Protective Mechanism of Adenosine to the Rat Arterial Endothelial Dysfunction Induced by Hydrogen Peroxide

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This study was designed to examine the in vitro effects of adenosine (Ado) on hydrogen peroxide-induced endothelial dysfunction in rats. Endothelial dysfunction was induced by exposing isolated rat mesenteric arteries to hydrogen peroxide (0.5 mM) for 12 h using an organ culture system. The protective effects of adenosine were tested by exposing isolated mesenteric arteries to adenosine (3×10⁻⁷ mol/l, 10⁻⁶ mol/l, 3×10⁻⁶ mol/l)+hydrogen peroxide (0.5 mM) for 12 h. This exposure to hydrogen peroxide induced a significant concentration-dependent inhibition of endothelium-dependent relaxation (EDR). Coculture of segments of mesenteric artery with adenosine (3×10⁻⁷, 10⁻⁶, and 3×10⁻⁶ mol/l) attenuated the hydrogen peroxide-induced impairment of vasorelaxation. This impairment was accompanied by a reduction in nitrite/nitrate, nitric oxide (NO) synthase (NOS), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities and an increasing in malondialdehyde (MDA) and lactate dehydrogenase (LDH) activities in the aorta. These results indicate that adenosine can be used to attenuate hydrogen peroxide-induced endothelial dysfunction, an effect that may be related to antioxidation, thus enhancing NO production by preventing the decrease in NOS.

Key words  hydrogen peroxide; adenosine; endothelium-dependent relaxation (EDR); nitric oxide (NO); nitric oxide synthase (NOS)

It is well known that normal vascular endothelium possesses several important physiological properties. Endothelial dysfunction plays a crucial role in the pathogenesis of vascular diseases such as atherosclerosis, hypertension, and diabetic vascular complications. Reversing endothelial dysfunction is an effective approach to preventing the development of these vascular diseases.

Increased production of reactive oxygen species (ROS) has been implicated in the pathogenesis of cardiovascular diseases such as myocardial ischemia, atherosclerosis, restenosis, hypertension, diabetic vascular complications and heart failure.¹,² One electron reduction in molecular oxygen forms a superoxide anion, which acts as a progenitor for hydrogen peroxide (H₂O₂). By rapidly inactivating the nitric oxide, the superoxide anion contributes to the endothelial dysfunction.³ In mammalian cells, potential enzymatic sources of ROS include the mitochondrial electron transport chain, the arachidonic acid metabolizing enzymes lipoxigenase and cyclooxygenase, the cytochrome P450s, xanthine oxidase, NAD(P)H oxidases, uncoupled nitric oxide synthase (NOS), peroxides, and other hemoproteins. Molecular oxygen undergoes one or two-electron reduction to form superoxide or hydrogen peroxide respectively. The majority of the bioactive hydrogen peroxide is, however, derived from spontaneous or SOD (superoxide dismutase)-catalyzed dismutation of superoxide.⁴ Some studies have shown that the hydrogen peroxide stimulates reactive oxygen species production via enhanced intracellular iron uptake, mitochondrial damage, and sources of vascular NAD(P)H oxidases, xanthine oxidase, and uncoupled endothelial nitric oxide synthase (eNOS). This self-propagating phenomenon likely prolongs H₂O₂-dependent pathological signaling in vascular cells, thus contributing to vascular disease development.³–⁵ When produced transiently at high concentrations, hydrogen peroxide and hydroxyl radicals are capable of oxidizing proteins and lipids directly, and causing the cell injury, including endothelial dysfunction.

Adenosine is a primordial signaling molecule that has evolved to modulate physiological responses in all mammalian tissues. Although myocardial protective effects of the adenosine have been extensively investigated,¹⁰–¹² relatively little is known regarding the effects of adenosine on ischemic coronary vascular function and injury. Furthermore, while adenosine has been shown to modify neutrophil-dependent damage,¹²,¹³ very little is known regarding the ability of adenosine to modify other aspects of the vascular injury process, such as the direct effect of ROS, which has been implicated in the pathogenesis of ischemic cell injuries. Therefore, the aim of the current study was to identify the effects of adenosine on the rat arterial endothelial dysfunction induced by the hydrogen peroxide.

