Acidic reoxygenation protects against endothelial dysfunction in rat aortic rings submitted to simulated ischemia

Diego López, Antonio Rodríguez-Sinovas, Luis Agulló, Javier Inserte, Alberto Cabestrero, and David García-Dorado


Acidic reoxygenation protects against endothelial dysfunction in rat aortic rings submitted to simulated ischemia. Am J Physiol Heart Circ Physiol 295: H2409–H2416, 2008. First published October 17, 2008; doi:10.1152/ajpheart.00409.2008.—Ischemia-reperfusion causes endothelial dysfunction. Prolongation of acidosis during initial cardiac reperfusion limits infarct size in animal models, but the effects of acidic reperfusion on vascular function are unknown. The present work analyzes the effects of acidic reoxygenation on vascular responses to different agonists in rat aortic rings.

Arterial rings obtained from Sprague-Dawley rat aorta were placed in organ baths containing a Krebs solution oxygenated at 37°C (pH 7.4).

Vascular dysfunction has been implicated in the pathogenesis of diseases such as atherosclerosis, hypertension, diabetes, coronary and cerebral vasospasm, and ischemia-reperfusion injury (15). Endothelial dysfunction after ischemia-reperfusion, denoted by attenuated responses to the endothelium-dependent vasodilator agonists acetylcholine, bradykinin, or ADP (7, 9, 10, 14, 20, 21, 27, 30, 33, 34) or by increased vascular permeability (8), has been demonstrated in several animal models, including rats, cats, dogs, and pigs (7–10, 14, 20, 21, 27, 30, 33, 34). The extent of injury may vary between microvessels and large vessels, as occurs between coronary microvessels and large coronary arteries (33). Moreover, reperfused arteries may also depict abnormal smooth muscle function, although existing data in this regard are controversial (13, 21, 34, 37). In fact, some studies have suggested that endothelium is more vulnerable to ischemia-reperfusion injury than smooth muscle cells and cardiomyocytes, functionally assessed as percent recovery of vasodilatory responses to 5-hydroxytryptamine and glyceryl trinitrate and of aortic flow, respectively (26). Several cardioprotective maneuvers, such as ischemic preconditioning (9), adenosine given during reperfusion (3), or ischemic postconditioning (25, 39), have been shown to reduce endothelial dysfunction after ischemia-reperfusion.

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ACIDIC REOXYGENATION AND AORTIC FUNCTION

Experimental Preparation

Thirty-eight rats were submitted to thoracotomy under pentobarbital anesthesia (1.5 g/kg ip). After heart extraction, the descending thoracic aorta was quickly excised and placed, for 5–10 min, in oxygenated (95% O2–5% CO2) Krebs solution at 4°C containing (in mM) 118.07 NaCl, 4.70 KCl, 1.77 CaCl2, 2 H2O, 1.17 KH2PO4, 1.17 MgSO4·7 H2O, 24.04 NaHCO3, and 12.2 glucose (pH 7.4). The aorta was carefully cleaned of debris and blood and cut into rings of 3 to 4 mm in length. Aortic rings were placed in 10-ml organ baths containing oxygenated Krebs solution at 37°C and were connected to horizontal isometric force transducers (FSG-01; Experimetria, London, UK). Changes in tension were amplified (SG-M DC bridge amplifier module; Experimetria) and stored in a computer for later analysis. All rings were equilibrated at a resting tension of 30 mN for 1 h. Tissues were exposed, after stabilization (pH 7.4), 3–5 min to 50 mM KCl, until the amplitude of the contractile response was similar in magnitude. To verify the functional state of endothelium, aortic rings were relaxed with the endothelial-dependent vasodilator acetylcholine (1 μM) after precontraction with 2·10–7 M norepinephrine. Aortic rings that relaxed less than 50% of previous precontraction were discarded.

Effects of Acidosis on Aortic Responses to Agonists During Normoxia

Thirty-two aortic rings were exposed, under normoxic conditions, successively to Krebs at pH 7.4 and Krebs at pH 6.4 or vice versa. This degree of acidosis was chosen since previous studies from our group have demonstrated, using NMR, that this is the minimum pH attained during ischemia (18). The composition of Krebs solution at pH 6.4 was the same described in Experimental Preparation, differing only on NaCl (140.5 mM) and NaHCO3 (2.4 mM). Resting tension and agonist-induced responses were determined before and 15 min after changing the Krebs solution. Rings were exposed to cumulative concentrations of norepinephrine (from 10–9 to 10–5 M) and acetylcholine (from 10–9 to 10–5 M). Responses to acetylcholine were determined after precontraction with 2·10–7 M norepinephrine.

