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Aspirin Prevents *Escherichia coli* Lipopolysaccharide- and *Staphylococcus aureus*-Induced Downregulation of Endothelial Nitric Oxide Synthase Expression in Guinea Pig Pericardial Tissue

Maria M. Arriero, Juan C. de la Pinta, Marta Escribano, Angel Celdrán, Luis Muñoz-Alameda, Joaquín García-Cañete, Ana M. Jiménez, Santos Casado, Jerónimo Farré, Antonio López-Farré

Abstract—The aim was to analyze whether pericardial tissue expresses endothelial NO synthase (eNOS) protein and to determine the presence of cytosolic proteins that bind to eNOS mRNA. The effect of aspirin on the above-mentioned parameters was also analyzed. eNOS protein was expressed in pericardial tissue from male guinea pigs. *Escherichia coli* lipopolysaccharide (LPS, 10 μ g/mL) and *Staphylococcus aureus* endotoxin (SA, 10 μ g/mL) reduced eNOS protein expression and shortened the half-life of the eNOS messenger. Under basal conditions, cytosolic extracts from pericardial samples bound to the 3'-untranslated region (3'-UTR) of eNOS mRNA, which was enhanced by LPS and SA. Proteinase K fully prevented the binding of cytosolic pericardial extracts to 3'-UTR of eNOS mRNA, suggesting the involvement of proteins that were further characterized as 60- and 51-kDa proteins. Aspirin (1 to 10 mmol/L) restored eNOS expression in either LPS- and SA-stimulated pericardial samples and reduced the binding activity of the pericardial cytosolic proteins to 3'-UTR of eNOS mRNA. Indomethacin also reduced the downregulation of eNOS by LPS and diminished the binding activity of the cytosolic proteins, although higher doses of indomethacin than of aspirin were needed to improve these parameters. In conclusion, eNOS protein is expressed in guinea pig pericardial tissue. LPS and SA stimulate the binding activity of pericardial cytosolic proteins to 3'-UTR of eNOS mRNA and reduce eNOS protein expression. High doses of aspirin and indomethacin protect eNOS protein expression and reduce the binding activity of the cytosolic proteins to 3'-UTR of eNOS mRNA, suggesting an inverse association between the presence of these cytosolic proteins and eNOS expression. (*Circ Res.* 2002;90:719-727.)

Key Words: aspirin ■ endotoxins ■ inflammation ■ nitric oxide ■ pericardium

Nitric oxide is a gas generated by the endothelium through the metabolic conversion of L-arginine to L-citrulline by endothelial NO synthase (eNOS).¹ Loss of endothelium-derived NO results in vascular abnormalities, including vasoconstriction, smooth muscle proliferation, activation of blood elements, and increase in extracellular matrix protein synthesis.²⁻⁵

Although eNOS protein was initially defined as a constitutive enzyme, it was later demonstrated that cytokines downregulate the expression of this enzyme by destabilizing eNOS mRNA.^{6,7} We recently reported that cytokines stimulated the binding activity of endothelial cytosolic proteins to a specific region within eNOS mRNA, the 3'-untranslated region (3'-UTR), which was associated with eNOS mRNA destabilization.^{8,9} These cytosolic proteins have been also detected in human neutrophils.¹⁰

The pericardial tissue provides a nonadhesive surface and gates the traffic of molecules and cells between the circula-

tion and the pericardial compartment. The pericardial tissue produces a number of substances, including vasoactive factors, such as cytokines, fibrinolytic agents, prostaglandins, and endothelin-1.¹¹⁻¹³ However, whether eNOS protein is expressed in the pericardium is not evident. In the present study, we show that an eNOS-like protein is expressed in the pericardial tissue.

Pericardial inflammation has been implicated as a major cause of morphological and functional alterations of the pericardium. Acute pericardial inflammation is usually of idiopathic etiology, but it may be also secondary to systemic infections and autoimmune disease.^{14,15} Moreover, symptomatic pericarditis may also occur days to weeks after myocardial infarction.¹⁶ The loss of eNOS expression in the pericardium during pericarditis may contribute to the alteration of its functional properties; therefore, in the present study, we analyzed the effect of bacterial endotoxins, ie, *Escherichia coli* lipopolysaccharide (LPS) and *Staphylococcus aureus*

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endotoxin (SA), on the expression of eNOS protein in pericardial tissue, determining the presence of the cytosolic proteins that bind to 3'-UTR of eNOS mRNA.

The most troublesome complication of acute pericarditis is the development of recurrent episodes of pericardial inflammation. The optimal method for preventing recurrences has not been established; however, one of the most frequently used treatments is aspirin, a drug with anti-inflammatory properties. The mechanism of action of aspirin remains to be fully known. In this sense, although aspirin inhibits the synthesis of prostaglandins, much higher doses of aspirin are needed to treat inflammatory diseases than are required to inhibit prostaglandin synthesis.¹⁷ Thus, the aim of the present study was to analyze the effect of aspirin on the expression of eNOS protein and the binding of cytosolic proteins to 3'-UTR of eNOS mRNA.

Material and Methods

Tissue Samples

Pericardial samples were obtained from 15 guinea pigs. Pericardial tissue was obtained under sterile conditions.

Pericardial samples were washed with isotonic saline to remove the remaining blood and cut into similar portions. The pericardial portions were preincubated in RPMI medium containing 10% FCS, 5 mmol/L glutamine, 2×10^{-5} U/L penicillin, and 2×10^{-5} μ g/L streptomycin for 1 hour. Afterward, the medium was removed and replaced with fresh RPMI medium. LPS (10μ g/mL, iserotype 0127:B8, Sigma Chemical Co) or SA (10μ g/mL, staphylococcal dried cells from *S aureus*, strain Newman D₂C, Sigma) was then added for different periods of time. Additional experiments in the presence of increasing concentrations of aspirin were also performed. Aspirin was added 10 minutes before the corresponding endotoxin. After the incubation period, the pericardial samples were frozen in liquid nitrogen for molecular biological determinations.

Western Blot Analysis

eNOS protein was analyzed in the pericardial samples by Western blotting after the tissues were homogenized and lysed in Laemmli buffer containing 2-mercaptoethanol.¹⁸ Equal amounts of protein (20 μ g per lane) estimated by bicinchoninic acid reagent (Pierce) were loaded. To verify that equal amounts of protein had been loaded in the gel, a parallel gel with identical samples was run and stained with Coomassie blue to compare the intensities of the protein bands. Western blot analysis was performed with a monoclonal antibody against eNOS (1:2500, Transduction Laboratories) as previously reported.^{10,19}

Dot-Blot Analysis

Tissue levels of eNOS mRNA stability were determined by dot-blot analysis. Because of the small size of the pericardial tissue, pericardial samples from three or four guinea pigs were pooled for each experiment. Guinea pig pericardial samples were preincubated in the presence and in the absence of the transcription inhibitor actinomycin D (10μ g/mL) for 1 hour. Then, the pericardial samples were incubated in the presence and in the absence of LPS (10μ g/mL) and SA (10μ g/mL) for 24 hours. Each experiment was performed four times. Total RNA was extracted according to the method of Chomczynski and Sacchi.²⁰ For dot-blot analysis, 10 μ g of RNA was then loaded onto the nitrocellulose membrane in a dot-blot filtration manifold apparatus and hybridized for 16 hours at 42°C with 500 000 cpm ³²P-labeled eNOS probe. The cDNA probe used was the *HindIII-BamHI* fragment of probe NOS (pNOS) UTR-L (where L indicates large) (see below). Hybridization was performed as reported.²¹ The same membranes were then rehybridized with a probe encoding GAPDH as a control for loading.

