extracts was determined by the bicin-

choninic acid reagent (Pierce) as described by the manufacturer. Cytoplasmic lysates (10 μg) were incubated with 5 to 10 × 10^4 cpm of radiolabeled UTR-L in 15 mM HEPES (pH 7.9), 10 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 μg of yeast tRNA per microliter, 40 U of RNAsin (Promega), and 10% glycerol in a total volume of 15 μl for 10 min at 25°C. Twenty units of RNase T1 per reaction (Life Technologies-BRL, Eggenstein, Germany) were then added, and the reaction mixtures were incubated for 30 min at 37°C. Samples were electrophoresed on 4% native polyacrylamide gel in 0.25 × TBE (Tris-Borate-ethylenediaminetetraacetate) as running buffer, dried, and autoradiographed with Kodak X-OMAT-S film (Madrid, Spain).

UV Cross-Linking of RNA-Protein Complex

Ten micrograms of the cytoplasmic lysates were incubated with 10^5 cpm of RNA in the same buffer described above in a total volume of 20 μl for 10 min at 25°C. The samples were transferred to 20-well U-bottom plates and UV irradiated in ice in a Stratalinker (Stratagene LTD, Cambridge, UK) for 20 min on the automatic setting, followed by RNase digestion (20 μg of RNase A and 20 U of RNase T1 for 30 min at 37°C). The samples were then heated for 10 min at 70°C in Laemmli buffer (20) without 2-mercaptoethanol and electrophoresed on denaturing SDS-polyacrylamide gel. After drying, the gels were exposed for 3 to 5 d to Kodak film with two intensifying screens.

Statistical Analyses

Results are expressed as means ± SEM. Unless otherwise stated, each value corresponds to a minimum of six different experiments. To determine the statistical significance of our results, we performed an ANOVA with Bonferroni's correction for multiple comparisons or a *t* test (paired or unpaired). A P value < 0.05 was considered statistically significant.

Results

eNOS Expression in Human Peritoneum: Effect of LPS

Western blot analysis using a specific monoclonal antibody against eNOS demonstrated the presence of a 140-kD band in the homogenate of human peritoneum (Figure 1), indicating the presence of eNOS protein. The specificity of the eNOS monoclonal antibody used was studied further. The monoclonal antibody did not cross react with the neuronal-type constitutive isoform (155 kD) because the band of eNOS protein was undetectable in a homogenate of rat pituitary (Figure 2, top), which was previously positively stained with a monoclonal antibody against the neuronal-type NOS isoform (Figure 2, bottom). The monoclonal antibody used specifically recognized the eNOS isoform (140 kD) expressed in homogenates of human umbilical endothelial cells (Figure 2, top).

LPS (10 μg/ml) reduced eNOS protein expression in human peritoneum (Figure 1). A marked decrease in eNOS protein expression was found 6 h after exposure to LPS, and the maximal decrease in eNOS protein expression was observed after 24 h (Figure 1).

Northern blot analysis of total RNA obtained from human peritoneal samples demonstrated the presence of eNOS mRNA (Figure 3). The eNOS mRNA half-life was determined in the presence of actinomycin D (10 μg/ml), an inhibitor of transcription. Under basal conditions, the eNOS mRNA half-life was higher than 24 h. Stimulation of human peritoneum with LPS (10 μg/ml) markedly shortened eNOS mRNA half-life to less than 6 h (Figure 3). A marked decrease in eNOS mRNA expression was in fact observed 6 h after LPS incubation with nearly no detectable message 24 h after LPS incubation (Figure 3).

