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

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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 µg/mL) and Staphylococcus aureus endotoxin (SA, 10 µg/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 protein. 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 stimulate 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 circulation 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...
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. 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 × 10⁻³ U/L penicillin, and 2 × 10⁻³ µg/L streptomycin for 1 hour. Afterward, the medium was removed and replaced with fresh RPMI medium. LPS (10 µg/mL, serotype O127:B8, Sigma Chemical Co) or SA (10 µg/mL, staphylococcal endotoxin (SA), strain Newman D 2 C, 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 blot analysis demonstrated the presence of a 140-kDa band in the homogenate of guinea pig pericardium, consistent with the presence of the cytosolic isoform of eNOS. Western blot analysis was performed with a monoclonal antibody against eNOS (1:2500, Transduction Laboratories) as previously reported. 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. Then, the gel was UV-irradiated in ice in a Stratalinker (Stratagene Ltd) for 20 minutes, followed by four freezing and thawing cycles, and frozen at −70°C until use. Cytoplasmic lysates (10 µg) were then incubated with 5 to 10 x 10⁵ cpm of the radiolabeled UTR-L in a total volume of 15 µL, for 10 minutes at 25°C. Then, 20 U of RNase T 1, per reaction (GIBCO-BRL) was added, and the mixture was incubated for 30 minutes at 37°C. The samples were electrophoresed on 4% native polyacrylamide gel in 0.25x Tris borate-EDTA as a running buffer, dried, and autoradiographed with Kodak X-OMAT-S film as previously described.

UV Cross-Linking of the RNA-Protein Complex

Ten micrograms of the cytoplasmic lysates from pericardial tissue were incubated with 10⁵ cpm of RNA in the buffer above described in a total volume of 20 µL for 10 minutes at 25°C. Samples were UV-irradiated in ice in a Stratalinker (Stratagene Ltd) for 20 minutes, followed by RNase digestion (20 µg 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 ± 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 (10 µg/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 (10 µg/mL) also reduced eNOS protein expression in a time-
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 \( \beta \)-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 \( \mu \)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 1. A, Representative Western blot demonstrating the expression of eNOS protein in guinea pig pericardial tissue after exposure to LPS (10 \( \mu \)g/mL) or SA (10 \( \mu \)g/mL) for different periods of time. The expression of \( \beta \)-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 to recognize the eNOS protein. 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).

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; \( \star \)\( P < 0.05 \) vs corresponding endotoxin alone.
mRNA levels were determined in the presence of actinomycin D (10 μg/mL), a transcriptional inhibitor. Twenty-four hours after LPS (10 μg/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 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 μg/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 μg/mL) before their incubation with UTR-L abolished the complex formation (Figure 5A). 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
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 μg/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
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. In this regard, Kaku et al 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 expression.
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. 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.

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, Searl et al. 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 penetration 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


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