Cardioprotective effects of nitric oxide-aspirin in myocardial ischemia-reperfused rats

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Fu Y, Wang Z, Chen WL, Moore PK, Zhu YZ. Cardioprotective effects of nitric oxide-aspirin in myocardial ischemia-reperfused rats. Am J Physiol Heart Circ Physiol 293: H1545–H1552, 2007. First published May 25, 2007; doi:10.1152/ajpheart.00064.2007.—In this study, the cardioprotective effects of nitric oxide (NO)-aspirin, the nitroderivative of aspirin, were compared with those of aspirin in an anesthetized rat model of myocardial ischemia-reperfusion. Rats were given aspirin or NO-aspirin orally for 7 consecutive days preceding 48 h of reperfusion (MI/R). Treatment groups included vehicle (Twee 80), aspirin (30 mg·kg−1·day−1), and NO-aspirin (56 mg·kg−1·day−1). NO-aspirin, compared with aspirin, displayed remarkable cardioprotection in rats subjected to MI/R as determined by the mortality rate and infarct size. Mortality rates for vehicle (n = 23), aspirin (n = 22), and NO-aspirin groups (n = 22) were 34.8, 27.3, and 18.2%, respectively. Infarct size of the vehicle group was 44.5 ± 2.7% of the left ventricle (LV). In contrast, infarct size of the LV decreased in the aspirin- and NO-aspirin-pretreated groups, 36.7 ± 1.8 and 22.9 ± 4.3%, respectively (both P < 0.05 compared with vehicle group; P < 0.05, NO-aspirin vs. aspirin ). Moreover, NO-aspirin also improved ischemia-reperfusion-induced myocardial contractile dysfunction on postischemic LV developed pressure. In addition, NO-aspirin downregulated inducible NO synthase (iNOS; 0.37-fold, P < 0.01) and cyclooxygenase (COX)-2 (0.61-fold, P < 0.05) gene expression compared with the vehicle group after 48 h of reperfusion. Treatment with N' nitro-l-arginine methyl ester (l-NNAME; 20 mg/kg), a nonselective NOS inhibitor, aggravated myocardial damage in terms of mortality and infarct size but attenuated effects when coadministered with NO-aspirin. l-NNAME administration did not alter the increase in iNOS and COX-2 expression but did reverse the NO-aspirin-induced inhibition of expression of the two genes. The beneficial effects of NO-aspirin appeared to be derived largely from the NO moiety, which attenuated myocardial injury to limit infarct size and better recovery of LV function following ischemia and reperfusion.

nitroaspirin; aspirin; cardioprotection; ischemia-reperfusion; infarct size

ISCHEMIC MYOCARDIAL TISSUE will inevitably induce necrosis if blood flow is not restored immediately. Early reperfusion after coronary obstruction is well established to recover injured myocardium; nevertheless, reperfusion itself is believed to bring about additional cellular injury (25, 30). During the last two decades, numerous studies have been done that focus on the roles of nitric oxide (NO) in the pathogenesis progress and pharmacological intervention of myocardial ischemia and reperfusion. Of them, exogenous NO donors may provide therapeutic benefit and are also a recent conceptual advance in the management of reperfusion damage (1, 17, 19, 39). However, conventional NO donors (e.g., organic nitrates) frequently result in unwanted hemodynamic effects due to the NO fast-releasing property and the development of clinical tolerance. Aspirin (acetylsalicylic acid), the most widely prescribed drug in the world for the treatment of inflammatory conditions, inhibits cyclooxygenase (COX) in platelets and therefore decreases thromboxane A2 and prostacyclin (PGI2) formation in infarcted heart. Although decreased thromboxane A2 synthesis is beneficial, a decline in PGI2 production is undesirable, because PGI2 possesses cardioprotective action by inhibiting platelet aggregation (50).