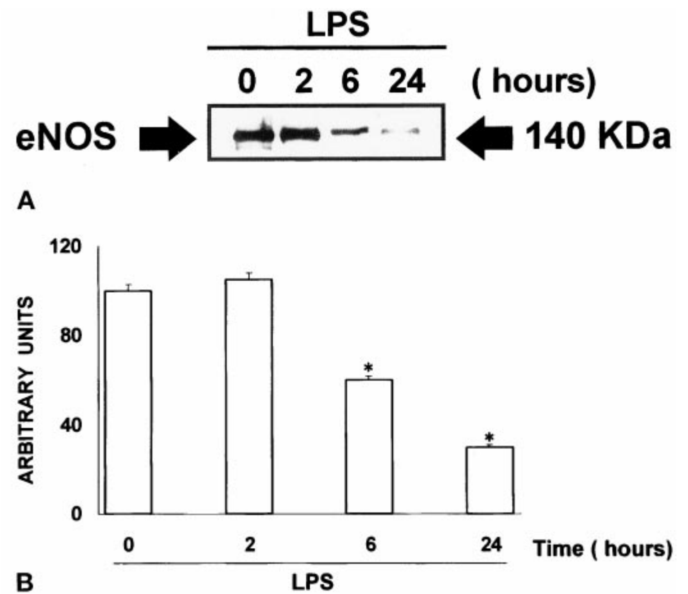

Figure 1. (A) Western blot demonstrating the expression of endothelial nitric oxide synthase (eNOS) protein in human peritoneal samples after exposure to lipopolysaccharide (LPS) (10 μg/ml) for different periods of time. (B) Bar graph showing the densitometric scanning of the Western blot. Results are represented as mean ± SEM of six different experiments. *$P < 0.05$ with respect to basal.

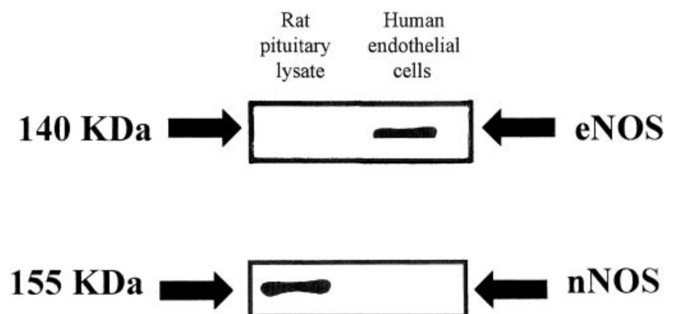

Figure 2. Western blot that demonstrates the specificity of the monoclonal antibody used in the experiments to recognize the eNOS protein. The monoclonal antibody did not recognize the neuronal-type NOS in rat pituitary homogenate, but it specifically recognized the eNOS isoform in homogenates of human umbilical vein endothelial cells (top). The bottom of the figure shows a Western blot that demonstrates the presence of the neuronal-type NOS (155 kD) in rat pituitary lysate, and it does not detect it in homogenates of human endothelial cells.

Immunolocalization of eNOS Protein

The distribution of eNOS protein was studied before and after 24 h of exposure to LPS (10 μg/ml). Positive immunostaining for eNOS protein was observed in peritoneal capillary endothelium and in the mesothelium (Figure 4, A and B). This