Plasmids and In Vitro Transcription

Oligonucleotides complementary to bovine eNOS cDNA were purchased from Bio-synthesis Inc. pNOS-UTR plasmids were made as previously reported.¹⁰ In brief, oligo 1 (5'-GGATCTAGAACGCTATCAGGAGGACATT-3') and oligo 2 (5'-AGGAAGCTTAGT-AGGTCCTAACTTCTG-3') were used to produce by reverse transcriptase-polymerase chain reaction (from bovine aortic endothelial cell 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, plasmid UTR-L). Oligo 3 (5'-GTTGGATCCCCTGTACTATCTC-ACCCT-3') and oligo 2 were used to make, by polymerase chain reaction, pNOS-UTR-S (where S indicates short) as described.^{8,9} To make plasmid pNOS-UTR-UC (where UC indicates uridine+cytidine), pNOS-UTR-S was cut with *AcsI*. To make pNOS-UTR-AU (where AU indicates adenine+uridine), *AcsI*-linearized pNOS-UTR-S was purified by agarose with the use of the different probes containing the different segments of the 3'-UTR of eNOS mRNA. To produce single-stranded RNA, the 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 Biotech) with [³²P]CTP (Amersham Iberica).

Band-Shift Assays

After incubation with LPS (10μ g/mL) or SA (10μ g/mL) in the presence and in the absence of aspirin, the pericardial samples were frozen in liquid nitrogen. Afterward, the samples were pulverized and resuspended in hypotonic buffer (25 mmol/L Tris-HCl, pH 7.9, 0.5 mmol/L EDTA, and 1 mmol/L phenylmethylsulfonyl fluoride), followed by four freezing and thawing cycles, and frozen at -70°C until use. Cytoplasmic lysates (10 μ g) were incubated with 5 to 10×10^4 cpm of the radiolabeled UTR-L in a total volume of 15 μ L for 10 minutes at 25°C. Then, 20 U of RNase T₁ per reaction (GIBCO-BRL) was added, and the reaction mixtures were incubated for 30 minutes at 37°C. The samples were electrophoresed on 4% native polyacrylamide gel in 0.25× Tris borate-EDTA as a running buffer, dried, and autoradiographed with Kodak X-OMAT-S film as previously described.^{8,9}

UV Cross-Linking of the RNA-Protein Complex

Ten micrograms of the cytoplasmic lysates from pericardial tissue was incubated with 10^5 cpm of RNA in the buffer above described in a total volume of 20 μ L for 10 minutes at 25°C. Samples were UV-irradiated in ice in a Stratallinker (Stratagene Ltd) for 20 minutes, followed by RNase digestion (20 μ g of RNase A and 20 U of RNase T₁ for 30 minutes at 37°C). Then, they were heated for 10 minutes at 70°C in Laemmli buffer without 2-mercaptoethanol and electrophoresed on denaturing SDS-PAGE. The gels were exposed for 3 to 5 days to Kodak film with two intensifying screens.

Statistical Methods

Results are expressed as mean \pm 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 the Bonferroni correction for multiple comparisons or a Student *t* test (paired or unpaired). A value of *P* < 0.05 was considered statistically significant.

Results

eNOS Expression in Guinea Pig Pericardial Tissues

Western blot analysis demonstrated the presence of a 140-kDa band in the homogenate of guinea pig pericardium, indicating the presence of eNOS protein (Figure 1A).

LPS (10μ g/mL) reduced eNOS protein expression in the guinea pig pericardial tissue in a time-dependent manner (Figure 1A). The maximal decrease in eNOS protein expression was observed 24 hours after LPS incubation. SA (10μ g/mL) also reduced eNOS protein expression in a time-

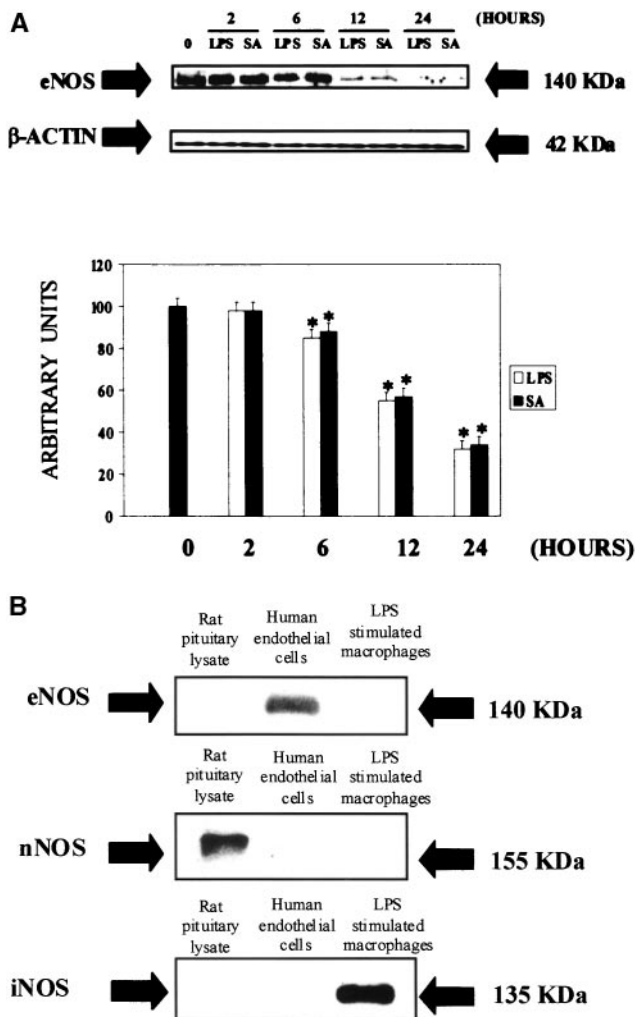


Figure 1. A, Representative Western blot demonstrating the expression of eNOS protein in guinea pig pericardial tissue after exposure to LPS (10 μ g/mL) or SA (10 μ g/mL) for different periods of time. The expression of β -actin protein is also shown. Bottom bar graph shows densitometric scanning of the Western blot. Results are represented as mean \pm SEM of 6 different experiments. * P < 0.05 with respect to basal. B, Western blot demonstrating the specificity of the monoclonal antibody used in the experiments. The monoclonal antibody did not recognize the neuronal-type NO synthase (nNOS) in rat pituitary homogenate and the inducible-type NO synthase (iNOS) in *E coli* LPS-stimulated macrophages, but it specifically recognized the eNOS isoform (140 kDa) in homogenates of human umbilical vein endothelial cells (top). In the middle is shown a Western blot demonstrating the presence of the nNOS (155 kDa) in rat pituitary lysate (detected by a specific anti-nNOS antibody, 1:2500, Transduction Laboratories), which is not detectable in homogenates of human endothelial cells. At the bottom is shown a Western blot demonstrating the presence of iNOS (135 kDa) in homogenates of *E coli* LPS-stimulated macrophages (detected by a specific anti-iNOS antibody, 1:2500, Transduction Laboratories).

dependent manner. As observed in LPS-incubated pericardial tissue, the maximal decrease in eNOS expression was observed 24 hours after SA incubation (Figure 1A). Therefore, most of the following experiments were performed at this time. No changes in β -actin expression were observed between the control and either LPS- or SA-incubated pericardial

samples (Figure 1A). The specificity of the monoclonal antibody used was assessed as demonstrated in Figure 1B.