Effects of Acidosis on Aortic Responses to Agonists After Hypoxia/Reoxygenation

Changes in aortic responses to agonists during reoxygenation. The effects of acidosis during hypoxia and/or reoxygenation on vascular responses to different agonists were analyzed in 40 aortic segments that were assigned to one of the following treatment groups: normoxia, hypoxia pH 7.4/reoxygenation pH 7.4, hypoxia pH 7.4/reoxygenation pH 6.4, hypoxia pH 6.4/reoxygenation pH 7.4, and hypoxia pH 6.4/reoxygenation pH 6.4. Hypoxia was conducted on a hypoxia chamber (1 h; 95% N2, 5% CO2, 0.3% O2) after removing the hooks from the organ bath. Reoxygenation (1 h) was performed after fixing the hooks again in the organ bath. Vascular responses to norepinephrine (2·10–7 M; endothelium-independent vasoconstrictor), acetylcholine (10–7 M; endothelium-dependent vasodilator), or the nitric oxide (NO) donor sodium nitroprusside (10–7 M; endothelium-independent vasodilator) were assessed before hypoxia and after 5, 15, 30, 45, and 60 min of reoxygenation. Responses to acetylcholine and sodium nitroprusside were tested after precontraction with 2·10–7 M norepinephrine.

Measurement of cGMP Content

To analyze the influence of the different treatments on the cGMP generating system, cGMP content was measured as previously described (1). Briefly, rat aortic segments (about 2 cm) were frozen in liquid N2 and homogenized in cold trichloroacetic acid. cGMP was measured using a radioimmunoassay method (1), and results were expressed as femtomoles of cGMP per milligram of tissue. cGMP content was analyzed after 30 min of reoxygenation or the corresponding period of normoxia in nonstimulated rings (n = 8/group) and in rings incubated for 5 min with acetylcholine (10–6 M) (n = 8/group). Eight additional aortic rings were submitted to normoxic incubation in the presence of the NO synthase inhibitor Nω-nitro-L-arginine (10–4 M) to determine the main source of aortic cGMP.

Analysis of Cell Death

Membrane rupture and cell death was analyzed by propidium iodide (PI) staining of nuclei and fluorescence microscopy. PI is a membrane-impermeant dye that stains the nuclei by intercalating into DNA molecules only when the plasmatic membrane is broken. Three aortic rings from each group were incubated after reoxygenation in the same buffer containing PI at 5 μg/ml for 5 min. Rings were fixed in 4% paraformaldehyde, cut into 2-μm sections, and analyzed by fluorescence microscopy. Positive control rings, after incubation with distilled water, were also analyzed.

Data Analysis

For cumulative concentration-response curves, increases in tension induced by norepinephrine were expressed as percentages of the postequilibration contraction to 50 mM KCl, and relaxations induced by acetylcholine or sodium nitroprusside were expressed as percentages of the precontraction to 0.2 μM norepinephrine. Data from concentration-response curves were fitted to a sigmoid function [y = y0 + a/l + exp(−x − xo)/b] to determine the maximal effect (Emax) and the concentration of agonist necessary to produce half-maximal response (EC50 value).

Statistics

All values are expressed as means ± SE. Tension responses and cGMP content for each of the five treatment groups were compared by ANOVA and Holmes-Sidak’s tests.