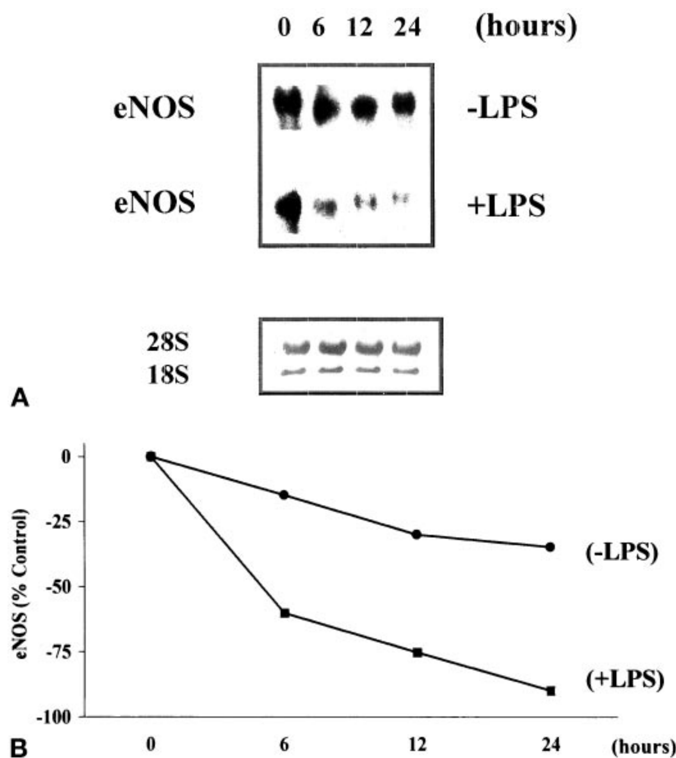

Figure 3. (A) Representative Northern blot analysis of eNOS mRNA expression in human peritoneal samples incubated in the presence and in the absence of LPS (10 μg/ml) for different periods of time. All of the experiments were performed in the presence of the transcriptional inhibitor actinomycin D (10 μg/ml). Equal loading of RNA was confirmed by the ethidium bromide staining of 28S and 18S rRNA. Each experiment was performed four times. (B) Autoradiographic mRNA signals were quantified by scanning densitometry and corrected relative to 18S rRNA. The density of the hybridization signal was presented on the ordinate as a percentage of the control values immediately before the addition of actinomycin D.

was confirmed by the fact that eNOS protein was co-localized in cells positively stained by HBME-1, an anti-human mesothelial cell antibody (Dako A/S, Glostrup, Denmark) which reacts with an incompletely characterized membrane antigen present on the microvillous surface of mesothelial cells (23). These cells were negatively stained by factor VIII (Dako A/S), suggesting the presence of mesothelial cells (Figure 4, C and D). Furthermore, eNOS protein was also expressed in cells positively stained by factor VIII and negatively by HBME-1, indicating the presence of endothelial cells (Figure 4, C and D). The histologic examination also revealed that LPS reduced eNOS protein expression in both peritoneal endothelial and mesothelial cells (Figure 5). In all experiments, control immunostaining in which the primary antibody was replaced with normal rabbit serum produced no positive signal (data not shown).

Importance of Tumor Necrosis Factor-α in the Effect of LPS on eNOS Expression

Resident peritoneal macrophages, although low in number, were present in human peritoneum, as demonstrated by the positive stain for CD68 antigen (Figure 6), a monocyte/macrophage marker (24). LPS-activated macrophages are a recognized source of cytokines such as tumor necrosis factor-α (TNF-α). Moreover, it has been recently demonstrated that TNF-α reduces eNOS expression in cultured bovine endothelial cells (9,10,13). Therefore, we analyzed the involvement of TNF-α in the abovementioned effects of LPS on eNOS protein expression. We first analyzed the ability of the peritoneal samples to produce TNF-α. TNF-α was measured in the RPMI medium obtained after the incubation of the peritoneal samples for 24 h at 37°C. As previously reported (25), TNF-α was determined by an enzyme-linked immunosorbent assay kit (Chromogenix, Mölndal, Sweden) in which the intra- and interassay variabilities were 1.1 and 4.7%, respectively. Under basal conditions, the production of TNF-α by human peritoneum was undetectable and it was significantly stimulated after incubation with LPS. We then determined whether an anti–TNF-α antibody could modify the decrease in eNOS expression mediated by LPS on human peritoneum. Addition of a polyclonal anti–TNF-α antibody (1:400) to 24-h LPS-incubated peritoneum slightly prevented the reduction of eNOS protein expression (Figure 7). This effect was not observed when a nonspecific IgG was used (Figure 7). The efficacy of the polyclonal antibody to inhibit TNF-α activity was previously demonstrated by its ability to prevent the reduction of eNOS protein expression produced by the addition of TNF-α ($<$500 pg/ml) to cultured endothelial cells (25). Moreover, an increased concentration of anti–TNF-α antibody (1:200) failed to demonstrate a greater level of prevention of eNOS expression than that obtained with 1:400 anti–TNF-α antibody (data not shown).