Pericardial samples were then stimulated with LPS (10 μ g/mL) for 24 hours in the presence and in the absence of increasing doses of aspirin (1 to 10 mmol/L). Aspirin prevented the reduction in eNOS protein expression induced by LPS in pericardial tissue. The protective effect of aspirin was dependent on its concentration. A slight increase in eNOS protein expression was observed at 1 mmol/L aspirin (Figure 2A). The maximal increase in eNOS protein expression achieved by aspirin was observed at 10 mmol/L (Figure 2A). Aspirin (10 mmol/L) also prevented the down expression of eNOS elicited by SA in pericardial tissue (Figure 2B).

A marked reduction in eNOS messenger was observed 24 hours after incubation of the pericardial tissue with LPS (Figure 3A). Aspirin (5 mmol/L) prevented the inhibitory effect of LPS on the eNOS mRNA levels (Figure 3A). eNOS

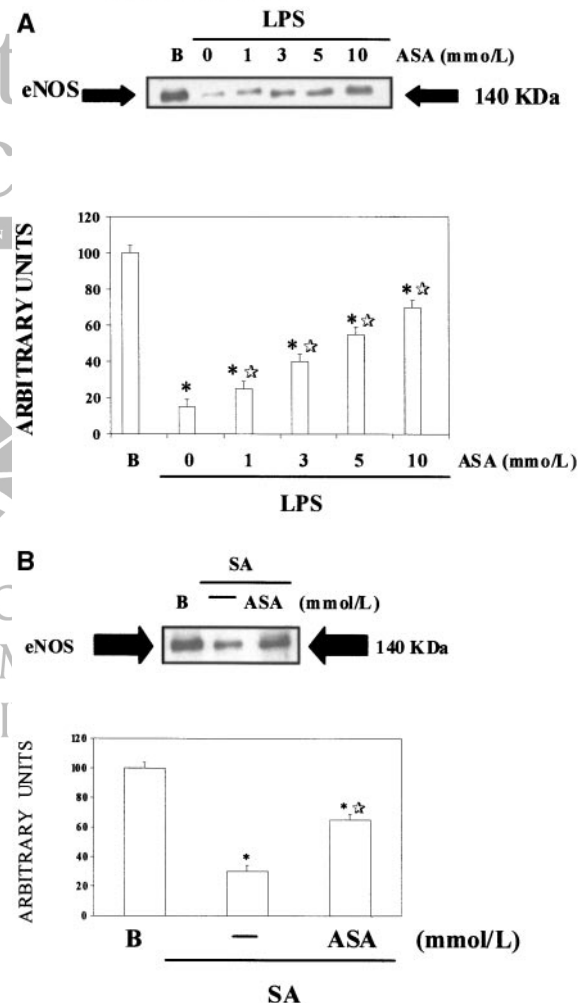


Figure 2. A, Western blot showing the effect of increasing doses of aspirin (ASA) on the LPS-induced downregulation of eNOS expression. Bottom bar graph shows the densitometric scanning of the Western blot. B, Western blot demonstrating the interaction of aspirin (10 mmol/L) with the effect of SA on eNOS protein expression. Bottom bar graph shows the densitometric scanning of the Western blot. Results are represented as mean \pm SEM of 6 different experiments. * P < 0.05 vs basal; ☆ P < 0.05 vs corresponding endotoxin alone.

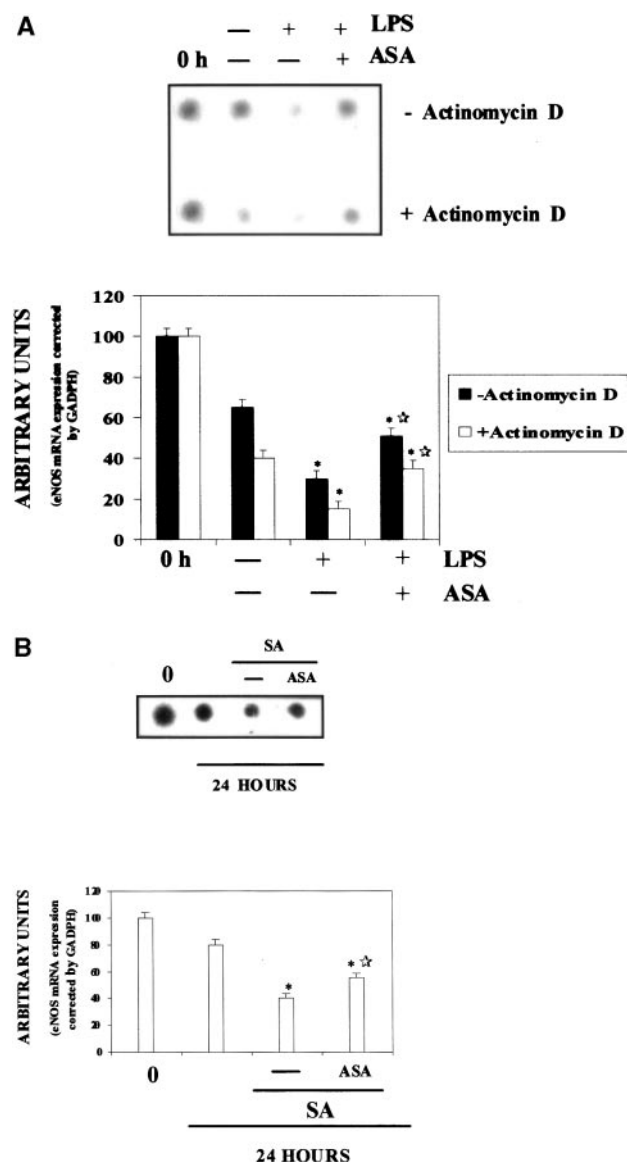


Figure 3. A, Representative dot-blot analysis of eNOS mRNA expression in pericardial samples 24 hours after LPS (10 μ g/mL) incubation. eNOS mRNA levels were also determined in the presence of 5 mmol/L aspirin. The experiments were performed in the absence and in the presence of the transcriptional inhibitor actinomycin D (10 μ g/mL). B, Representative dot-blot analysis showing the effect of 24-hour incubation with SA (10 μ g/mL) on eNOS mRNA levels in the presence of actinomycin D (10 μ g/mL). The effect of aspirin (5 mmol/L) is also shown. Equal loading of RNA was confirmed by incubation with a probe encoding GAPDH as a control for loading. At the bottom is shown the corresponding densitometric analysis corrected by GAPDH expression. Results are represented as mean \pm SEM of 6 different experiments. * P <0.05 vs 24 hours in the absence of LPS; ☆ P <0.05 vs LPS alone.