We examined whether adenosine exerts beneficial effects on the hydrogen peroxide-induced impairment of EDR in rat mesenteric arteries in vitro and found the protective action of adenosine. We then explored the possible mechanisms.

MATERIALS AND METHODS

Tissue Preparation and Organ Culture Procedure

Adult Sprague-Dawley rats of both genders (body weight: 250–300 g), which were supplied by the Medical Experimental Animal Center of Xi’an Jiaotong University, China, were used in accordance with the Guidelines on the Care and Use of Laboratory Animals issued by the Chinese Council on Animal Research and the Guidelines of the Animal Care. The study was approved by the ethical committee of Xi’an Jiaotong University. Rats were anesthetized with an i.p. injection of sodium pentobarbital (17 mg/kg).¹⁴,¹⁵ The artery
was gently removed, immediately immersed in 4 °C Krebs solution (mM: NaCl 119, NaHCO₃ 15, KCl 4.6, MgCl₂ 1.2, NaH₂PO₄ 1.2, CaCl₂ 1.5, glucose 5.6), and dissected free of adhering tissue with the aid of a light microscope. The vessels were then cut into 1-mm-long cylindrical segments that were incubated for 24 h at 37 °C in humidified 5% CO₂ in O₂. The segments that were destined for organ culture were placed in a 96-well plate, one segment in each well, in 300 μl Eagle’s Minimum Essential Medium (EMEM) containing 1-glutamine (584 mg/l) and supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml).

The thoracic aorta was gently removed for biochemical assay, and the superior mesenteric artery was immediately isolated for vascular function assay. Segments of artery from the control, hydrogen peroxide, hydrogen peroxide + adenosine groups were cultured for 12 h with vehicle (saline), hydrogen peroxide (0.5 mM), and hydrogen peroxide (0.5 μM) in the presence of adenosine (3×10⁻⁷ mol/l, 10⁻⁶ mol/l, 3×10⁻⁶ mol/l), respectively.

**Functional Studies** Fresh or incubated mesenteric artery segments with one or other combination of saline, hydrogen peroxide, and adenosine, the segments were immersed in temperature-controlled (37 °C) tissue baths containing Krebs solution. The solution was continuously aerated with 5% CO₂ tissue baths containing Krebs peroxide, and adenosine, the segments were immersed in segments with one or other combination of saline, hydrogen peroxide (0.5 mM), and hydrogen peroxide (0.5 μM) in the presence of adenosine (3×10⁻⁷ mol/l, 10⁻⁶ mol/l, 3×10⁻⁶ mol/l), respectively.

**Biochemical Assays** The thoracic aorta samples cultured for 12 h (see ‘Tissue Preparation and Organ Culture Procedure’) were manually homogenized, using a Potter homogenizer with a glass pestle, in 19 volumes of ice-cold 100 mM Tris–HCl buffer containing 0.1 mM ethylenediaminetetraacetic acid and 0.1% (v/v) Triton X-100, pH 7.8. All procedures were performed on ice. Homogenates were centrifuged at 1500×g at 4 °C for 15 min (TGL-16G centrifuge, Shanghai Scientific Instrument Factory, Shanghai, China), and the resultant supernatants were aliquoted and stored at −70 °C until analysis.

The NO levels in the aorta were determined indirectly as the contents of nitrite and nitrate. Levels of nitrite and nitrate were measured as described previously. Briefly, nitrate was converted into nitrite with aspergillus nitrite reductase, and the total nitrite level was then measured with the Griess reagent. The absorbance was determined at 550 nm using a spectrophotometer (UV-260, Shimadzu Co., Kyoto, Japan).

NOS activities in aorta homogenates were measured using a NOS-detection assay kit according to the manufacturer’s instructions. This kit measures levels of nitrite. The quantity of serum or aorta homogenates sample is 30 μl or 100 μl, respectively. The presence of protein in the aorta homogenates was detected by the Coomassie brilliant blue method. The absorbance was determined at 530 nm by a spectrophotometer. The results are expressed as nanomoles of NO released per milliliter of serum (nmol NO/ml serum) or per milligram of protein (nmol NO/mg protein). All measurements were performed in triplicate.