Peritoneal Cytosolic Proteins and 3′UTR of eNOS mRNA

In a previous work, we demonstrated that bovine aortic endothelial cells contain cytosolic proteins that form complexes with 3′-UTR eNOS mRNA, which could be involved in destabilization of eNOS mRNA (13). Addition of peritoneal cytoplasmic extracts to a labeled probe containing the entire 3′-UTR eNOS mRNA, the UTR-L probe, resulted in a gel-shifted band (Figure 8A, lane 1).

Cytosolic extracts from human peritoneum were isolated further after *in vitro* exposure to LPS for different periods of time. These cytosolic extracts showed an increased time-dependent binding to the labeled UTR-L probe (Figure 8A, lanes 2, 3, and 4). Complex formation showed a significant increase after 6 h of LPS incubation and demonstrated the highest binding activity at 24 h (Figure 8A, lanes 2, 3, and 4).

The complex between UTR-L and the cytosolic proteins obtained from LPS-incubated human peritoneal samples was prevented by an excess (1000 ng) of unlabeled UTR-L (Figure 8B, lane 2). Treatment of peritoneal cytosolic extracts with proteinase K (87 μg/ml) before their incubation with the UTR-L abolished the complex formation, thus indicating the involvement of cytosolic proteins in the eNOS mRNA peritoneal cytosolic extract interactions (Figure 8B, lane 3).

To characterize the peritoneal cytosolic proteins that interacted with 3′-UTR eNOS mRNA, we performed UV cross-linking