mRNA levels were determined in the presence of actinomycin D (10 μ g/mL), a transcriptional inhibitor. Twenty-four hours after LPS (10 μ g/mL) incubation, eNOS mRNA levels were reduced in pericardial tissue compared with the experiments performed in the absence of LPS, suggesting a reduction in eNOS mRNA half-life (Figure 3A). The presence of aspirin (5 mmol/L) prolonged the half-life of eNOS

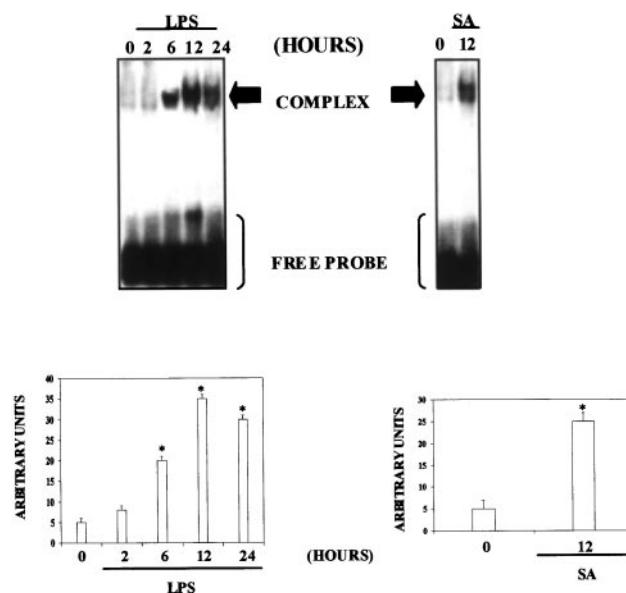


Figure 4. Regulation of the cytosolic protein binding activity by LPS. Pericardial tissues were stimulated with LPS (10 μ g/mL) for different periods of time, and their cytosolic extracts were analyzed by a gel mobility shift assay with the use of the UTR-L probe (left). On the right are the gel-shifted bands in cytosolic extracts obtained from pericardial tissue incubated during 12 hours with SA (10 μ g/mL). At the bottom is a densitometric analysis of the corresponding gel mobility shift assay. * P <0.05 vs experiments in the absence of endotoxins.

mRNA in LPS-stimulated pericardial samples. SA also destabilized eNOS mRNA similarly to LPS, an effect that was prevented by 5 mmol/L aspirin (Figure 3B).

Pericardial Cytosolic Proteins and 3'-UTR of eNOS mRNA

The addition of pericardial cytoplasmic extracts to a labeled probe containing the entire 3'-UTR eNOS mRNA, the UTR-L probe, resulted in a gel-shifted band (Figure 4, top left).

Cytosolic extracts from LPS-incubated pericardial samples showed an increased time-dependent binding to the labeled UTR-L probe (Figure 4, bottom left). The complex formation was significantly increased after 6 hours of LPS incubation and demonstrated the highest binding activity at 12 hours (Figure 4, bottom left). Incubation of pericardial tissue with SA (10 μ g/mL) for 12 hours also increased the binding activity of pericardial cytosolic proteins to the UTR-L RNA probe (Figure 4, bottom right).

Treatment of pericardial cytosolic extracts from LPS-incubated pericardium with proteinase K (87 μ g/mL) before their incubation with UTR-L abolished the complex formation (Figure 5A). Similar results were obtained in cytosolic extracts from SA-incubated pericardial samples (data not shown).

Unlabeled UTR-L prevented the binding of cytosolic extracts to labeled UTR-L in a concentration-dependent manner (Figure 5B, left).

Computer analysis of the nucleotide sequence of the 3'-UTR of eNOS mRNA shows two different regions: an AU-rich region with two AUUUA pentamers in the 3' half of

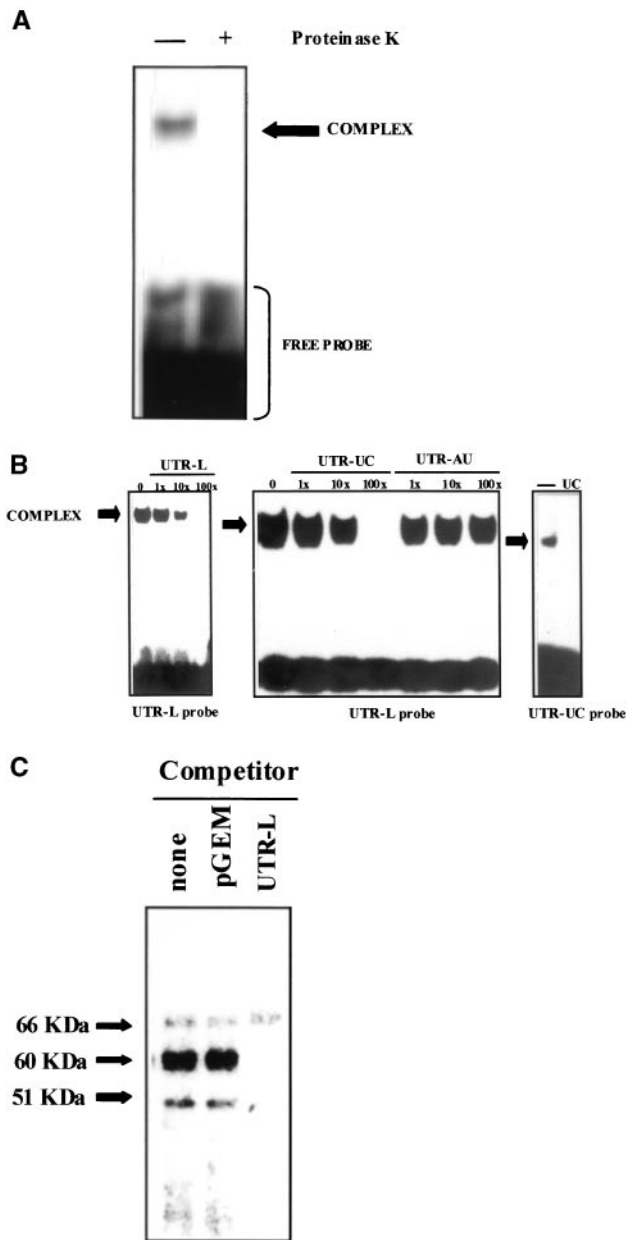


Figure 5. A, Effect of proteinase K (87 $\mu\text{g/mL}$) on the binding activity of pericardial cytosolic extracts from 24-hour LPS-incubated pericardium to UTR-L RNA. B, Left, Comparative experiments using increasing concentrations of unlabeled UTR-L RNA. B, Middle, Prevention of binding activity of cytosolic extracts from 24-hour LPS-incubated pericardial tissue to UTR-L RNA by an RNA probe containing a cytidine-rich region but not an RNA probe containing an adenine-rich region. B, Right, Gel mobility shift assay using labeled UTR-UC RNA probe incubated with pericardial cytosolic extracts from 24-hour LPS-stimulated pericardium. Specificity of the binding was demonstrated with $\times 100$ unlabeled UTR-UC RNA (1000 ng) ($\times 1 = 10$ ng RNA). C, UV cross-linking of UTR-L RNA probe-protein complex showing 3 bands with apparent molecular masses of 66, 60, and 51 kDa. Before extracting the cytosol, the pericardial tissue was incubated for 24 hours in the presence of LPS (10 $\mu\text{g/mL}$). The presence of the 60- and 51-kDa proteins was prevented when an excess of unlabeled UTR-L (1000 ng) was used as competitor. No effect was observed by using an excess of an unrelated RNA, pGEM (1000 ng).