Recently, a new family of NO-donating moiety to nonsteroidal anti-inflammatory drugs (NSAIDs) to improve organ tolerability and add new pharmacological profiles via the NO slow-releasing property, has been extensively investigated (21). One of these compounds, 2-acetoxybenzoate 2-(2-nitroxyethyl)phenyl ester (NO-aspirin), has been shown to be easily hydrolyzed by ubiquitous esterases in vivo into acetylsalicylic acid and an unchanged NO-donating moiety in the circulatory system (6), and NO release has been shown at a constant rate from NO-aspirin metabolite in the same cell compartments as the endogenous NO derived from l-arginine at the cellular level (11). NO can inhibit platelet aggregation (34), thus counteracting the inhibition of PGI2 by the aspirin moiety. Anti-inflammatory effects of NO-aspirin have been studied in vitro and in animal models. NO-aspirin has been found to be involved in the inflammatory process of several cells (such as platelets, monocytes/macrophages, leukocytes, endothelial cells, and smooth muscle cells) and to interact with different inflammatory targets (3). Moreover, this compound exerts arrhythmogenic and infarct-limiting activity through inhibition of neutrophil invasion and platelet aggregation in normal/diabetic rats and pigs following myocardial ischemia-reperfusion (MI/R) (5, 36, 46). However, these cardioprotective effects against postischemic reperfusion injury are limited to 2–3 h, as shown by employing triphenyltetrazolium chloride (TTC) staining to assess infarcted myocardium. Our present interest is whether NO-aspirin only delayed the progression of reperfusion injury during early reperfusion in the studies reported (5, 36, 46), because, to our knowledge, rats subjected to acute myocardial infarction (AMI) develop severe inflammation a few hours postsurgery and reach maximum injury/mortality by the next morning. Furthermore, in the setting of brief ischemia followed by reperfusion, of the vehicle group was 44.5.
by reperfusion, we are interested in whether the reduction in TTC-negative tissue observed in early reperfusion signifies genuine reduction of eventual infarct size following extended reperfusion. Because the stained myocardium consists of a complex mixture of necrotic and surviving myocytes in the early reperfusion, at that time the method of TTC staining has limitations in its accuracy for evaluation of cell necrosis (24); furthermore, inducible NO synthase (iNOS) induced by proinflammatory cytokines occurring during the late phase of postischemic infarction could increase infarct size (47–49).

Our preliminary study (data not shown) showed that rats treated daily with NO-aspirin for 2 wk (1 wk before AMI and 1 wk after AMI; the same dosage as this study) displayed some, but not significant, cardioprotection compared with the control group. The beneficial effects were evidenced by decreased infarct size/mortality to some extent; however, NO-aspirin appeared to exacerbate cardiac dysfunction: a significantly higher hypertrophy index occurred than in the control group ($P < 0.05$). We hypothesized that this is likely NO accumulation during the myocardial infarction due to the excessive supplement of NO released from the NO moiety plus a large amount of NO induced from endogenous iNOS, where a high concentration of NO is believed to be detrimental to cardiac tissue (8, 44, 48).

Interestingly, Liang et al. (22) demonstrated that l-arginine (a substrate for NO production) administered at different time points during ischemia-reperfusion exerted different effects on postischemic myocardial injury. Thus, in the present study, we limited the treatment period of NO-aspirin before the induction of MI/R and evaluated its cardioprotective effect by evaluating infarct size and left ventricular (LV) function recovery at an extended reperfusion time (48 h) compared with those of vehicle- and aspirin-treated groups against lethal reperfusion injury, as well as the possible biochemical and molecular mechanisms involved in endogenous NO production blocked by NOS inhibitor to define the NO role from exogenous NO donor in ischemia-reperfusion rats.

**MATERIALS AND METHODS**

**Animals and Drug Administration**

Wistar rats (148 males, 200–250 g) were obtained from the Laboratory Animal Centre, National University of Singapore (NUS). Animals were housed under standard conditions, maintained with a diurnal 12-h light-dark cycle, and fed standard rat chow and water ad libitum according to regulations for animal care by NUS. The project conforms with the *Guide for the Care and Use of Laboratory Animals* [DHEW Publication No. (NIH) 85-23, Revised 1985; http://grants1.nih.gov/grants/olaw/references/phspol.htm], which were approved by the animal ethics committee of NUS.