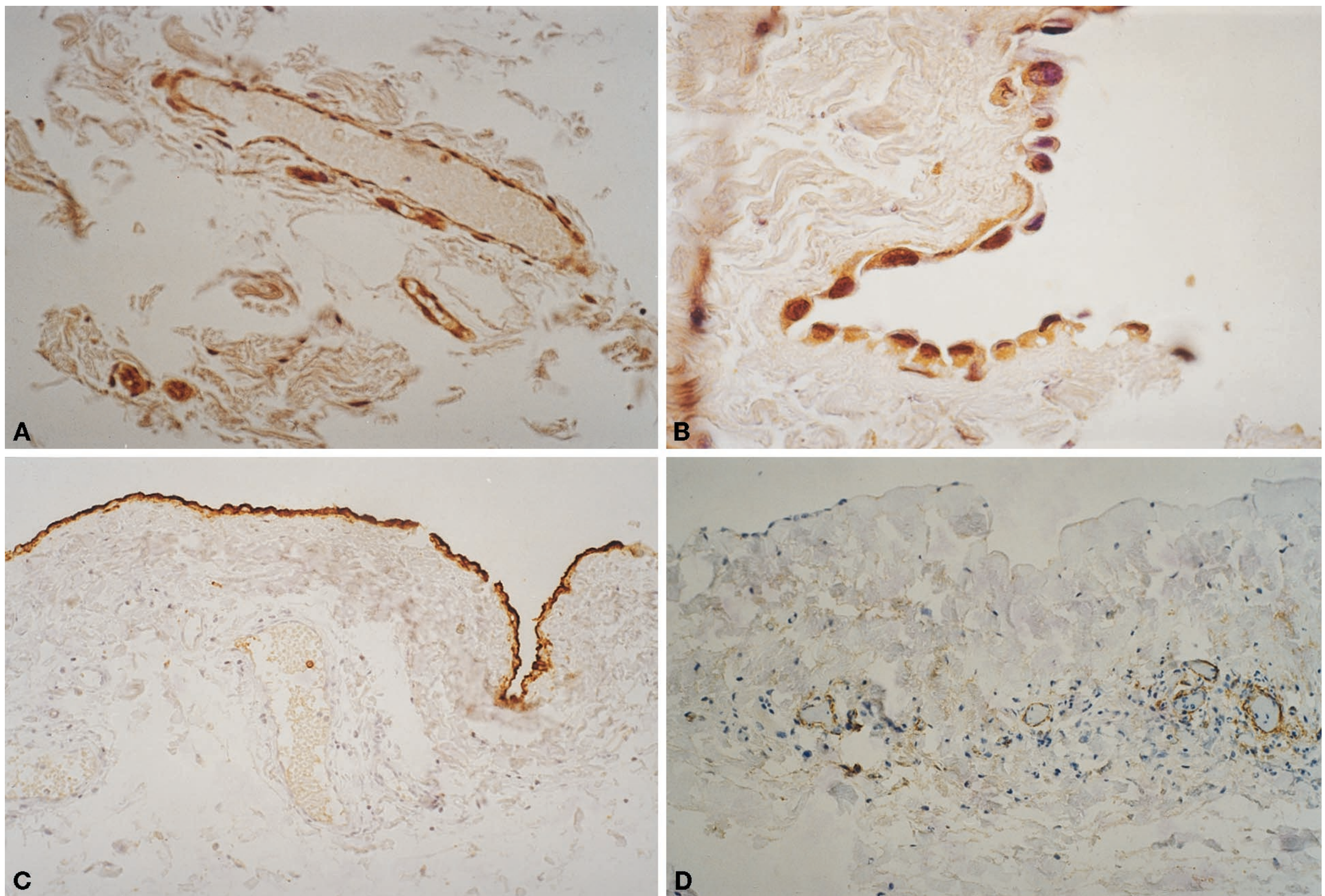

Figure 4. Cross sections of human peritoneum. The expression of eNOS protein was detected by a monoclonal antibody against eNOS in paraffin sections. eNOS protein was detected in peritoneal endothelial cells (A; magnification, 400×) and mesothelial cells (B; magnification, 400×), because eNOS protein staining was co-localized in cells showing positive immunostaining for an antihuman mesothelial antibody, HBME-1 (C; magnification, 200×) and in cells showing positive immunostaining for factor VIII (D; magnification, 200×).

experiments followed by SDS-polyacrylamide gel electrophoresis analysis. A band with an apparent molecular weight of 60 kD was observed in the mixtures of labeled 3-UTR-L and peritoneal cytosolic extracts exposed to UV light (Figure 9, lane 1).The intensity of the 60-kD band was markedly enhanced after 24 h in LPS-incubated human peritoneal samples (Figure 9, lane 2). The 24-h incubation time was chosen because the maximal binding activity induced by LPS was observed at this time (Figure 8A, lane 4).

Discussion

In the present study we demonstrate that both human peritoneal mesothelial and endothelial cells express eNOS protein that is modulated by LPS. Furthermore, we also show that peritoneal cytosolic extracts contain a 60-kD protein that interacts with the 3′-UTR of eNOS mRNA. The precise identification of the cells expressing eNOS protein in the human peritoneum demonstrated positive eNOS immunostaining in both HBME-1–positive mesothelial and factor VIII–positive endothelial cells. The immunohistochemical study also demonstrated that LPS reduced eNOS protein in both endothelial and mesothelial cells.

Various studies have demonstrated a potent destabilization of eNOS mRNA in TNF-α–stimulated endothelial cells, (9,10,13). In the present work, LPS reduced eNOS mRNA in human peritoneal tissue. This effect was observed in the presence of actinomycin D, suggesting that a change in stability of the eNOS message may be the predominant mechanism for the LPS-induced downregulation of its expression. In addition, a correlative decrease in eNOS protein expression was observed in the LPS-treated peritoneal samples. It is of note that under basal conditions, eNOS mRNA was very stable. A similar observation was previously reported for cultured endothelial cells (10). However, the present experiments will not allow us to rule out a possible effect of TNF-α on eNOS gene transcription in human peritoneum until formal experimental runoff studies are performed.

