Involvement of nitric oxide in cardioprotective effect of endothelin receptor antagonist during ischemia-reperfusion

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Gourine, Andrey V., Adrian T. Gonon, and John Pernow. Involvement of nitric oxide in cardioprotective effect of endothelin receptor antagonist during ischemia-reperfusion. Am J Physiol Heart Circ Physiol 280: H1105–H1112, 2001.—The interaction between the cardioprotective effect of endothelin (ET) receptor blockade and nitric oxide (NO) during ischemia-reperfusion injury was investigated. Anesthetized pigs were subjected to 45 (protocol 1) or 30 min (protocol 2) coronary artery ligation and 4 h reperfusion. In protocol 1, five groups were given vehicle, the ET<sub>A</sub> receptor antagonist LU-135252 (LU), the NO synthase (NOS) inhibitor N<sup>ω</sup>-nitro-L-arginine (L-NNA), L-NNA in combination with LU, or L-NNA in combination with the NO precursor L-arginine (L-Arg) and LU intravenously before ischemia. In protocol 2, two groups were given vehicle or L-NNA. In protocol 1, the infarct size (IS) was 79 ± 5% of the area at risk in the vehicle group and 93 ± 2% in the L-NNA group. LU reduced the IS to 43 ± 7% (P < 0.001). The cardioprotective effect of LU was abolished in the presence of L-NNA (IS 76 ± 6%), whereas addition of L-Arg restored its cardioprotective effect (IS 56 ± 2%; P < 0.05 vs. vehicle and L-NNA + LU groups). In protocol 2, the IS was 49 ± 6% in the vehicle group and 32 ± 4% in the L-NNA group (P = not significant). Myocardial ET-like immunoreactivity (ET-LI) increased in the vehicle group of protocol 1. ET-LI in the ischemic-reperfused myocardium was lower in the groups given LU (P < 0.01) and L-NNA + L-Arg + LU (P < 0.05) but not in the group given L-NNA + LU compared with the vehicle group. These results suggest that the cardioprotective effect of the ET<sub>A</sub> receptor antagonist is mediated via a mechanism related to NO.

reperfusion injury

THE EXTENT OF MYOCARDIAL INJURY after coronary artery occlusion and reperfusion depends on several factors, including rate-pressure product (RPP), myocardial blood flow, duration of ischemia, generation of free radicals, and accumulation of neutrophils during reperfusion (16). Much attention has recently been focused on the role of the vascular endothelium during ischemia-reperfusion. The endothelium produces several different relaxing and contracting factors, such as nitric oxide (NO), prostacyclin, endothelium-derived hyperpolarizing factor, endothelin-1 (ET-1), and ANG II (23). Endothelial NO is formed from L-arginine (L-Arg) by the constitutive form of the enzyme NO synthase (NOS). The enzyme can be inhibited competitively by L-Arg analogs such as N<sup>ω</sup>-nitro-L-arginine (L-NNA). NO plays a crucial role not only in the regulation of vascular tone but also in the prevention of platelet and leukocyte adherence and the inhibition of superoxide accumulation (25, 26). Endothelial dysfunction is an early event in various pathological cardiovascular conditions, including myocardial ischemia-reperfusion (33). The dysfunction is characterized by an impairment of endothelium-dependent relaxation due to reduced bioavailability of endothelial NO. Administration of L-Arg or NO donors reduce the extent of ischemia-reperfusion injury (21, 27). Thus NO appears to be a crucial factor in protecting the myocardium from ischemia-reperfusion injury. By contrast, several studies indicate that the endothelium-derived contracting factor ET-1 may contribute to the development of the ischemia-reperfusion injury (32, 35). ET-1 mediates its effects through the two receptor subtypes, ET<sub>A</sub> and ET<sub>B</sub> (29). Activation of the ET<sub>A</sub> receptor (1) evokes severe coronary constriction, whereas activation of the endothelial ET<sub>B</sub> receptor (30) leads to vasodilation via release of NO or prostacyclin (7, 8). The production (32) and the vasoconstrictor activity (38) of ET-1 are upregulated during myocardial ischemia-reperfusion. It has been demonstrated that both ET<sub>A</sub> and ET<sub>B</sub> (5, 6, 36) and selective ET<sub>A</sub> receptor antagonists (13, 14) effectively protect the myocardium from ischemia-reperfusion injury. The mechanism behind this cardioprotective effect is still unclear. In isolated hearts subjected to ischemia-reperfusion, the ET-receptor antagonist bosentan not only improved myocardial function but also preserved endothelial function (36). This may indicate that ET receptor blockade maintains endothelial NO production, which may contribute to tissue protection. It was also recently demonstrated that administration of an ET<sub>A</sub> receptor antagonist preserves endothelial function in atherosclerotic mice (2).