RESULTS

Effects of Acidosis on Aortic Responses to Agonists During Normoxia

Resting tension of rat aortic rings was slightly, but significantly, reduced when bathed with Krebs at pH 6.4 compared with pH 7.4 (29.96 ± 0.03 vs. 28.71 ± 0.12 μN at pH 7.4 and 6.4, respectively; P < 0.05; Fig. 1A). In contrast, acidosis did not modify concentration-response curves to norepinephrine [Emax (in percentage), 131 ± 14 vs. 113 ± 8 at pH 7.4 and 6.4, respectively; EC50 (−log M), 7.19 ± 0.11 vs. 7.14 ± 0.12; p-NS; Fig. 1B] or acetylcholine [Emax (in percentage), 69 ± 3 vs. 67 ± 2; EC50 (−log M), 7.33 ± 0.12 vs. 7.45 ± 0.11; p-NS; Fig. 1C]. Similarly, acidosis during normoxia did not modify contractile responses to 50 mM KCl (2.37 ± 0.34 vs. 2.26 ± 0.21 mM at pH 7.4 and 6.4, respectively) or 0.2 μM norepinephrine (2.19 ± 0.18 vs. 1.89 ± 0.20 mM).

Effects of Acidosis on Aortic Responses to Agonists After Hypoxia/Reoxygenation

All groups of aortic rings showed similar contractile responses to KCl or norepinephrine (Table 1). Thus they were...
used to normalize responses to norepinephrine, acetylcholine, or sodium nitroprusside in the different experiments.

Control rings, incubated for 2 h in normoxic Krebs (pH 7.4), did not show any change in the responses to norepinephrine, acetylcholine, or sodium nitroprusside (Fig. 2, A–C). In contrast, hypoxia/reoxygenation, independently of pH, significantly lowered norepinephrine-induced contractions ($2 \times 10^{-7}$ M) to about 25% of prehypoxia levels, with a progressive recovery of function during reoxygenation (about 50% at 60 min of reoxygenation), with no significant differences between groups ($n = 8$).

Table 1. Contraction to 50 mM KCl, measured after stabilization, and to 0.2 µM norepinephrine, determined before treatments with vasodilator agonists, in the different experimental groups

<table>
<thead>
<tr>
<th></th>
<th>KCl, mN</th>
<th>Norepinephrine, mN</th>
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<tbody>
<tr>
<td></td>
<td>For Norepinephrine Responses</td>
<td>For Acetylcholine Responses</td>
</tr>
<tr>
<td>Normoxia</td>
<td>2.21±0.19</td>
<td>2.08±0.21</td>
</tr>
<tr>
<td>Hypoxia pH 7.4/ reoxygenation pH 7.4</td>
<td>2.47±0.27</td>
<td>1.99±0.22</td>
</tr>
<tr>
<td>Hypoxia pH 7.4/ reoxygenation pH 6.4</td>
<td>2.30±0.18</td>
<td>2.17±0.18</td>
</tr>
<tr>
<td>Hypoxia pH 6.4/ reoxygenation pH 7.4</td>
<td>2.33±0.20</td>
<td>2.02±0.14</td>
</tr>
<tr>
<td>Hypoxia pH 6.4/ reoxygenation pH 6.4</td>
<td>2.22±0.15</td>
<td>1.97±0.16</td>
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</tbody>
</table>

Values are means ± SE; $n$, number of aortic rings/group. These contractions were used to normalize responses to norepinephrine and to acetylcholine or sodium nitroprusside, respectively. No differences were observed between groups ($n = 8$).
groups (Fig. 2A). Concentration-response curves to norepinephrine, performed 30 min after reoxygenation, were also similarly modified during hypoxia/reoxygenation independently of group allocation. However, in rings submitted to hypoxia at pH 7.4 and reoxygenation at pH 6.4, this difference did not reach statistical significance. Hypoxia/reoxygenation elicited in all cases a reduction of $E_{\text{max}}$, compared with normoxic control rings, but did not modify $EC_{50}$ values (Fig. 3 and Table 2). A nonsignificant trend toward higher $E_{\text{max}}$ values in groups reoxygenated at pH 6.4 was observed compared with its respective pairs reoxygenated at pH 7.4.

Acetylcholine ($10^{-6}$ M)-induced relaxation was decreased to $10 \pm 2\%$ of baseline relaxation by hypoxia/reoxygenation at pH 7.4, with a poor recovery during the entire reperfusion period. A similar aortic dysfunction was observed in rings submitted to hypoxia at pH 6.4 and reoxygenation at pH 7.4. However, rings reoxygenated at pH 6.4 showed partially preserved relaxations to acetylcholine (Fig. 2B). Similarly, acidosis during reoxygenation also improved cumulative concentration-response relaxations to acetylcholine 30 min after reoxygenation. As shown in Fig. 4 and Table 2, hypoxia/reoxygenation at pH 7.4 significantly shifted the $EC_{50}$ of acetylcholine from $7.4 \pm 0.1$ (log M) to $6.8 \pm 0.1 (P < 0.05)$ and reduced the $E_{\text{max}}$ (from $68 \pm 7\%$ to $24 \pm 1\%; P < 0.001$). Acidosis only during hypoxia did not improve relaxations to acetylcholine. However, acidosis during reoxygenation improved them, as observed by a significant increase in $E_{\text{max}}$ values.