The SOD activities in aorta homogenates were determined by monitoring the inhibition of the autoxidation of hydroxy-lamine by previously described methods; the presence of protein in the supernatant was detected by the Coomassie brilliant blue method.

The levels of MDA in aorta, reflecting the level of lipid peroxide, were determined spectrophotometrically by measuring the content of thiobarbituric acid reactive substance as previously described. The MDA equivalents could be reflected by thiobarbituric acid reactive substances (TBARS).

The activity of LDH in aorta, as an indicator of cell cytotoxicity, was measured spectrophotometrically using a commercially available assay kit.

The activity of GSH-Px in aorta was measured spectrophotometrically by DTNB method using a commercially available assay kit.

**Drugs and Reagents** Adenosine, EMEM and acetylcholine chloride were purchased from Sigma (St. Louis, MO, U.S.A.). Norepinephrine was obtained from Tianjin Heping Pharmaceutical Factory (Tianjin, China). Assay kits for Coomassie brilliant blue, nitrite/nitrate, NOS, SOD, MDA, LDH and GSH-Px were obtained from Nanjing Jiancheng Bioengineering institute (Nanjing, China). All other reagents and solvents used in experiments were of analytical grade. A stock solution (1 μM) of acetylcholine chloride was prepared daily and diluted using 0.9% NaCl solution to the desired concentration as required.

**Statistics** Relaxation responses in each segment was expressed as the percentage of the norepinephrine-induced contraction. Data are expressed as mean±S.E.M. The data were analyzed by the analysis of variance (ANOVA) followed by post hoc test appropriately. Differences were considered significant at p<0.05.

**RESULTS**

**Functional Studies** There were no differences in the relaxation responses to acetylcholine in mesenteric artery segments before culture between any of the experimental groups (data not shown). After culture with vehicle for 24 h, acetylcholine (10⁻⁸ to 10⁻⁵ M) still evoked a significant concentration-dependent relaxation, whereby the maximal relaxation (Fₘᵡₐₓ) reached 89.71±1.31%. External exposure of mesenteric artery segments to hydrogen peroxide (0.5 mM) for 6 or 12 h significantly attenuated the acetylcholine-induced relaxation in a time-dependent manner compared with the control (saline only) segments (Table 1, n=6. *p<0.05 compared
Table 1. Inhibitory Effects of Hydrogen Peroxide (H$_2$O$_2$) on the Endothelium-Dependent Relaxation Induced by Acetylcholine (ACh) in the Rat Mesenteric Artery Cultured for 6 or 12 h Precontracted with 1 $\mu$m Norepinephrine (%; Mean±S.E.M.)

<table>
<thead>
<tr>
<th>Group</th>
<th>$n$</th>
<th>Relaxation induced by different contraction of ACh (mol/l)</th>
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<tr>
<td></td>
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<td>$1\times10^{-3}$</td>
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<tr>
<td>Control</td>
<td>6</td>
<td>14.01±2.69</td>
</tr>
<tr>
<td>H$_2$O$_2$ 6 h</td>
<td>6</td>
<td>6.61±1.71</td>
</tr>
<tr>
<td>H$_2$O$_2$ 12 h</td>
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<td>5.09±0.43</td>
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* $p<0.05$ vs. control.

Table 2. Effects of Adenosine on the Endothelium-Dependent Relaxation Induced by Acetylcholine (ACh) in the Rat Mesenteric Artery Cultured for 12 h with Hydrogen Peroxide (H$_2$O$_2$) Precontracted with 1 $\mu$m Norepinephrine (%; Mean±S.E.M.)