The rats were randomly assigned to six different treatment groups: *group 1*, sham-operated animals with open thorax without left descending coronary artery (LCA) occlusion, treated with Tween 80 (vehicle) (sham, $n = 14$); *group 2*, animals treated with vehicle and subjected to 25 min of LCA occlusion followed by 48 h of reperfusion (vehicle + MI/R, $n = 23$); *group 3*, animals treated with aspirin (30 mg·kg$^{-1}$·day$^{-1}$) and subjected to MI/R (ASA + MI/R, $n = 22$); *group 4*, animals treated with NO-aspirin (56 mg·kg$^{-1}$·day$^{-1}$; NicOx, Sophia-Antipolis, France) and subjected to MI/R (NOA + MI/R, $n = 22$) [the dose of NO-aspirin was chosen to provide a dose equimolar with that employed for aspirin and was based on reports showing cardioprotective effects at this dose (36, 46)]; *group 5*, animals treated with N$^\omega$-nitro-l-arginine methyl ester (l-NNAME; 20 mg·kg$^{-1}$·day$^{-1}$), a nonspecific inhibitor of endogenous NOS, and subjected to MI/R (l-NNAME + MI/R, $n = 23$); and *group 6*, animals treated with NO-aspirin (56 mg·kg$^{-1}$·day$^{-1}$) followed 1 h later by l-NNAME (20 mg·kg$^{-1}$·day$^{-1}$) and subjected to MI/R (NOA + l-NNAME + MI/R, $n = 20$). All drugs were dissolved in Tween 80 and were administered orally (2 ml/kg) once a day for 7 consecutive days. On the seventh day, the last dose of drugs was administered 3 h before the surgical operation was started. The tissue samples were collected 48 h after MI/R (day 9) for biochemical and molecular studies.

**Animal Model of MI/R**

The procedure for animal heart exposure was introduced as described earlier (16, 52, 54). In brief, under anesthesia, rats were intubated with a cannula connected to a rodent ventilator and were artificially ventilated with room air. The rectal temperature was monitored, and core temperature was maintained between 36.7 and 37.3°C by placing animals on a controlled heating pad throughout the experiment. The left thoracotomy was performed at the third and fourth ribs; the ribs were gently spread using a small-sized retractor, and then the heart was exposed. For temporary occlusion of the LCA, a 6/0 silk suture (Ethibond; Ethicon, Norderstedt, Germany) was placed around the artery 2–3 ml from the aortic root. The ends of the ligature were threaded through a polyethylene tube to produce a snare. After completion of the surgical procedure, the heart was returned to its normal position in the thorax. After 20 min of stabilization, regional ischemia was induced by tightening the snare against the ventricular wall and successful ischemia was verified by change in the color of the LV myocardium. After 25 min of ischemia, the snare was released and myocardial reperfusion was verified by change in the color of the ventricular myocardium. The thoracic cavity was then closed in layers by using 3/0 silk suture. Animals were killed after 48 h for biochemical and histological studies. Hearts were collected, immediately immersed in liquid nitrogen, and stored at $-80°C$ for further studies.

**In Vivo Hemodynamic and Regional LV Function Measurement**

**Systemic blood pressure.** Blood pressure (BP) was determined using the NIBP (noninvasive blood Pressure) system (ML125/R PowerLab System; ADInstruments, Mountain View, CA), and electrocardiogram (ECG) was monitored by applying the Animal BioAmp amplifier (ML 136 PowerLab System; ADInstruments) as described previously (52). BP and ECG readings were measured three times per randomly selected rat before the start of the treatment on day 1, subsequently before the surgical operation on day 7, and finally, before the death of the animal on day 9.

**LV function measurements.** Animals were anesthetized after BP measurement on day 9 and artificially ventilated while placed on controlled heating pads to maintain core temperature between 36.7 and 37.3°C. LV hemodynamics were measured using a pressure transducer (MLT 844 high-precision BP transducer; ADInstruments). After stabilization for 20 min, the pressure signal was continuously recorded using a MacLab analog-to-digital converter (ADInstruments) and stored and displayed on a computer. The peak LV systolic pressure (LVSP) and LV developed pressure (LVDp) were measured, and the maximal slopes of systolic pressure increment (LV dP/dt$\text{max}$) and diastolic pressure decrement (LV dP/dt$\text{min}$), indexes of contractility and relaxation, respectively, were analyzed.