the UTR-S probe and a UC-rich region in the 5' half of the UTR-S probe. Complex formation between labeled UTR-L and the LPS-stimulated pericardial extracts was prevented in a dose-dependent manner by unlabeled UTR-UC but not by unlabeled UTR-AU (Figure 5B, middle). It is noteworthy that the prevention of complex formation was very similar with the use of unlabeled UTR-L and unlabeled UTR-UC (Figure 5B, left and middle). These results suggested that no additional binding sites were present in the 3'-UTR of eNOS mRNA outside the UTR-UC fragment. In this regard, a gel-shifted band similar to that with labeled UTR-L was observed when labeled UTR-UC was used as probe, and it was also prevented by an excess (1000 ng) of unlabeled UTR-UC (Figure 5B, right).

Mixtures of 3'-UTR and UV light-exposed, LPS-incubated, pericardial cytosolic extracts showed bands with apparent molecular masses of 66, 60, and 51 kDa (Figure 5C). The bands of 60 and 51 kDa were absent when an excess of unlabeled UTR-L (1000 ng) was used. However, no effect was obtained by using an excess (1000 ng) of an unrelated RNA probe, pGEM, as a competitor, indicating that only the 60- and 51-kDa bands bound specifically to the 3'-UTR of eNOS mRNA (Figure 5C).

Effect of Aspirin on Binding Activity of Pericardial Cytosolic Proteins

The addition of aspirin to LPS-incubated pericardial tissue inhibited the binding activity of the cytosolic pericardial extracts to UTR-L in a dose-dependent manner (Figure 6A). A significant decrease in the binding activity of the cytosolic extracts to 3'-UTR was observed at 1 mmol/L aspirin (Figure 6A). The maximal effect of aspirin was found at 10 mmol/L (Figure 6A). Doses of aspirin >10 mmol/L did not demonstrate a higher ability to reduce the binding activity of the pericardial cytosolic extracts than that observed with 10 mmol/L aspirin (data not shown). Aspirin (10 mmol/L) also reduced the SA-induced binding activity of the cytosolic pericardial extracts to 3'-UTR of eNOS mRNA (Figure 6B).

Indomethacin also prevented eNOS protein expression in LPS-incubated pericardial tissue and the binding activity of the pericardial cytosolic proteins to 3'-UTR of eNOS mRNA (Figures 7A and 7B). The minimal dose of indomethacin that consistently prevented eNOS expression and the binding activity of the pericardial cytosolic proteins was greater (3 mmol/L) than that of aspirin (1 mmol/L) (Figures 2A, 6A, 7A, and 7B).

We then analyzed whether aspirin could directly affect the binding activity of the pericardial cytosolic proteins to 3'-UTR of eNOS mRNA. For this purpose, we added aspirin (0.5 and 10 mmol/L) to the in vitro incubation of cytosolic extracts from LPS-stimulated (10 $\mu\text{g/mL}$) pericardial samples with labeled UTR-UC probe. As shown in Figure 8, aspirin reduced the binding ability of the cytosolic pericardial proteins to labeled UTR-UC probe. This effect was observed with 0.5 and 10 mmol/L aspirin (Figure 8).

Discussion

In the present study, we have shown that eNOS protein is expressed in the pericardium. eNOS protein expression is

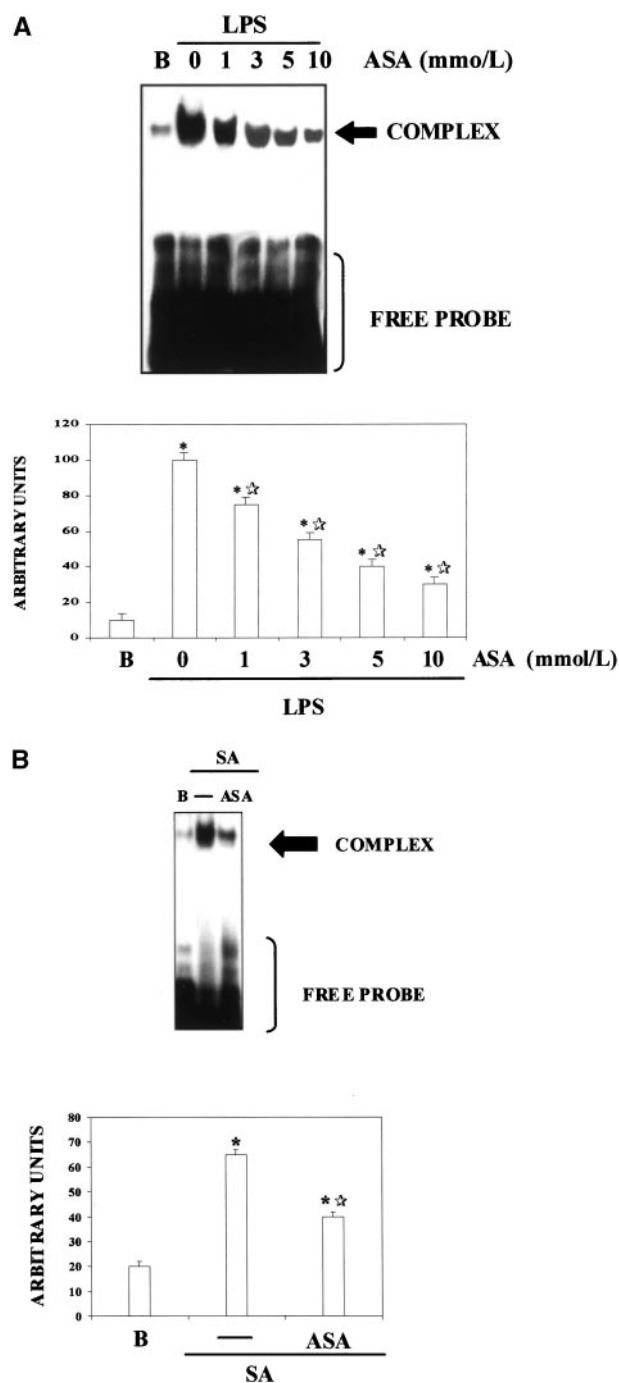


Figure 6. A, Gel mobility shift assay demonstrating the effect of increasing concentrations of ASA on the binding activity of cytosolic extracts to labeled UTR-L. The extracts were obtained from 24-hour LPS-incubated pericardial samples. At the bottom is a densitometric analysis of the gel-shifted bands. B, Gel mobility shift assay showing the effect of 10 mmol/L ASA on the binding activity of cytosolic extracts obtained from 24-hour SA-incubated guinea pig pericardial tissue. At the bottom is a densitometric analysis of the gel-shifted bands. Results are represented as mean \pm SEM of 6 different experiments. * P < 0.05 vs basal; ☆ P < 0.05 vs corresponding endotoxin alone.

downregulated by Gram-positive and Gram-negative endotoxins, ie, SA and *E coli* LPS, respectively. Furthermore, we have also shown that pericardial cytosolic extracts contain proteins that interact with the 3'-UTR of

eNOS mRNA, particularly with a cytidine-rich region within 3'-UTR. The interaction of the cytosolic proteins with 3'-UTR of eNOS mRNA was associated with eNOS mRNA destabilization and the downregulation of eNOS protein expression.