Table 2. $E_{\text{max}}$ and concentration of agonist necessary to produce $EC_{50}$ value for the different agonists norepinephrine, acetylcholine, and sodium nitroprusside and conditions tested in rat aortic rings submitted to hypoxia/reoxygenation

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Normoxia</th>
<th>Hypoxia pH 7.4/ reoxygenation pH 7.4</th>
<th>Hypoxia pH 7.4/ reoxygenation pH 6.4</th>
<th>Hypoxia pH 6.4/ reoxygenation pH 7.4</th>
<th>Hypoxia pH 6.4/ reoxygenation pH 6.4</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$E_{\text{max}}, %$</td>
<td>$EC_{50}, \log M$</td>
<td>$E_{\text{max}}, %$</td>
<td>$EC_{50}, \log M$</td>
<td>$E_{\text{max}}, %$</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>133\pm17</td>
<td>7.26\pm0.14</td>
<td>72\pm6*</td>
<td>7.04\pm0.24</td>
<td>106\pm12</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>68\pm7</td>
<td>7.38\pm0.07</td>
<td>24\pm1*</td>
<td>6.79\pm0.11*</td>
<td>66\pm8</td>
</tr>
<tr>
<td>Sodium nitroprusside</td>
<td>71\pm4</td>
<td>8.66\pm0.16</td>
<td>82\pm4</td>
<td>8.54\pm0.19</td>
<td>70\pm5</td>
</tr>
</tbody>
</table>

Values are means $\pm$ SE. $*P < 0.05$, significant differences vs. normoxic rings. $E_{\text{max}}$, maximal effect.
In contrast to those of acetylcholine, relaxation responses to sodium nitroprusside were not affected by hypoxia/reoxygenation in any of the groups analyzed (Figs. 2C and 5 and Table 2).

Aortic cGMP

The cGMP content of nonstimulated aortic rings incubated in normoxic Krebs was 32.4 ± 4.2 fmol/mg of tissue (Fig. 6). When the NO synthase inhibitor Nω-nitro-L-arginine was added to the incubation medium, cGMP content decreased to 11.8 ± 7.4, showing that most of the cGMP measured was synthetized by the soluble or NO-dependent guanylyl cyclase. Changes in pH during hypoxia or reoxygenation did not modify cGMP content in nonstimulated aortic rings (Fig. 6). However, when normoxic rings were stimulated with acetylcholine, a marked increase in cGMP synthesis was observed that was reduced in rings submitted to hypoxia and reoxygenated at pH 7.4. However, reoxygenation under acidic conditions restored the increase in cGMP content seen in normoxic stimulated rings (Fig. 6).

Analysis of Cell Death

PI staining depicted, in positive control rings incubated with distilled water, extensive cell death both at the smooth muscle layer and at the endothelium (Fig. 7, arrowheads). In contrast, both in normoxic rings and in aortic rings submitted to hypoxia/reoxygenation, smooth muscle staining was almost absent, indicating the lack of plasmatic membrane rupture at the media layer. Moreover, endothelial cell death, depicted as nuclear PI staining, was also scant with no differences between groups. Only some PI-stained nuclei were observed in most cases in the adventitia layer (Fig. 7, arrows).

DISCUSSION

The present study demonstrates in rat aortic rings submitted to hypoxia, in a model causing very little cell death, that acidosis during reoxygenation enhances recovery of endothelial function, assessed as vasodilation to acetylcholine. This protective effect is associated with preserved cGMP synthesis in response to acetylcholine in aortic rings reoxygenated at a low pH. In contrast to this improvement in endothelial reactivity, smooth muscle vasoconstrictor function, as assessed by norepinephrine-induced contractions, is reduced during hypoxia and reoxygenation in a pH-independent manner. Responses to sodium nitroprusside, an endothelial-independent vasodilator, were not modified by hypoxia/reoxygenation. These results are consistent with a beneficial effect of acidic reoxygenation on endothelial function.