The aim of the present study was to elucidate the possible interaction between ET receptor blockade and NO production during myocardial ischemia-reperfusion. We therefore evaluated the cardioprotective effect of the ET<sub>A</sub> receptor antagonist LU-135252 (LU) in the absence and presence of NOS inhibition.
MATERIALS AND METHODS

Animal preparation. The study was approved by the regional ethical committee for laboratory animal experiments. Pigs of either sex (32 ± 1 kg wt) were premedicated with ketamine hydrochloride (20 mg/kg im) and atropine sulfate (0.1 mg/kg im). Anesthesia was induced by pentobarbital sodium (20 mg/kg iv) and maintained by a continuous infusion (2–4 mg·kg⁻¹·h⁻¹ iv). The animals were intubated and mechanically ventilated with air and oxygen. Respiratory rate and tidal volume were adjusted to keep arterial blood pH, PO₂, and PCO₂ within the physiological range. The rectal temperature was kept at 38.5–39.0°C with the use of a heated operating table. A 7-Fr catheter was positioned in the superior caval vein through the internal jugular vein for fluid and drug administration. Another 7-Fr catheter was positioned in the descending aorta via the left femoral artery for sampling of blood and for measurement of mean arterial pressure (MAP) via a Statham P23 Db transducer. Heart rate (HR) was determined from the arterial pressure curve. All of the variables were continuously recorded on a polygraph (model 7, Grass Instruments). The heart was exposed via a sternotomy. A ligature was placed around the artery just proximal to the ligature. An ultrasonic flow probe (Transonic Systems, Ithaca, NY) was placed around the artery just proximal to the snare for measurement of blood flow. The flow probe was connected to a Transonic 208 blood flowmeter. After 30 min of stabilization after the preparation, the pigs were randomized into seven groups. In protocol 1, five groups were subjected to 45 min of coronary artery ligation, followed by 4 h of reperfusion (Fig. 1). In protocol 2, two additional groups were subjected to 30 min of coronary artery ligation, followed by 4 h of reperfusion (Fig. 1). The five groups in protocol 1 were given vehicle (n = 11), the ET₄ receptor antagonist LU (5 mg/kg iv) 10 min before ischemia (n = 8), the NOS inhibitor L-NNA (10 mg/kg iv) 30 min before ischemia (n = 6), L-NNA in combination with LU (L-NNA + LU) 30 and 10 min before ischemia (n = 6), or L-NNA in combination with the NO precursor L-Arg and LU (L-NNA + L-Arg + LU) 30, 20, and 10 min before ischemia, respectively (n = 8). The two groups in protocol 2 were given vehicle (n = 6) or L-NNA (n = 7) as stated above (Fig. 1). The dose of LU was given on the basis of a previous study (13) in which this dose was demonstrated to reduce infarct size (IS) in pigs. The dose of L-NNA has been shown (3) to inhibit endothelium-dependent vasodilation in vivo.

Determination of IS. At the end of the experiment, LAD was reoccluded and 2% Evans blue was injected into the left atrium to outline the ischemic myocardium. The pigs were then euthanized by injection of a high dose of potassium chloride into the left atrium. The heart was rapidly extirpated. The atria and the right ventricle were removed. The left ventricle was cut into 1-cm-thick slices perpendicular to the heart base-apex axis. The slices were then incubated in 0.8% triphenyltetrazolium chloride at 37°C, which stained the viable myocardium red to measure the extent of myocardial necrosis (10). The extent of myocardial necrosis and the area at risk was determined by planimetry.