Various studies have demonstrated destabilization of eNOS mRNA by cytokines, such as tumor necrosis factor- α .^{6–10} In this regard, Kaku et al²² have suggested that LPS in combination with other cytokines increased eNOS protein expression in cultured bovine endothelium. However, most of the studies have demonstrated that LPS alone or in combination with other cytokines downregulated eNOS protein ex-

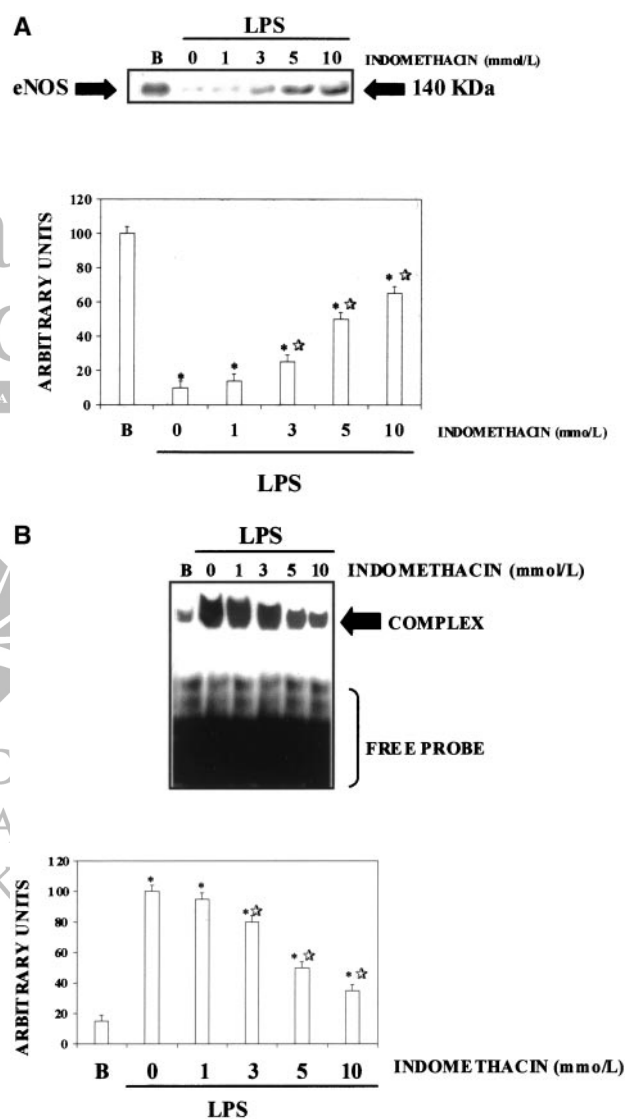


Figure 7. A, Representative Western blot showing the effect of increasing doses of indomethacin on the LPS-induced downregulation of eNOS expression. Bottom bar graph shows densitometric scanning of the Western blot. B, Representative gel mobility shift assay showing the effect of increasing concentrations of indomethacin on the binding activity of cytosolic extracts obtained from 24-hour LPS-incubated pericardial samples to labeled UTR-L. At the bottom is a densitometric analysis of the shifted bands. Results are represented as mean \pm SEM of 6 different experiments. * P < 0.05 vs basal; ☆ P < 0.05 vs LPS alone.

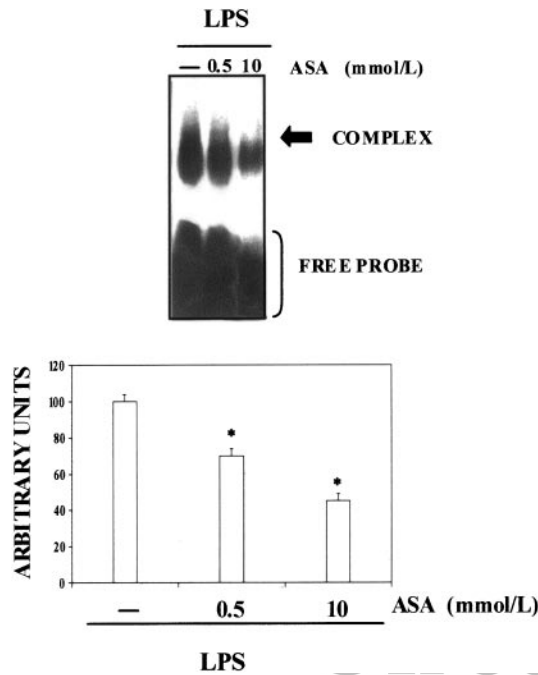


Figure 8. Gel mobility shift assay demonstrating the direct effect of ASA on the binding activity of the pericardial cytosolic proteins obtained from LPS (10 μ g/mL)-incubated pericardial samples to labeled UTR-UC probe. ASA (0.5 and 10 mmol/L) was added in vitro at the time of incubation of the cytosolic extracts with labeled UTR-UC. At the bottom is a densitometric analysis of the shifted bands. Results are represented as mean \pm SEM of 6 different experiments. * P < 0.05 vs experiments in the absence of ASA.

pression in cultured bovine and human endothelial cells by shortening the eNOS mRNA half-life through an undescribed mechanism.^{23,24} In the present work, LPS and SA reduced eNOS mRNA levels in pericardial tissue incubated in the absence and in the presence of actinomycin D, a transcriptional inhibitor, suggesting that a change in the stability of the eNOS messenger may be the predominant mechanism for LPS-induced downregulation of its expression. However, the present experiments do not allow us to rule out a possible effect of LPS on eNOS gene transcription in the pericardial tissue until formal experimental runoff studies have been performed.