**Infarct size determination.** Infarct size was identified using 2,3,5-triphenyltetrazolium chloride (TTC) as we previously reported (16). In brief, the infarcted area was judged from both epicardial and endocardial sides and then outlined on paper, cut, and weighed. The infarct size is defined as the ratio of the LV infarct area to the whole LV area (41). The sizes of the LV and the infarct area were evaluated using the software Scion Image.
Measurement of Plasma Nitrate/Nitrite

Nitrate/nitrite (NOx) levels were determined in plasma collected at the end of 48 h immediately after surgery. Briefly, intracardiac blood samples (0.5 ml) were collected. The blood was centrifuged for 15 min at 2,400 g at 4°C, and the plasma supernatant was removed and stored frozen at −80°C until assay. NOx levels were determined spectrophotometrically in aliquots (80 μl) of plasma by using the Greiss reagent as described previously (52). In brief, aliquots of plasma were incubated (37°C, 30 min) with nitrate reductase (10 μU) in the presence of NADPH (100 μM) to reduce nitrate to nitrite and then centrifuged (14,000 g, 25 min, 4°C). The resulting supernatant and sodium nitrite standard (100 μl; 0.125–75 μM) were added in duplicate to 96-well microtiter plates. Thereafter, Greiss reagent [containing 0.2% N-1-naphthylethlenediamine dihydridor chloride and 2% (wt/vol) sulfanilamide in 5% (vol/vol) H3PO4] was added to the above mixture in a ratio of 1:1 (vol/vol) and incubated for 10 min at room temperature, after which absorbance was determined at 550 nm in a 96-well microplate reader (Tecan Systems). The concentration of nitrate (indicative of NOx in the original samples) was calculated from a standard curve of NaNO2 (0.125–75 μM) and expressed as micromolar nitrite.

RNA Extraction and RT-PCR Amplification

Total RNA was extracted according to standard protocol (53). Total RNA (1 μg) of each sample was reverse-transcribed into first-strand cDNA and amplified using a OneStep RT-PCR kit (Qiagen). Briefly, 1 μg of RNA from each pooled sample was used in RT-PCR. The RT-PCR was carried out in a total volume of 20 μl, containing 4 μl of Qiagen OneStep RT-PCR buffer: 0.8 μl of dNTP, 1.2 μl of sense primer, 1.2 μl of antisense primer, and 0.8 μl of Qiagen OneStep RT-PCR enzyme mix. RT-PCR was carried out in a thermocycler (GeneAmp PCR System 2700). First, the samples were incubated at 50°C for 30 min to allow reverse transcription for the synthesis of cDNA. Next, the samples were subjected to PCR amplification with primers specific for COX-1, COX-2, endothelial NOS (eNOS), neuronal NOS (nNOS), iNOS, and GAPDH. Three-step PCR of denaturing and extension was carried out at 94°C for 30 s, 55°C for 45 s, and 72°C for 30 s, respectively. The PCR products were in a linear relationship and had not yet reached plateau. The annealing temperature and PCR cycle for different genes were set as follow: GAPDH, 55°C for 30 s, 25 cycles; COX-1, COX-2, eNOS, nNOS, and iNOS, 55°C for 30 s, 35 cycles. The primer sequences and their product sizes are given in Table 1.

Statistical Analysis

Statistical comparisons between groups were analyzed using one-way analysis of variance (ANOVA) and post hoc unpaired, two-sided Student’s t-test with a Bonferroni adjustment. All data are means ± SE. Significant differences among the groups were defined by a P value < 0.05.

Results

Mortality and Infarct Size

The results for infarct size and mortality in MI/R rats are reported in Table 1. Mortality rates for vehicle (n = 23), aspirin (n = 22), and NO-aspirin (n = 22)-treated groups were 34.8%, 27.3%, and 18.2%, respectively. Although no significant difference was found when any two groups were compared, NO-aspirin and aspirin tended to reduce the mortality rate, and more rats in the NO-aspirin-treated group survived than in the aspirin-treated group. In contrast, the l-NAME-treated group had a higher mortality rate (45.0%) than the vehicle-treated group (34.8%); however, with coadministration of NO-aspirin and l-NAME, the higher mortality of l-NAME was abrogated (34.8%).

There were no significant differences in body weight (BW), heart weight (HW), and LV weight (LVW) among all groups, although a corresponding reduction in infarct size in the groups with better survival rates was noted. Thus, infarct size (n = 5) for the vehicle group was 44.5 ± 2.7% of the LV, whereas infarct size for aspirin and NO-Aspirin groups was reduced to 36.7 ± 1.8 and 22.9 ± 4.3% of the LV, respectively. Infarct sizes were significantly decreased in both aspirin- and NO-aspirin-treated groups compared with the vehicle group (P < 0.05). There was a significant difference observed between aspirin and NO-aspirin-treated groups (P < 0.05). Compared with the vehicle group, treatment with l-NAME increased infarct size (P < 0.05) and completely blunted the NO-aspirin-induced reduction in infarct size when combined.