It has been demonstrated that LPS stimulated TNF-α production by macrophages (26). In this regard, we recently demonstrated that TNF-α reduces eNOS mRNA stability in cultured endothelial cells (13). Therefore, TNF-α could be involved in the observed reduction of eNOS expression elicited

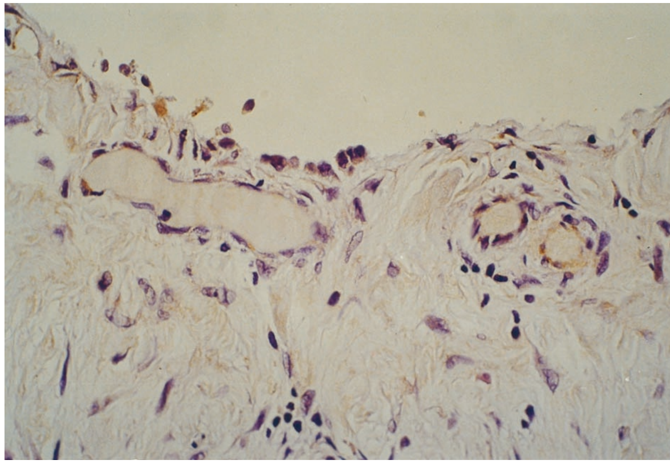

Figure 5. Cross sections of human peritoneum. The expression of eNOS was detected by a monoclonal antibody against eNOS protein in paraffin sections previously incubated in the presence and in the absence of LPS (10 μg/ml) for 24 h. Magnification, 200×.

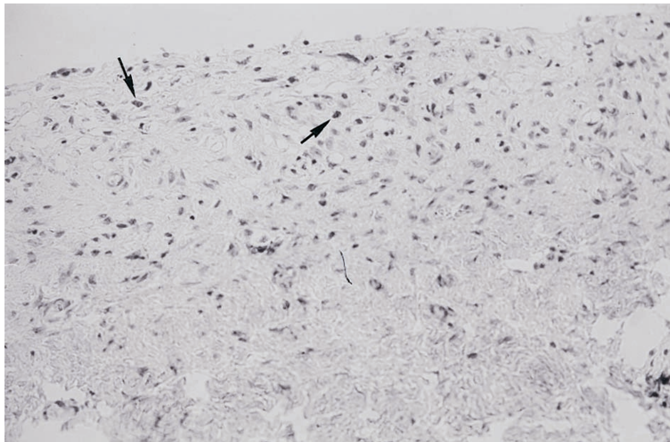

Figure 6. Cross section of human peritoneum showing the presence of macrophages that were positively stained with CD68 antigen (arrows).

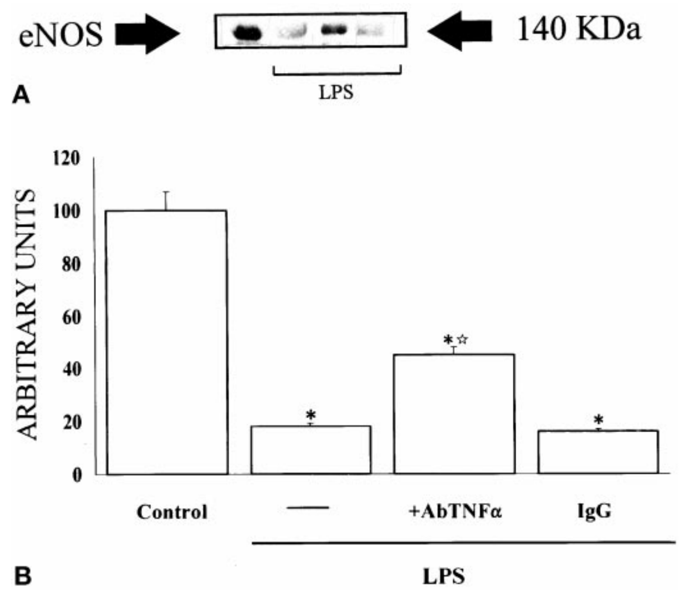

Figure 7. (A) Representative Western blot that shows the eNOS protein expression in 24-h LPS (10 μg/ml)-incubated peritoneal samples. The experiments were performed in the presence and in the absence of a polyclonal anti–tumor necrosis factor-α (TNF-α) antibody (AbTNF-α, 1:400) and a nonspecific IgG. (B) Bar graph showing the densitometric scanning of the Western bot. Results are expressed as mean ± SEM of six different experiments. * $P < 0.05$ with respect to basal (control). ☆$P < 0.05$ with respect to LPS-incubated peritoneal samples.

by LPS in the human peritoneal samples. Despite that immunohistochemistry examination demonstrated the presence of macrophages in the peritoneal tissue, the addition of an anti–TNF-α antibody to LPS-incubated peritoneal samples slightly prevented the decrease in eNOS protein expression. These results suggest that TNF-α has a modest participation in the effect of LPS on eNOS expression.