<table>
<thead>
<tr>
<th>Group</th>
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<th>Relaxation induced by different concentration of ACh (mol/l)</th>
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<tr>
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<tr>
<td>Control</td>
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<td>14.01±2.69</td>
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<td>H$_2$O$_2$, 0.5 mmol/l</td>
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<tr>
<td>Ado 3 $\times 10^{-7}$ mol/l</td>
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<td>4.76±0.79</td>
</tr>
<tr>
<td>Ado 3 $\times 10^{-6}$ mol/l</td>
<td>6</td>
<td>9.61±2.53</td>
</tr>
<tr>
<td>Ado 3 $\times 10^{-5}$ mol/l</td>
<td>6</td>
<td>10.09±2.67</td>
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</tbody>
</table>

* $p<0.05$ vs. control.

with the control group). However, endothelium-dependent relaxation (EDR) in response to hydrogen peroxide (0.5 mm) for 24 h disappeared (data not shown).

Treatment of mesenteric artery segments with hydrogen peroxide (0.5 mm) for 12 h in the presence of adenosine (3 $\times 10^{-7}$ mol/l, 10 $\times 10^{-6}$ mol/l, 3 $\times 10^{-6}$ mol/l) resulted in a concentration-dependent attenuation of the hydrogen peroxide-induced inhibition of EDR. The hydrogen peroxide+adenosine groups showed more enhanced $E_{\text{max}}$ compared with the hydrogen peroxide group (0.5 mm, Table 2, $n=6$. * $p<0.05$ compared with the hydrogen peroxide group).

**Biochemical Assays** Culturing of the thoracic aorta samples with hydrogen peroxide (0.5 mm) for 6 or 12 h significantly decreased the content of nitrate/nitrite (the stable end products of NO) in aorta homogenates when compared with that of the control group (Fig. 1A: control, 20.34±6.29 mmol/l; 6 h, 10.30±2.50 mmol/l; 12 h, 5.19±1.09 mmol/l; $n=6$, * $p<0.05$, ** $p<0.01$ compared with the control group). Adenosine (3 $\times 10^{-4}$ mol/l, 10 $\times 10^{-5}$ mol/l, 3 $\times 10^{-6}$ mol/l) prevented this reduction in nitrate/nitrate levels (Fig. 1B: control, 20.34±6.29 mmol/l; 12 h, 5.19±1.09 mmol/l; Ado-low, 8.40±3.28 mmol/l; Ado-medium, 12.55±2.74 mmol/l; Ado-high, 17.81±3.30 mmol/l; * $p<0.05$, ** $p<0.01$ compared with the hydrogen peroxide group).

NOS (Fig. 2A: control, 16.22±2.04 U/mg protein; 6 h, 9.74±1.05 U/mg protein; 12 h, 8.82±1.75 U/mg protein; $n=6$. * $p<0.05$ compared with the control group), SOD (Fig. 2B: control, 106.15±10.26 U/mg protein; 6 h, 80.38±12.18 U/mg protein; 12 h, 63.08±4.87 U/mg protein; $n=6$. * $p<0.05$ compared with the control group), and GSH-Px (Fig. 2C: control, 394.08±33.11 U/mg protein; 6 h, 302.98±34.77 U/mg protein; 12 h, 180.81±32.21 U/mg protein; $n=6$. * $p<0.05$ compared with the control group) activities in aorta homogenates were significantly decreased in the hydrogen peroxide-treated group compared with the control group.

Adenosine (hydrogen peroxide+adenosine group) resulted in a reversal of the hydrogen peroxide-induced reduction of NOS (Fig. 3A: control, 16.22±2.04 U/mg protein; 12 h, 8.82±1.75 U/mg protein; Ado-low, 11.34±1.19 U/mg protein; Ado-medium, 14.33±1.67 U/mg protein; Ado-high, 17.48±0.65 U/mg protein; $n=6$. * $p<0.05$ compared with the hydrogen peroxide group), SOD (Fig. 3B: control, 106.15±10.26 U/mg protein; 12 h, 63.08±4.87 U/mg pro-
Ado-low, 82.41±6.65* U/mg protein; Ado-medium, 97.26±4.24* U/mg protein; Ado-high, 101.66±9.50* U/mg protein; n=6, *p<0.05 compared with the hydrogen peroxide group)