Hemodynamic Measurements

Systemic BP and heart rate. Six to eight rats from each treatment group were randomly selected for the measurement of systemic BP. Systemic BP and heart rate (HR) were measured (Table 2) at three time points: just before start of drug treatment (baseline), 1 h after the last drug treatment on day 7, and 48 h after the onset of reperfusion (end of reperfusion). Baseline BP values were in the same range in all treatment groups. After 7 days of treatment, BP in the NO-aspirin-treated group showed a slight but not significant decrease compared with that in the vehicle group.

In the l-NAME-treated group, systemic BP showed a significant increase after 7 days of treatment but a marked decrease at the end of 48 h of reperfusion compared with the vehicle group (P < 0.01). When l-NAME was coadministered with NO-aspirin, NO-aspirin abolished the l-NAME-induced systemic BP increase (not significant, NS) and attenuated the

| Table 1. Mortality, body weight, heart weight, LV weight, and infarct size in rats subjected to MI/R |

<table>
<thead>
<tr>
<th>Group</th>
<th>BW, g</th>
<th>HW, g</th>
<th>LVW, g</th>
<th>Infarct Size, % of LV</th>
<th>Mortality, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>259.5 ± 4.3</td>
<td>0.78 ± 0.018</td>
<td>0.49 ± 0.022</td>
<td>0</td>
<td>0 (14)</td>
</tr>
<tr>
<td>Vehicle</td>
<td>255.0 ± 7.5</td>
<td>0.86 ± 0.037</td>
<td>0.53 ± 0.035</td>
<td>44.5 ± 2.7</td>
<td>34.8 (23)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>255.4 ± 4.4</td>
<td>0.80 ± 0.026</td>
<td>0.50 ± 0.020</td>
<td>36.7 ± 1.8</td>
<td>27.3 (22)</td>
</tr>
<tr>
<td>NOA</td>
<td>244.5 ± 4.3</td>
<td>0.80 ± 0.040</td>
<td>0.51 ± 0.035</td>
<td>22.9 ± 4.3</td>
<td>18.2 (22)</td>
</tr>
<tr>
<td>NOA + l-NAME</td>
<td>248.0 ± 10.3</td>
<td>0.83 ± 0.031</td>
<td>0.53 ± 0.020</td>
<td>42.0 ± 4.2</td>
<td>34.8 (23)</td>
</tr>
<tr>
<td>l-NAME</td>
<td>257.9 ± 7.5</td>
<td>0.79 ± 0.030</td>
<td>0.46 ± 0.030</td>
<td>55.0 ± 2.6</td>
<td>45.0 (20)</td>
</tr>
</tbody>
</table>

Values are means ± SE of body weight (BW), heart weight (HW), left ventricular weight (LVW), infarct size, and mortality rate in rats subjected to 25 min of coronary artery occlusion followed by 48 h of reperfusion (MI/R). NOA, NO-aspirin; l-NAME, N⁵-nitro-l-arginine methyl ester. Numbers in parentheses for mortality indicate the number of rats. *P < 0.05 compared with vehicle-treated group. †P < 0.05 compared with aspirin-treated group.
day 7 (after 7 consecutive days of treatment), and compared with sham group. NOA, nitric oxide (NO)-aspirin; L-NAME, L-NAME-induced BP decrease at 48 h of reperfusion (P < 0.05 in the NO-aspirin group; P < 0.01 in the L-NAME group).

Baseline HR were in the same range and remained unchanged after treatment and at 48 h of reperfusion in rats treated with vehicle, aspirin, and NO-aspirin. L-NAME also had no effect on HR.

LV mechanic function parameters. Figure 1 and Table 3 depict the LV mechanic function parameters at 48 h of reperfusion, including LVEDP, LVSP, LV dp/dt max, and LV dp/dt min. Compared with the sham group, significant decreases in LVEDP, LV dp/dt max, and LV dp/dt min were noted in rats subjected to MI/R. Figure 1 shows that NO-aspirin significantly improved LVEDP from reperfusion injury compared with the sham group (NS), whereas other treatment had no such influence (P < 0.05 vs. sham group). NO-aspirin and aspirin appeared to increase, although not significantly, LVSP, LV dp/dt max, and LV dp/dt min compared with the vehicle group, demonstrating that the LV mechanic function recovered partly from reperfusion injury (Table 3). However, L-NAME tended to decrease LVSP, LV dp/dt max, and LV dp/dt min compared with the vehicle group, and NO-aspirin combined with L-NAME exerted results similar to those of L-NAME.