Determination of ET-like immunoreactivity. Pieces of myocardium from ischemic and nonischemic areas of the left ventricle were frozen in liquid nitrogen and thereafter stored at −80°C. The tissues were cut into small pieces in the frozen state and transferred to glass tubes containing 10 vol preheated distilled water and were then heated for 10 min at 95°C. The samples were chilled in an ice-water bath and homogenized. The homogenates were centrifuged for 10 min at 1,200 g, +4°C, and the supernatants were transferred to new test tubes and evaporated in a vacuum centrifuge. The samples were stored at −20°C and subsequently dissolved in assay buffer. ET-like immunoreactivity (ET-LI) was then analyzed by radioimmunoassay with the use of a commercially available antiserum (rabbit anti-ET-1 6901, Peninsula), according to a previously described method (13). The cross-reactivity of the antiserum used is 7% with ET-2, 7% with ET-3, and 17% with Big ET-1 when cross-reactivity with ET-1 is set at 100%. The intra-assay and interassay variations are 8 and 18%, respectively.

Chemicals. Ketamine hydrochloride was purchased from Parke-Davis, pentobarbital sodium was from Apotekbolaget (Sweden), atropine sulfate and heparin sodium were from Lovens (Denmark), and L-NNA and L-Arg from

![Figure 1](http://ajpheart.physiology.org/). Illustration of the two experimental protocols. In protocol 1, 45 min of myocardial ischemia was followed by 4 h of reperfusion. Five groups of pigs were given the following drugs: 1) vehicle; 2) the ET₄ receptor antagonist LU-135252 (LU), 5 mg/kg iv 10 min before ischemia; 3) the nitric oxide (NO) synthase inhibitor NG-nitro-L-arginine (L-NNA), 10 mg/kg iv 30 min before ischemia; 4) L-NNA with LU (L-NNA + LU), 30 and 10 min before ischemia; and 5) L-NNA in combination with L-arginine and LU (L-NNA + L-Arg + LU), 30, 20, and 10 min before ischemia, respectively. In protocol 2, two groups were given: 1) vehicle or 2) L-NNA, as in protocol 1.
Table 1. Hemodynamic data before drug administration, before ischemia, at the end of ischemia, and during reperfusion

<table>
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<th>Group</th>
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<th>Preinfusion, basal level</th>
<th>Preischemia</th>
<th>Ischemia, end</th>
<th>Reperfusion, min</th>
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<tr>
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Protocol II

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<td>115 ± 9</td>
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<td>127 ± 4</td>
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Values are means ± SE; n, no. of pigs in each group. Protocol I, 45 min ischemia and 4 h reperfusion; protocol 2, 30 min ischemia and 4 h reperfusion. MAP, mean arterial pressure (in mmHg); LAD flow, left anterior descending coronary artery flow (in ml/min); and HR, heart rate (in beats/min). Significant differences from preinfusion values, *P < 0.05, †P < 0.01; and from the vehicle group, ‡P < 0.05, are shown.

Sigma. LU was supplied by Manfred Raschack (Knoll, Germany). LU was dissolved in 1 M NaOH and saline and adjusted with 0.1 M HCl to obtain pH 7.4. Further dilutions were made in saline. L-NNA was dissolved in saline by increasing the pH to 9 by adding NaOH. The pH was then readjusted to 7.4 by HCl. L-Arg was dissolved in saline.

Calculations and statistical analysis. All values are presented as means ± SE. Statistically significant differences were calculated with the use of Friedman’s test or Kruskal-Wallis nonparametric ANOVA for multiple paired and unpaired observations, respectively, followed by the Bonferroni test. P < 0.05 was considered statistically significant.

RESULTS

Mortality and exclusions from the study. Of the 52 randomized pigs, 6 pigs were excluded because they developed irreversible ventricular fibrillation during ischemia. In protocol 1, one animal was excluded in the vehicle group, two animals in the LU group, and two animals in L-NNA + L-Arg + LU-treated groups. In protocol 2, one animal was excluded in the L-NNA group. The remaining 46 pigs were included in the final analysis of the study.