Both TBARS (Fig. 4A1: control, 1.85±0.52 nmol/mg protein; 6 h, 2.11±0.38 nmol/mg protein; 12 h, 3.56±0.40* nmol/mg protein; n=6, *p<0.05 compared with the control group) and LDH (Fig. 4A2: control, 2792.10±508.40 U/g protein; 6 h, 3997.76±978.78 U/g protein; 12 h, 5386.59±666.67* U/g protein; n=6, *p<0.05 compared with the control group) activities in aorta homogenates were significantly increased in the hydrogen peroxide-treated group compared with the control group. Treatment with adenosine (hydrogen peroxide+adenosine group) resulted in a reversal of the hydrogen peroxide-induced increasing of TBARS (Fig. 4B1: control, 1.85±0.52 nmol/mg protein; 12 h, 3.56±0.40 nmol/mg protein; Ado-low, 2.69±0.21* nmol/mg protein; Ado-medium, 2.16±0.16* nmol/mg protein; Ado-high, 1.82±0.21* nmol/mg protein; n=6, *p<0.05 compared with the hydrogen peroxide group) and LDH (Fig. 4B2: control, 2792.10±508.40 U/g protein; 12 h, 5386.59±666.67* U/g protein; Ado-low, 4114.12±591.89* U/g protein; Ado-medium, 3908.23±678.49* U/g protein; Ado-high, 3983.34±449.67* U/g protein; n=6, *p<0.05 compared with the hydrogen peroxide group) activities in aorta.

DISCUSSION

The major findings of the present study are as follows.

(1) Hydrogen peroxide inhibited the EDR in isolated rat mesenteric arteries in a time-dependent manner, which was partially abolished by simultaneous treatment with adenosine.

(2) Adenosine reversed the hydrogen peroxide-induced changes in nitrite/nitrate levels and enzyme activities of NOS, SOD, GSH-Px, MDA and LDH in aorta homogenates.

These results suggest that adenosine can attenuate hydrogen peroxide-induced endothelial dysfunction, an effect that may be attributed to antiooxidation which can prevent the hydrogen peroxide-induced reduction in NOS, thus enhancing NO production in endothelial cells.

Hydrogen Peroxide-Induced Endothelial Dysfunction
The term endothelial dysfunction has been used to refer to several pathological conditions, including altered anticoagulant and anti-inflammatory properties of the endothelium, impaired modulation of vascular growth, and dysregulation of vascular remodeling. In the literature, however, this term has been used to refer to an impairment of endothelium-dependent vasorelaxation caused by a loss of nitric oxide bioactivity in the vessel wall. Ambient production of hydrogen peroxide at low levels, likely maintained by pre-assembled NAD(P)H oxidases, is necessary for endothelial cell growth and proliferation. Under pathological conditions, however, agonists-provoked activation of vascular NAD(P)H oxidases produces hydrogen peroxide in large quantities, which in turn amplifies its own production, resulting in compensatory or detrimental consequences. An excess in hydrogen peroxide activity accelerates NO inactivation and impairs eNOS activity, resulting in decreased bioactive NO and increased vascular resistance.

Acetylcholine-induced vasorelaxation is mediated by the stimulation of endothelial NO activity, which leads to the synthesis of NO. The NO produced subsequently diffuses to the adjacent smooth muscle cells to activate soluble guanylate cyclase, which in turn leads to smooth muscle relaxation. The release of NO by endothelial cells is important in the mediation of endothelium-dependent vasorelaxation. It also interferes with key events in the development of atherosclerosis, inducing, for example, oxidative damage to the endothelium, monocyte adhesion and migration to vessel walls, platelet aggregation, and vascular smooth muscle proliferation. Although the precise mechanisms underlying the hydrogen peroxide-induced impairment of endothelium-dependent vasodilatation are not fully understood, increasing the degradation of NO and reducing endothelium-derived NO synthesis could be implicated in this dysfunction. The present study demonstrates that hydrogen peroxide impairs the EDR of the rat mesenteric artery in vitro, and that hydrogen peroxide decreases not only NOS activity and levels of nitrite/nitrate, stable end products of NO that reflect NO synthesis, but also SOD and GSH-Px activity and increases MDA equivalents and LDH activity in aorta homogenates. Taken together, these results support the proposal that hydrogen peroxide contributes to endothelial dysfunction by increasing NO degradation and reducing endothelium-derived NO synthesis by decreasing levels of NOS.