Plasma NOx Concentration

The concentration of NOx, which is the stable end product of NO in plasma, is shown in Fig. 2. Treatment for 7 consecutive days with NO-aspirin caused a marked increase of plasma NOx (P < 0.05 vs. sham and vehicle groups). Rats subjected to MI/R (vehicle group, 33.3 ± 1.5 μM) showed a higher NOx level compared with the sham group (17.6 ± 2.4 μM, P < 0.05). Aspirin (25.6 ± 2.1 μM) and NO-aspirin (17.3 ± 1.6 μM) groups showed attenuation of NOx level compared with the vehicle group, but the only significant difference occurred in the NO-aspirin-treated group (P < 0.01).

Gene Expression of NOS and COX

The gene expressions for NOS and COX are shown in Fig. 3. All drug-treated groups had no significant influence on COX-1, eNOS, and nNOS mRNA expression compared with vehicle-treated animals after 48 h of reperfusion. The level of iNOS mRNA expression increased significantly in the vehicle group compared with the sham group (2.6-fold), but compared with that in the vehicle group, the level of iNOS mRNA decreased in the aspirin (0.64-fold, P < 0.05) and NO-aspirin groups (0.37-fold, P < 0.01). The COX-2 mRNA level also increased significantly (2.5-fold) in the vehicle group compared with the sham group. There were significantly lower levels of COX-2 production in the aspirin- and NO-aspirin-treated groups (P <

Fig. 1. Left ventricular developed pressure (LVEDP; in mmHg) in rats subjected to 25 min of coronary artery occlusion (myocardial ischemia) followed by 48 h of reperfusion (MI/R). Values are means ± SE. *P < 0.05 compared with sham group. NOA, nitric oxide (NO)-aspirin; L-NAME, Nω-nitro-l-arginine methyl ester.

Fig. 2. Plasma nitrate/nitrite (NOx) concentration (in μM) in rats subjected to MI/R. Values are means ± SE; n = 4–6. *P < 0.05 compared with vehicle-treated group just before MI/R. +P < 0.01 compared with vehicle-treated group 48 h after MI/R.
0.01) compared with the vehicle group. L-NAME administration did not alter the increase in iNOS and COX-2 expression but did reverse the NO-aspirin-induced inhibition of expression of these genes.

**DISCUSSION**

The present results clearly demonstrate that the 7-consecutive-day oral administration of NO-aspirin before myocardial ischemia offers noticeable cardioprotection by significantly limiting infarct size and/or lowering the mortality rate caused by 25 min of LCA occlusion followed by 48 h of reperfusion, which confirms previous infarct-limiting effects during early reperfusion (5, 36, 46). Moreover, the NO-aspirin improved part of cardiac dysfunctional recovery was noted at 48 h of reperfusion.

**Cardioprotection of Pretreatment NO-Aspirin Against Cell Damage**

In the present study, compared with an equimolar dose of aspirin, NO-aspirin displayed a marked infarct-limiting effect; the mechanism(s) most likely involved is that enhanced NO bioavailability initiates a preconditioning (PC)-like phenomenon. Based on previous studies reported, exogenous NO (NO donor) could trigger and/or mediate cardiac adaptive protection against reperfusion injury from the first 2–3 h to a few days (28, 33, 43), although the exact targets or effectors of NO in PC are open to investigation (17). Given the multifarious nature of the chemical and physiological actions of NO, supplementation with NO could also exert inhibition of platelet aggregation and neutrophil adhesion during reperfusion (34, 36, 37), and we believe that the enhanced NO bioavailability by NO-aspirin pretreatment elicits salubrious effects. Of note, NO-aspirin was not sufficient to cause any appreciable systemic BP and HR changes compared with baseline level, which might be due to the NO slow-releasing property; therefore, any difference in the NO-aspirin-treated group cannot be attributed to hemodynamic alterations.