Hemodynamics. MAP, HR, and LAD blood flow before drug administration, before ischemia at the end of ischemia, and during reperfusion are presented in Table 1. There were no significant differences in hemodynamics before drug administration between the groups. In protocol 1, MAP decreased significantly during ischemia-reperfusion in the LU and vehicle groups. However, there were no significant differences in MAP between the LU group and the vehicle group. L-NNA increased MAP before the onset of ischemia, and this increase persisted until 4 h of reperfusion. Administration of L-Arg after L-NNA reduced MAP from 118 ± 10 to 110 ± 2 mmHg (P < 0.05). Administration of LU reduced MAP further to 99 ± 3 mmHg (P < 0.01). HR tended to increase in all groups, but the increase reached statistical significance only in the vehicle and L-NNA + L-Arg + LU groups. RPP was significantly higher in the L-NNA than in the LU group and vehicle group (Fig. 2A). When LU or L-Arg and LU were given after L-NNA, RPP were not different from the vehicle group during ischemia-reperfusion. Thus LU and the combination of L-Arg and LU reversed the increase in MAP and RPP evoked by L-NNA (Table 1, Fig. 2A). A hemodynamic pattern similar to that in protocol 1 was observed in animals included in protocol 2. Thus MAP was higher after drug administration in animals given L-NNA than in those given vehicle (Table 1). RPP increased significantly in the L-NNA group.
but not in the vehicle group (Fig. 2B). LAD blood flow increased at the onset of reperfusion in all groups of animals in both protocols (Table 1). The degree of hyperemia varied between 200 and 250% of basal flow, and there were no significant differences between the groups. At the end of reperfusion, LAD blood flow had returned to preischemic levels.

Infarct size. Figure 3 shows the IS expressed as a percentage of the area at risk in all experimental groups. In protocol 1, the IS was 79 ± 5% of the area at risk in the vehicle group and 93 ± 2% in the l-NNA group, which was not significantly different from the vehicle group. LU reduced the IS to 43 ± 7% (P < 0.001 vs. vehicle). When l-NNA was administered before LU, the IS (76 ± 6%) was not different from that of the vehicle group but significantly larger than that of the group given LU only (P < 0.001). The addition of L-Arg restored the cardioprotective effect of LU (IS 56 ± 2%; P < 0.05 vs. vehicle and l-NNA+LU groups). In protocol 2, the IS in the vehicle group was 49 ± 6% and 32 ± 4% in the l-NNA group (P = not significant). These infarcts in protocol 2 were significantly smaller than those of the corresponding groups in protocol 1. No significant differences in the areas at risk were observed between the groups (data not shown).

Myocardial ET-LI. The tissue level of ET-LI was not significantly different in the ischemic-reperfused myocardium compared with the nonischemic myocardium in animals subjected to 30 min of ischemia (Fig. 4). However, in animals subjected to 45 min of ischemia myocardial ET-LI was four times higher in the ischemic-reperfused myocardium than in the nonischemic myocardium (P < 0.001, Fig. 4). In addition, ET-LI was significantly higher in ischemic-reperfused myocardium from animals subjected to 45 min of ischemia than from animals subjected to 30 min of ischemia (P < 0.001, Fig. 4). The tissue level of ET-LI in the ischemic-reperfused myocardium of the LU-treated animals in protocol 1 was almost three times lower than that of the vehicle group (P < 0.01, Fig. 5). ET-LI in the ischemic-reperfused myocardium of the groups given l-NNA before LU or l-NNA only were not significantly different from that of the vehicle group (Fig. 5). However, when L-Arg was given together with l-NNA and LU, ET-LI in the ischemic-reperfused myocardium was lower than in the vehicle group (Fig. 5).