In previous studies, we have demonstrated that the cytosol of bovine endothelial cells, neutrophils, and mononuclear cells contains proteins that interact with 3'-UTR of eNOS mRNA.^{8–10,25} In the present study, we have demonstrated that pericardial cytosolic lysates form complexes with the complete in vitro-transcribed 3'-UTR of eNOS mRNA. Furthermore, cytosolic lysates obtained from LPS- and SA-incubated pericardial samples showed an increased binding activity to 3'-UTR of eNOS mRNA, which was associated with a decreased level of eNOS protein and eNOS mRNA expression. The experiments with proteinase K suggested that the cytosolic components of the pericardium that had bound to 3'-UTR of eNOS mRNA were proteins. UV cross-linking and SDS-PAGE identified a 60- and a 51-kDa protein that specifically interacted with the 3'-UTR of eNOS mRNA. These proteins are within the range of other 3'-UTR-binding proteins.^{26,27}

The gel-shift analysis demonstrated that the cytosolic proteins bind to a cytidine-rich region within the 3'-UTR of eNOS mRNA. Although the AUUUA pentamer is a common sequence that binds proteins within other 3'-UTRs, other authors have also demonstrated proteins that interact with the 3'-UTR mRNAs independently of these AUUUA sequences.²⁶ It is noteworthy that although in the present study we used probes from bovine 3'-UTR of eNOS mRNA, a cytidine-rich region has been also identified in guinea pig, rabbit, and even human 3'-UTRs of eNOS mRNA. Therefore, the *cis*-acting sequences in the 3'-UTR, which potentially participate in the regulation of eNOS mRNA stability, have probably been conserved during evolution. As a hypothesis, the binding of the cytosolic proteins to this specific region may modify the spatial configuration of this region, showing an RNase active site, as has been reported for iron-regulated transferrin receptor mRNA stability.²⁷

In the present study, we have not shown functional evidence demonstrating the involvement of the cytosolic proteins in eNOS mRNA destabilization. Therefore, we may establish an association only between the presence of the cytosolic proteins and eNOS mRNA destabilization. Accordingly, Searls et al²⁸ have recently reported that deletion of 3'-UTR of eNOS mRNA stabilized eNOS mRNA, supporting the importance of 3'-UTR in the regulation of eNOS stability.

However, in an attempt to study the correlation between the binding of pericardial cytosolic proteins to eNOS mRNA and the level of expression of eNOS protein, we performed experiments in the presence of aspirin. Although aspirin is a useful therapeutic tool for treating inflammatory reactions, such as pericarditis, its mechanism of action still remains unknown. In the present study, we have shown that aspirin prevents the downregulation of eNOS protein expression in pericardial tissue, which is associated with an increased eNOS mRNA stability and a reduced binding activity of the pericardial cytosolic proteins to 3'-UTR of eNOS mRNA. These results suggest an association between the binding activity of the cytosolic proteins and the level of expression of eNOS protein.

The mechanisms by which aspirin reduced the binding activity of the pericardial cytosolic proteins to 3'-UTR of eNOS mRNA are out of the scope of the present work. However, we analyzed whether the effect of aspirin on the binding activity of the pericardial cytosolic proteins was specific for aspirin or also occurred with other nonsteroidal anti-inflammatory agents. Indomethacin reduced the downregulation of eNOS expression by LPS and diminished the binding activity of the pericardial cytosolic proteins to eNOS mRNA. However, these pericardial parameters were improved with lower doses of aspirin than of indomethacin. This may be due to a different tissue penetrance of the two drugs, or perhaps aspirin and indomethacin have different potencies for cyclooxygenase inhibition. However, we could not discard the possibility that aspirin could exert further effects on these pericardial parameters beyond its anti-cyclooxygenase and anti-inflammatory properties. We then analyzed whether, inde-

pendent of its anti-cyclooxygenase ability, aspirin could also directly affect the binding activity of the pericardial cytosolic proteins to 3'-UTR of eNOS mRNA. Aspirin reduced in vitro the binding ability of the pericardial cytosolic proteins to an RNA probe containing the cytidine-rich region of 3'-UTR of eNOS mRNA. Other authors have demonstrated that aspirin also acetylates other proteins further to cyclooxygenase-1,^{29,30} modifying their activity.

Loss of eNOS expression in pericardial cells could compromise different cellular functions. Pericardial inflammation is associated with an increased infiltration of blood cells, and it has been demonstrated that NO limits the expression of several adhesion molecules.³¹ Recent studies have also demonstrated that NO contributes to the regulation of fibrinolytic activity.^{32,33} Pericardial tissue injury and subsequent inflammation causes a reduction in fibrinolytic activity, resulting in the presence of fibrin and a rise in fibrinous adhesions.³⁴ Therefore, a situation of pericardial dysfunction, in terms of a reduced ability of the pericardium to express eNOS, could favor changes in the activity of the pericardial fibrinolytic system. In addition, because the pericardium could alter the function of the adjacent myocardium,³⁵ a reduced expression of eNOS protein in the pericardium could also contribute to modification of the function of the myocardium during pathological situations, particularly those associated with inflammation.

The fact that Gram-negative and Gram-positive bacteria products reduced eNOS protein expression and increased the binding activity of the cytosolic proteins to 3'-UTR of eNOS mRNA suggests a common pathway by which endotoxins downregulate eNOS expression in the pericardium. A better knowledge of the mechanisms by which aspirin and indomethacin protect the expression of eNOS protein in the pericardium may lead to new pharmacological strategies in the prevention of the pericardial dysfunction associated with pericardial inflammation.