Pretreatment of L-NAME, the nonselective NOS inhibitor, before ischemia increased systemic BP compared with baseline level and exacerbated the extent of myocardial reperfusion.
injury compared with the vehicle group, but coadministration of NO-aspirin attenuated the hypertensive state and worsening effect in terms of infarct size/mortality rate induced by L-NAME; these findings also agree with those of previous studies (27, 36). The mechanism likely is that exogenous NO supplement constitutes the reduced endogenous NO production by chronic L-NAME treatment before MI/R. In the normal physiological condition, eNOS displayed more infarct-limiting effect than nNOS, determined by comparing the infarct size among the wild-type, eNOS, and nNOS knockout mice subjected to global MI/R (40). Jones et al. (18) also demonstrated that endothelial cell-derived NO played a vital role in vascular homeostasis within the coronary circulation and displayed intrinsic defenses against MI/R-induced cardiac myocyte injury in eNOS knockout mice. The beneficial effects of eNOS were supported by applying transgenic (TG) mice that overexpress eNOS exclusively in cardiac myocytes; these authors (40) reported that high levels of NO/cGMP strongly protect against ischemia-reperfusion injury related to reduced preischemic performance compared with wild-type mice. NO-aspirin, unlike conventional NO donors, releases NO intracellularly at a rate similar to that generated by endogenous eNOS (12, 14, 20). Thus we propose that NO donated by NO-aspirin maintains or augments eNOS-exerting vasodilatory tone and defensive functions against reperfusion injury and abolishes the endothelial dysfunction and injury exacerbation by L-NAME in combined administration with NO-aspirin.

Cytotoxicity of iNOS-Derived NO Exacerbates LV Dysfunction

Rossini et al. (36) reported that NO-aspirin did not change the cardiac mechanic parameters (LVP, LVDevP, HR) in rats subjected to 2 h of reperfusion following myocardial ischemia (36), and our preliminary studies showed that hypertrophy occurred in NO-aspirin-treated rats after AMI; however, our present study demonstrates that NO-aspirin recovered LV function partly at 48 h after reperfusion, but the decrease of infarct size in NO-aspirin-treated groups were observed in all three studies. Moreover, it is interesting in the present study that plasma total NOx measured at 48 h of reperfusion was significantly lower when treated with NO-aspirin compared with that of vehicle-treated animals despite the 7-day NO supplement. We speculate that iNOS-derived NO plays a vital role in NO accumulation during 2 days of reperfusion and also results in the discrepancy of LV function among the three studies.

Different from constitutive NO (eNOS and nNOS), iNOS can be induced by proinflammatory substances such as cytokines and lipopolysaccharide (LPS) or pathological conditions such as stroke, trauma, infection, and a variety of cardiovascular diseases (13) and produces higher levels of NO than normal for a longer period (32); Wildhirt et al. reported that iNOS activity increased and reached maximal level range from 48 to 72 h after initiation of AMI (48) and MI/R in rabbits (49), which was also supported by significant augmentations of plasma NOx level and cardiac iNOS mRNA expression in the control group compared with those of the sham group. Although considerable evidence exists that NO itself has low reactivity with most biological molecules and is cytoprotective, the large amount of NO produced by iNOS is recognized as a mediator and regulator of inflammatory responses (39, 55). Because overproduction of NO can combine with superoxide to form the potent oxidant peroxynitrite (ONOO−) at a rate far exceeding that which endogenous SOD can compete with, peroxynitrite is a highly cytotoxic molecule that contributes to cardiac dysfunction and myocardial injury under a variety of cardiovascular diseases, including MI/R (10, 35). Inhibition of iNOS can recover the LV function and protect the salvaged reperfused myocardium against postinfarction dysfunction (48, 49).

Therefore, the timing of exogenous NO supplement, the enzymatic source of endogenous NO generation in physiological/pathological conditions, and the concurrent formation of the toxic reaction product between NO and reactive oxygen species (ROS) could explain the discrepancy between the present and the preliminary study results against cardiac mechanical dysfunction. Of note, whether NO released from NO-aspirin interacted with the endogenous NOS before and during the MI/R needs further investigation, which is beyond the scope of the present study.