DISCUSSION

The aim of the present study was to investigate whether the cardioprotective effect of a selective ET<sub>A</sub> receptor antagonist during ischemia-reperfusion was dependent on the production of NO. Administration of the ET<sub>A</sub> receptor antagonist LU significantly reduced IS compared with the vehicle group after 45 min of ischemia and 4 h of reperfusion. The most important finding of the present study is that the cardioprotective effect of the ET<sub>A</sub> receptor antagonist was abolished by the NOS inhibitor by L-NNA and that it was restored by addition of the NO precursor L-Arg. This suggests that the cardioprotective effect of LU is related to maintained production of NO. A second important finding is that blockade of NOS by L-NNA did not significantly affect IS per se in pigs subjected to 30 or 45 min of ischemia. It is well documented that ischemia-reperfusion results in impaired endothelium-dependent vasodilation, which is the result of reduced NO formation or rapid inactivation of NO by oxygen-derived free radicals (20, 33). Myocardial ischemia-reperfusion is also associated with enhanced production and coronary constrictor activity of ET-1 (32, 38). The increased activity of the ET system seems to be deleterious during ischemia-reperfusion because administration of ET receptor antagonists have been demonstrated to protect from ischemia-reperfusion in-
jury in the present as well as in previous studies (12–14, 35). The mechanisms behind the cardioprotective effect of ET receptor antagonists have not been clarified, however. In a study (36) on isolated rat hearts the mixed ETA and ETB receptor antagonist not only improved myocardial function, but it also preserved endothelium-dependent relaxation. Furthermore, long-term administration of LU preserved endothelial function in atherosclerotic mice (2). These findings indicate that ET-1 may interfere with NO bioavailability. The present finding that the cardioprotective action of LU was abolished after blockade of NOS is in accordance with this hypothesis and suggests that the protection is related to maintained production of NO. The relation of the cardioprotective effect of LU to NO production was further evaluated by administration of a high dose of the NO precursor L-Arg after NOS inhibition by L-NNA. L-Arg reversed the increase in MAP induced by L-NNA, indicating that the inhibition of NOS was reversed. The addition of L-Arg was found to restore the infarct-limiting effect of LU. This finding further supports the notion that the infarct-limiting effect of the ETA receptor antagonist is dependent on NO production.

Because we used a selective ETA receptor antagonist in the present study, endogenous ET-1 may stimulate unblocked ETB receptors leading to release of NO, which causes cardioprotection. However, this seems less likely because the mixed ETA and ET B receptor antagonist bosentan evokes the same degree of cardioprotection as LU and prevents endothelial dysfunction after ischemia-reperfusion (36). Furthermore, the pig coronary vascular bed contains little or no endothelial ETB receptors (35). Therefore, other mechanisms underlying the interaction between ET receptor blockade and NO production during ischemia-reperfusion are likely. For instance, ET-1 stimulates leukocytes that are known to induce endothelial damage (17, 22). It has been demonstrated that ET-1 induced activation of neutrophils results in tissue destruction of umbilical cords (15). Another possibility is that ET-1 via activation of protein kinase C increases the production of oxygen free radicals, which will cause endothelial dysfunction (24). Thus ET-1 may via direct or indirect actions cause endothelial damage and reduce the bioavailability of endothelial NO. However, the exact mechanism by which ET-1 may interact with NO production under the present experimental situation remains to be explored in future studies.

Increased RPP may elevate myocardial oxygen demand and thereby increase IS during ischemia-reperfusion. In our study, NOS blockade by L-NNA increased MAP and RPP in both protocols, which could result in a reversal of the infarct limitation by LU. It could therefore be expected that L-NNA would increase IS per se due to its effect on RPP. In protocol 1 (45 min of ischemia), administration of l-NNA resulted in a trend toward but not significantly increased IS compared with the vehicle group. However, because the IS

![Graph of infarct size](image-url)

Fig. 3. Infarct size expressed as percentage of the area at risk after 45 or 30 min of ischemia, followed by 4 h of reperfusion. The pigs were given either vehicle, LU, L-NNA, L-NNA + LU, or L-NNA + L-Arg + LU before ischemia. Data are means ± SE, n = 6–10 pigs in groups. Significant differences between the groups are shown: *P < 0.05; **P < 0.01; and ***P < 0.001.

![Graph of ET-LI](image-url)