Several proteins that bind to specific sequences in the 3′-UTR of many mRNAs have been implicated in the regulation of their half-life (11,12). In a previous study, we demonstrated that the cytosol of bovine endothelial cells contains proteins that interact with 3′-UTR of eNOS mRNA (13). In the present study, we demonstrated that human peritoneal cytosolic lysates form complexes with the complete *in vitro* transcribed 3′-UTR eNOS mRNA. Furthermore, cytosolic lysates obtained from LPS-incubated human peritoneal samples showed an increased binding activity to the 3′-UTR eNOS mRNA. The enhanced binding activity of the cytosol obtained from LPS-stimulated human peritoneal samples was associated with a decreased level of eNOS protein and eNOS mRNA expression.

The cytosolic components of human peritoneum that bind to the 3′-UTR of eNOS mRNA were proteins. This was suggested by the fact that preincubation of the human peritoneal cytosolic extracts with proteinase K fully inhibited complex formation with the 3′-UTR of eNOS mRNA. The UV cross-linking and SDS-polyacrylamide gel electrophoresis experiments using the labeled 3′-UTR eNOS mRNA showed a band with an apparent molecular weight of 60 kD. These results suggested that a 60-kD protein binds specifically to 3′-UTR eNOS mRNA.

Functional evidence demonstrating the involvement of the 60-kD protein in eNOS mRNA destabilization is not shown in the present work. Therefore, we may establish only an association between the presence of the 60-kD protein induced by LPS and the eNOS mRNA destabilization. In this regard, the activity of other reported 3′-UTR binding proteins correlated inversely with the destabilization of mRNA (27,28). Moreover, the molecular weight of the 60-kD eNOS mRNA binding protein was within the range of other previously reported 3′-UTR binding proteins. In this regard, proteins of apparent molecular weights between 30 kD and 141 kD have been reported to bind to 3′-UTR of different mRNA reducing their half-life (29,30).

The data presented here could not elucidate whether the LPS-dependent increase in the binding activity of the 60-kD

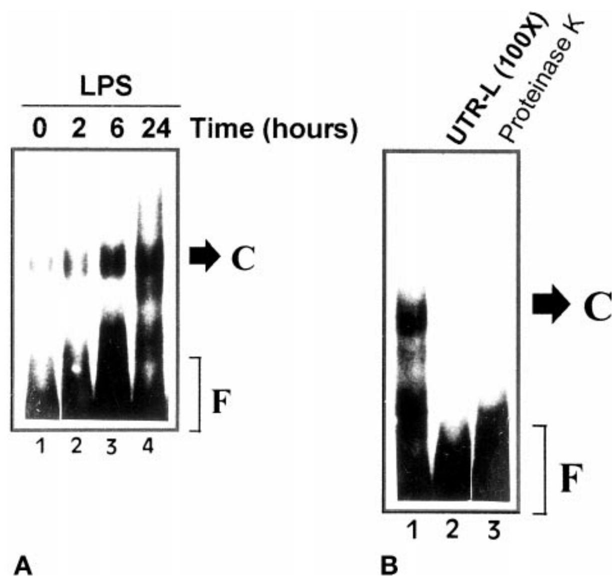

Figure 8. (A) Gel mobility-shift assay with the 3′-untranslated region (3′-UTR) of eNOS mRNA. Human peritoneal samples were stimulated with LPS (10 μg/ml) for different periods of time. (B) Gel mobility-shift assay showing a competition experiment using labeled UTR-L with unlabeled UTR-L (lane 2). It is also shown that the treatment of peritoneal cytosolic extracts with proteinase K before incubation with the UTR-L fully abolishes the gel-shifted band (lane 3).