Adenosine and Endothelial Function Adenosine, an endogenous nucleoside, is an important biochemical intermediate in cellular metabolism and has protective effects in cardiovascular system. In the present study we have demonstrated that adenosine can also prevent the hydrogen peroxide-induced impairment of EDR in isolated rat mesenteric artery segments. To determine whether this beneficial effect is due to its antioxidant property and enhancement of NO production, we examined NO, NOS, SOD, GSH-Px, MDA and LDH activities in aorta. The results demonstrate that adenosine treatment in aorta that are being administered by hydrogen peroxide not only improves the hydrogen peroxide-induced impairment of EDR, but also prevents the hydrogen peroxide-induced reduction of NO content as well as the diversification of NOS, SOD, GSH-Px, MDA and LDH activities in aorta homogenates. These results indicate that adenosine may inhibit the hydrogen peroxide-induced endothelial dysfunction partly by preventing the...

Fig. 4. Effect of Hydrogen Peroxide (H2O2) (A1, A2) and Adenosine (Ado) Treatment (B1, B2) on MDA Equivalents (TBARS) and LDH Activity in Aorta Homogenates of Rats

Data are expressed as mean±S.E.M., n=6. *p<0.05 compared with the control group; #p<0.05 compared with the hydrogen peroxide group.
hydrogen peroxide-induced reduction in NOS activity, thus enhancing NO production, and by scavenging the oxygen-free radicals, which may cause a deterioration of endothelial function during exposure to hydrogen peroxide. It is not certain whether adenosine can improve the hydrogen peroxide-induced structural impairment in endothelial cells. Thus, the morphological and functional aspects of the protective effect of adenosine should be examined.

In heart, A₁ adenosine receptor activation protects via enhancing antioxidant defense.26) Adenosine receptor activation reduces mitochondrial radical formation27) and oxidant injury,28) and increases cellular antioxidant capacity.29)

Adenosine receptors have been shown to be involved in endothelial protection with preconditioning.20,31) A₂ adenosine receptor activation was found to protect against postischemic vascular endothelial injury.10,32,33) Protection against this hydrogen peroxide-induced injury, and (iii) the beneficial effect of adenosine is achieved by scavenging oxygen radicals and preventing a reduction in NOS activity, (i) treatment with hydrogen peroxide results in impairment of NO production during exposure to hydrogen peroxide, and (ii) adenosine protects rat mesenteric endothelium via A₁ adenosine receptor activation further limits dysfunction and improves post-ischemic vasoregulation.34) The mechanism of adenosinergic “vasoprotection” is unclear. Maczewski and Beresewicz31) argue that PKC dependent reductions in endothelial function during exposure to hydrogen peroxide. It is not certain whether adenosine can improve the hydrogen peroxide-induced structural impairment in endothelial cells. Thus, the morphological and functional aspects of the protective effect of adenosine should be examined.

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CONCLUSION

In conclusion, the findings of the present study show that:
(i) treatment with hydrogen peroxide results in impairment of EDR function, an effect that may be partly due to the suppression of NO production through the endothelial dysfunction caused by the superoxide anions contained in hydrogen peroxide, (ii) adenosine protects rat mesenteric endothelium against this hydrogen peroxide-induced injury, and (iii) the beneficial effect of adenosine is achieved by scavenging oxygen-free radicals and preventing a reduction in NOS activity, thus enhancing NO production in endothelial cells. Adenosine is therefore an effective pharmacological approach to the prevention of ROS-induced vascular diseases.

Vascular endothelial injury and dysfunction may underlie more diseases than currently known.34) Clarification of the above processes will undoubtedly provide important insights into the intrinsic response of the cardiovascular system to acute ischemic insult, and whereas “adenosinergic therapy” itself may prove problematic, this knowledge may aid in developing novel therapeutic strategies that target selected aspects of ischemic and reperfusion injuries in man.

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