Table 4. Gene sequences and corresponding product sizes of GADPH, COX-1, COX-2, iNOS, nNOS, and eNOS

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene</th>
<th>Nucleotide Sequence</th>
<th>Product Size, bp</th>
<th>GenBank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-1</td>
<td>Sense</td>
<td>5' - CGAGGATCTCATCAAGGAG - 3'</td>
<td>350</td>
<td>S67721</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5' - TCAAGGAGCTGTTGTTAAC - 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COX-2</td>
<td>Sense</td>
<td>5' - CTGCTGACCTGCTGCTG - 3'</td>
<td>282</td>
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</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5' - ACTGCGGTCTGTTGTTAAC - 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eNOS</td>
<td>Sense</td>
<td>5' - CTGGCAAGCAGTTACAGGA - 3'</td>
<td>423</td>
<td>NM_021838</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>5' - CGGAACTGAGTCCGAAATAAT - 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iNOS</td>
<td>Sense</td>
<td>5' - CTACCTACCTGGGGAACCTGGG - 3'</td>
<td>442</td>
<td>NM_012611</td>
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<td></td>
<td>Antisense</td>
<td>5' - GGAGGACGGTGAGTAATGC - 3'</td>
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<tr>
<td>nNOS</td>
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<td>381</td>
<td>NM_052799</td>
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<td>5' - CATGCTCTAGCTCTCCAGT - 3'</td>
<td>349</td>
<td>XM_221353</td>
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<td>Antisense</td>
<td>5' - GCCTAAGCAGTTGCTGAG - 3'</td>
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</table>

COX, cyclooxygenase; eNOS, endothelial NO synthase; iNOS, inducible NO synthase; nNOS, neuronal NO synthase.
COX-2 Expression

Another point of interest was to evaluate the level of COX mRNA isoforms at 48 h of reperfusion. The two distinct COX isoforms, namely, COX-1 and COX-2, have been characterized. COX-1 is constitutively expressed in most cells, mostly in platelets and endothelial cells, and is responsible for homeostatic prostanooid formation, and COX-2, which is almost undetectable or lower in basal conditions, is dramatically upregulated in response to stress, such as inflammatory cytokines and ischemia, and often involves harmful effects including trauma, LPS-induced inflammation, and I/R injury. Several reports have suggested that the inhibition of COX-2 ameliorates tissue (lung, kidney, and liver) damage triggered by I/R injury (9, 15, 51). Oshima et al. (31) reported that inhibition of COX-2 (FK3311) significantly improved cardiac function in dogs following transplantation. Bouchard et al. (4) showed that COX-2 inhibitors protected the endothelial function against the deleterious effect of ischemia and reperfusion in isolated rat hearts. Therefore, we deduced that pretreatment with NO-aspirin might attenuate COX-2 expression, and in turn, contribute to ameliorating cardiac malfunction and damage in this study.

Another reason to investigate COX-2 mRNA expression is that COX-2 is closely related with iNOS. First, COX-2 is found to be coinduced together with iNOS in cardiac myocytes and many other cell types during inflammatory cytokine injury and ischemia (23, 29, 45). Furthermore, the signaling molecules that induce the expression of COX-2 in response to stress are similar to those that activate iNOS expression, which includes nuclear factor-κB (NF-κB) (7, 38) and protein kinase C (PKC) (2, 26). The COX-2 mRNA expression results validate our hypothesis: NO-aspirin exhibited a significant decrease in COX-2 mRNA expression, just like the resulting decrease in iNOS mRNA, compared with vehicle (P < 0.01). As a consequence of our study, pretreatment with NO-aspirin attenuated MI/R injury by possibly blunting COX-2 induction during reperfusion.

In conclusion, NO-aspirin exerts a better cardioprotective effects than aspirin in the rat model of ischemia and 48 h of reperfusion, which attenuates myocardial injury evidence as infarct-limiting and partial LV function recovery following ischemia and reperfusion. Thus the beneficial effects of NO-aspirin to decrease iNOS and COX-2 mRNA levels seems to involve a NOS-dependent pathway. Whether other mechanisms or molecular targets of NO are involved in the protection of postischemic injury needs to be explored. The findings of this study, however, offer a new possibility for NO-aspirin pretreatment in management of reperfusion injury. The cardioprotective effect of NO-aspirin also would benefit the aged and endothelial function-impaired patients for whom myocardial dysfunction is a common life-threatening complication.

GRANTS

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9. Feitoza CQ, Camara NO, Pinheiro HS, Goncalves GM, Cenedeza MA, Pacheco-Silva A, Santos OF. Cyclooxygenase 1 and/or 2 blockade ameliorates the renal tissue damage triggered by ischemia and reperfusion injury. Int Immunopharmacol 5: 79–84, 2005.