Fig. 4. Endothelin-like immunoreactivity (ET-LI) in myocardial tissue from the nonischemic area and the ischemic area of vehicle pigs subjected to 30 or 45 min of ischemia, followed by 4 h of reperfusion. Data are means ± SE, n = 6 pigs in all groups. Significant difference from the vehicle group: ***P < 0.001.
was close to 80% in the vehicle group, any further increase in IS induced by L-NNA is difficult to detect. We therefore investigated this further in protocol 2 (30 min of ischemia), which resulted in an IS that was significantly smaller than that of the vehicle group and comparable with that of the LU-treated group in protocol 1. By using this protocol, L-NNA did not increase the final IS compared with the vehicle group, despite increased RPP in the L-NNA group, which is in accordance with the results using low-flow ischemia reported by Post et al. (28). In addition, there was no significant difference in RPP between the L-NNA + LU group and the LU group during ischemia-reperfusion in protocol 1. Collectively, these data suggest that L-NNA did not increase IS per se but rather abolished the infarct-limiting effect of LU in protocol 1 and that other factors than altered hemodynamics are involved in the increase in IS by L-NNA in the presence of LU. It is also interesting to note that administration of LU completely abolished the increase in MAP and RPP evoked by L-NNA. This finding is in agreement with previous results (11, 31) from conscious and anesthetized rats in which the increase in blood pressure induced by NOS blockade was attenuated by ET receptor antagonists. Furthermore, the mixed ET_A and ET_B receptor antagonist inhibited the coronary constrictor response to L-NNA in the isolated rat heart (33a). These findings indicate that endogenous ET at least partly mediates the vasoconstrictor responses observed after NOS inhibiton.

One limitation of the present study is that quantitative measurement of NO formation was not performed and related to the effect on IS. NO is rapidly oxidized to nitrite/nitrate (39). Because total nitrite/nitrate originates from several sources other than endothelial NO, determination of plasma nitrite/nitrate poorly reflects NO production in vivo. Furthermore, because the plasma half-life of nitrate is 7–8 h (18), a reduction in plasma nitrate due to attenuated NO production under the present experimental condition is unlikely to occur. Another possibility would be to determine cGMP levels. However, myocardial cGMP levels have been repeated not to be reduced by administration of NOS inhibitors (9). Thus methodological problems limit the possibilities to quantitate NO production under the present experimental condition.

The tissue levels of ET-LI were markedly elevated in the ischemic compared with the nonischemic myocardium in protocol 1. These data are in agreement with a previous report (34) on the same animal model and seem to reflect enhanced production of ET-1, on the basis of the finding that the expression of mRNA for prepro ET-1 is enhanced (32). The myocardial level of ET-LI was significantly higher after 45 min of ischemia than after 30 min of ischemia. Because the reperfusion time was the same in these two groups, this finding indicates that ET levels increase substantially during prolonged ischemia and that the ET levels are mainly dependent on the duration of ischemia. The increase in tissue ET-LI was markedly attenuated in animals given LU, which is similar to what was found after administration of the mixed ET_A and ET_B receptor antagonist bosentan to pigs subjected to ischemia-reperfusion (35). The mechanism behind the attenuated increase in tissue ET levels in the presence of ET receptor antagonists is not fully clarified. It has previously been shown that NO inhibits production of ET-1 (4). Thus when NO activity is reduced after ischemia-reperfusion, this inhibition of ET production is attenuated, which may lead to the enhancement of tissue ET levels in the present study. The finding that the myocardial ET levels were unaffected by ischemia-reperfusion in the presence of LU might be related to preserved NO production, which results in intact NO-mediated inhibition of ET production. This suggestion is supported by the findings that the increase in ET tissue levels was not inhibited by LU in the presence of the NOS inhibitor L-NNA and that the increase in myocardial ET was attenuated after administration of L-Arg together with L-NNA.

In conclusion, myocardial ischemia-reperfusion results in a time-dependent increase in myocardial levels of ET. Administration of an ET_A receptor antagonist limits the extent of the ischemia-reperfusion injury as well as the enhancement of tissue ET levels. Both these effects are abolished by a NOS blocker and restored by addition of the NO precursor L-Arg, suggesting that they are mediated via a NO-dependent mechanism.

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**Fig. 5.** ET-LI in myocardial tissue from the nonischemic area and the ischemic area of pigs subjected to 45 min of ischemia, followed by 4 h of reperfusion. The pigs were given either vehicle, LU, L-NNA, L-NNA + LU, or L-NNA + L-Arg + LU before ischemia. Data are means ± SE, n, 6 pigs in all groups. Significant differences from the ischemic vehicle group are shown: *P < 0.05; **P < 0.01; and ***P < 0.001.
Further work is needed to elucidate the mechanism by which blockade of ET\textsubscript{A} receptors is coupled to NO.

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REFERENCES


ENDOTHELIN AND ISCHEMIA-REPERFUSION

H1111