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References

1. Sessa WC. The nitric oxide synthase family of proteins. *J Vasc Res*. 1994;31:131-143.
2. Moncada S, Higgs A. L-Arginine-nitric oxide pathway. *N Engl J Med*. 1993;329:2002-2012.
3. López-Farré A, Caramelo C, Esteban A, Arberola ML, Millás I, Montón M, Casado S. Effects of aspirin on platelet-neutrophil interactions: role of nitric oxide and endothelin-1. *Circulation*. 1995;91:2080-2088.
4. Kubes P, Suzuki M, Granger DN. Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci U S A*. 1991;88:587-590.
5. Trachtman H, Futterweit S, Shingal P. Nitric oxide modulates the synthesis of extracellular matrix proteins in cultured mesangial cells. *Biochem Biophys Res Commun*. 1995;207:120-125.
6. Mohamed F, Monge JC, Gordon A, Cernacek P, Blais D, Stewart DJ. Lack of role of nitric oxide (NO) in the selective destabilization of endothelial NO synthase mRNA by tumor necrosis factor alpha. *Arterioscler Thromb Vasc Biol*. 1995;15:52-57.
7. Yoshizumi M, Perella MA, Burnett JC Jr, Lee ME. Tumor necrosis factor downregulates an endothelial nitric oxide synthase mRNA by shortening its half-life. *Circ Res*. 1993;73:205-209.
8. Alonso J, Sánchez de Miguel L, Montón M, Casado S, López-Farré A. Endothelial cytosolic proteins bind to 3'-untranslated region of endothelial nitric oxide synthase mRNA: regulation by tumor necrosis factor- α . *Mol Cell Biol*. 1997;17:5719-5726.
9. Sánchez de Miguel L, Alonso J, Gonzalez-Fernandez F, de la Osada J, Montón M, Rodriguez-Feo JA, Guerra JI, Arriero MM, Rico L, Casado S, López-Farré A. Evidence that an endothelial cytosolic protein binds to the 3'-untranslated region of endothelial nitric oxide synthase mRNA. *J Vasc Res*. 1999;36:201-208.
10. De Frutos T, Sánchez de Miguel L, Gómez J, Romero J, Marcos-Alberca P, Nuñez A, Rico L, López-Farré A. Expression of an endothelial-type nitric oxide synthase isoform in human neutrophils: modification by tumor necrosis factor- α and acute myocardial infarction. *J Am Coll Cardiol*. 2001;37:800-807.
11. Satoh K, Prescott SM. Culture of mesothelial cells from bovine pericardium and characterization of their arachidonate metabolism. *Biochim Biophys Acta*. 1987;930:283-296.
12. Kuwahara M, Kuwahara M. Pericardial mesothelial cells produce endothelin-1 and possess functional endothelin ETB receptors. *Eur J Pharmacol*. 1998;347:329-335.
13. Nkere MM, Whawell SA, Thompson EM, Thompson JN, Taylor KM. Changes in pericardial morphology and fibrinolytic activity during cardiopulmonary bypass. *J Thorac Cardiovasc Surg*. 1993;106:339-345.
14. Shabetai R. Diseases of the pericardium In: Bennet JC, Plum F, eds. *Cecil Textbook of Medicine*. 20th ed. Philadelphia, Pa: WB Saunders; 1996: 336-342.
15. Moraes AN, Ferreira AG, Ferreira SM. Left ventricular pseudoaneurysm complicating infective pericarditis. *Heart*. 1999;82:393-394.
16. Lichstein E, Liu HM, Gupta P. Pericarditis complicating acute myocardial infarction: incidence of complications and significance of electrocardiogram on admission. *Am Heart J*. 1974;87:246-252.
17. Insel P. Analgesics-antipyretics and antiinflammatory agents: drugs employed in the treatment of rheumatoid arthritis and gout. In: Gilman AG, Rall T, Nies AS, Taylor P, eds. *The Pharmacological Basis of Therapeutics*. 8th ed. New York, NY: McGraw Hill Publishing; 1990: 638-681.
18. Laemmli NK. Changes of structural protein during the assembly of the head of bacteriophage T4. *Nature*. 1970;227:680-685.
19. Cernadas MR, Sánchez de Miguel L, García-Durán M, Gonzalez-Fernandez F, Millas I, Montón M, Rodrigo J, Rico L, Fernández P, de Frutos T, Rodriguez-Feo JA, Guerra J, Caramelo C, Casado S, López-Farré A. Expression of constitutive and inducible nitric oxide synthases in the vascular wall of young and aging rats. *Circ Res*. 1998;83:279-286.
20. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*. 1987;162:9940-9945.
21. Anderson KM, Berrebi-Bertrand I, Kirkpatrick RB, McQueney MS, Underwood DC, Rouanet S, Chabot-Fletcher M. cDNA sequence and characterization of the gene that encodes human myotrophin/V-1 protein, a mediator of cardiac hypertrophy. *J Mol Cell Cardiol*. 1999; 31:705-719.
22. Kaku Y, Naarri H, Sakimura T, Ejima K, Kuroiwa A, Ikeda M. Differential induction of constitutive and inducible nitric oxide synthases by distinct inflammatory stimuli in bovine aortic endothelial cells. *Biochim Biophys Acta*. 1997;1356:43-52.
23. Lu JL, Schmiede LM, Kuo L, Liao JC. Downregulation of endothelial constitutive nitric oxide synthase expression by lipopolysaccharide. *Biochem Biophys Res Commun*. 1996;225:1-5.
24. MacNaull KL, Hutchinson NI. Differential expression of iNOS and cNOS mRNA in human vascular smooth muscle cells and endothelial cells under normal and inflammatory conditions. *Biochem Biophys Res Commun*. 1993;196:1330-1334.
25. Jimenez A, Arriero MM, López-Blaya A, Gonzalez-Fernandez F, García R, Fortes J, Millas I, Velasco S, Sanchez de Miguel L, Rico L, Farré J, Casado S, López-Farré A. Regulation of endothelial nitric oxide synthase expression in the vascular wall and in mononuclear cells from hypercholesterolemic rabbits. *Circulation*. 2001;104:1822-1830.

26. Dodson RE, Shapiro DJ. An estrogen-inducible protein binds specifically to a sequence in the 3'-untranslated region of estrogen-stabilized vitellogenin mRNA. *Mol Cell Biol.* 1994;14:3130-3138.
27. Müller EW, Kühn LC. A stem-loop in the 3'-untranslated region mediates iron-dependent regulation of transferrin receptor mRNA stability in the cytoplasm. *Cell.* 1989;53:815-825.
28. Searls CD, Miwa Y, Harrison DG, Remasanny S. Posttranscriptional regulation of endothelial nitric oxide synthase during cell growth. *Circ Res.* 1999;85:588-595.
29. Pinckard RN, Hawkins D, Farr RS. In vitro acetylation of plasma proteins, enzymes and DNA by aspirin. *Nature.* 1968;219:68-69.
30. Bjornsson TD, Schneither DE, Berger H. Aspirin acetylates fibrinogen and enhances fibrinolysis: fibrinolytic effect is independent of changes in plasminogen activator levels. *J Pharmacol Exp Ther.* 1989;250:154-160.
31. De Caterina R, Libby P, Peng HB, Thannickal VJ, Rajavashisth TB, Gimbrone MA Jr, Shin WS, Liao JK. Nitric oxide decreases cytokine-induced endothelial activation: nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J Clin Invest.* 1995;96:60-68.
32. Newby DE, Wright RA, Dawson P, Ludlam CA, Boon NA, Fox KA, Webb DJ. The L-arginine/nitric oxide pathway contributes to the acute release of tissue plasminogen activator in vivo in man. *Cardiovasc Res.* 1998;38:485-492.
33. Korbut R, Warner TD, Gryglewski RJ, Vane JR. The effect of nitric oxide synthase inhibition on the plasma fibrinolytic system in septic shock in rats. *Br J Pharmacol.* 1994;112:289-291.
34. Leak LV, Ferrans VJ, Cohen SR, Eidbo EE, Jones M. Animal model of acute pericarditis and its progression to pericardial fibrosis and adhesions: ultrastructural studies. *Am J Anat.* 1987;180:373-393.
35. Mebajaa A, Wetzeln RC, Dodd-o JM, Redmond EM, Shab AM, Maeda K, Maistre G, Lakatta EG, Robotharm JL. Potential paracrine role of the pericardium in the regulation of cardiac function. *Cardiovasc Res.* 1998;40:332-342.



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Notification of Prior Publication

A study by Arriero et al (*Circ Res.* 2002;90:719–727) contained an instance of duplicate publication, in violation of the journal's editorial policy (*Circulation Research* Instructions to Authors, July 12, 2002 issue). Figure 5B, left panel, displayed a gel that had been previously published in at least three other studies (Alonso et al. *Mol Cell Biol.* 1997;17:5719–5726; Sánchez de Miguel et al. *J Vasc Res.* 1999;36:201–208; Jimenez et al. *Circulation.* 2001;104:1822–1830). Moreover, the origin of the materials in the various studies reportedly differed (bovine aortic endothelial cells [Alonso et al. *Mol Cell Biol.* 1997, and Sánchez de Miguel et al. *J Vasc Res.* 1999], rabbit aorta [Jimenez et al. *Circulation.* 2001], and guinea pig pericardium [Arriero et al. *Circ Res.* 2002]).

In response to this violation of journal policy and American Heart Association standards, the author's institution has been informed, and editorial sanctions have been imposed by the journal after an investigation including requesting an explanation from the authors.