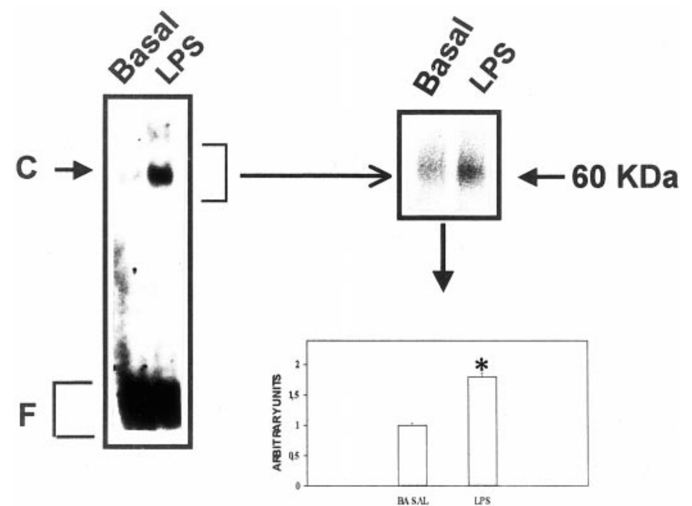

Figure 9. UV-cross linking of RNA protein complex. Binding assays were performed with UTR-L RNA, and the peritoneal cytosolic extracts obtained from human peritoneum incubated in the presence and in the absence of LPS (10 μg/ml) for 24 h. The complex was exposed to UV light for 20 min and digested with RNase. Cross-linked proteins to labeled RNA probe were run through a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A band with an apparent molecular weight of 60 kD was seen. In the lower panel, the bar graph shows the densitometric scanning of the UV cross-linking. Results are represented as mean ± SEM of six different experiments. $^{*}P < 0.05$ with respect to basal.

cytosolic protein was due to a greater binding of the protein to 3′-UTR eNOS mRNA or to the synthesis of new protein or both. In this regard, Mohamed *et al.* (9) and Yoshizumi *et al.* (10) showed that destabilization of the eNOS message by cytokines was largely prevented by coincubation with cycloheximide, suggesting that synthesis of new proteins was required for this effect. It is also noteworthy that although the immunohistochemical study showed that LPS reduced eNOS protein expression in both mesothelial and peritoneal endothelial cells, our present experimental design did not allow us to establish whether the 60-kD binding protein is expressed in endothelial, mesothelial, or both cell types. Additional studies with isolated cultured cells are needed to elucidate this question.

Loss of eNOS expression in human peritoneal cells could compromise different cellular functions, favoring the presence of peritoneal dysfunction. In this regard, peritoneal inflammation results in the expression of adhesion molecules in endothelial and mesothelial cells, and it has been demonstrated that NO limits the expression of several adhesion molecules (31–34). Moreover, recent evidence demonstrated that NO contributes to the regulation of human fibrinolytic activity (35,36). In this sense, the blockade of NO synthesis by L-arginine competitive inhibitors stimulated the generation of the plasminogen activator inhibitor (36). It is interesting that peritoneal inflammation causes a reduction in abdominal fibrinolytic activity, resulting in the persistence of intra-abdominal fibrin with subsequent adhesion and abscess formation (37,38). Therefore, a situation of mesothelial and endothelial dysfunction, in terms of a reduced ability to express eNOS, could favor changes in the activity of the peritoneal fibrinolytic system.

In summary, in the present study, we describe that eNOS protein is expressed in peritoneal capillary endothelial and mesothelial cells. The expression of eNOS protein was downregulated by LPS. In addition, we observed that a 60-kD peritoneal cytosolic protein binds to the 3′-UTR of eNOS mRNA, which increased after LPS stimulation, suggesting an association between the presence of the cytosolic protein and eNOS protein expression in human peritoneum. A better understanding of the regulatory mechanism of eNOS expression in peritoneal cells may lead to new strategies in the prevention of peritoneal dysfunction during peritonitis.

Acknowledgments

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