Protective Effect of Acidosis During Reoxygenation on Endothelial Hypoxia/Reoxygenation Injury

Attenuated responses to different endothelial-dependent vasodilators, such as acetylcholine (27, 30, 37), ADP (14, 30), or bradykinin (14, 21, 27, 34), have been described in reperfused coronary arteries. Endothelial dysfunction is also manifested during reperfusion by increased microvascular permeability, demonstrated by an enhanced extravascular accumulation of radiolabeled proteins (8). Our present results are in agreement with this previously described endothelial injury, since all groups of aortic rings showed a reduced vasodilation to acetylcholine during initial reoxygenation. However, aortic rings
reoxygenated at pH 6.4 showed an enhanced recovery in endothelial function. Importantly, this effect is not restricted to rings submitted to hypoxia at pH 7.4 but also to rings submitted to simulated ischemia (hypoxia plus acidosis), stressing the potential interest of this therapeutic approach.

The protective effect of prolongation of acidosis during reperfusion has been clearly demonstrated in cardiac preparations. Acid reoxygenation prevented cell death, assessed by PI staining, after prolonged exposure to simulated ischemia or anoxia in cardiomyocytes (5, 35). Similarly, acid reoxygenation reduced lactate dehydrogenase release during initial reoxygenation in isolated rat hearts submitted to 120 min global hypoxia (35). Moreover, periods of acid reperfusion as short as 3 min have been shown to be protective against cell injury in the isolated rat heart model, as determined by enzyme release (18, 29), whereas longer periods may exacerbate reper-

Fig. 7. Sections of rat aortic rings incubated with propidium iodide (fluorescence microscopy). Rings were stained after incubation in distilled (Dist) water (positive control) or after normoxia or hypoxia/reoxygenation at different pH. Arrowheads indicate nuclei stained in the endothelial layer. Arrows indicate cell death at the adventitia. Scale bar, 100 μm. Bottom: quantification of stained endothelial nuclei expressed per millimeter of endothelial circumference. *P < 0.05, significant differences vs. the positive control group (distilled water). L, lumen.
fusion injury. Finally, in situ dog hearts, submitted to coronary occlusion for 40 or 90 min, showed lower infarct size after 30–60 min of reperfusion under conditions of metabolic (HCl intracoronary infusion) (22, 32) or respiratory (22) acidosis. Moreover, the protective effect was not restricted to cell death, since acidosis during reperfusion also attenuates stunning after periods of ischemia short enough to avoid cell death (23).

However, the effects of prolongation of acidosis during reperfusion on endothelial function after ischemia were unknown. Indirect evidence using Na+ /H+ exchanger inhibitors previously suggested that a delay in intracellular pH recovery after ischemia can reduce endothelial activation or improve endothelial-dependent vasorelaxation (4, 17, 38). Moreover, Nishimura et al. (28) demonstrated that simulated reperfusion at pH 6.2 reduced cell death in isolated rat liver sinusoidal endothelial cells exposed to prolonged periods of chemical hypoxia. Our study shows that acidic reoxygenation has a protective effect on endothelial function. However, and although in general, vascular tone responses to conditions like hypoxia or acidosis are similarly affected in different vessels of the systemic circulation, we acknowledge that our present findings may not reflect changes occurring in other arteries, such as coronary arteries. In fact, others have reported that hypercapnic acidosis causes a vasodilatory effect in mice coronary arteries, an effect mediated through NO and ATP-sensitive K+ channels (16), whereas in the present study acidosis causes only a small decrease in tone. Moreover, vessel tone has been suggested to be differently regulated in mice coronary arteries and aorta (6).

**Effect of Hypoxia/Reoxygenation on Smooth Muscle Function**

In addition to endothelial injury, ischemia-reperfusion may also cause abnormal smooth muscle function. However, studies analyzing this possibility have not afforded conclusive data. Some authors have shown that coronary arteries preexposed to ischemia-reperfusion depict normal responses to endothelium-independent vasodilators, such as NO donors, adenosine, or isoproterenol (14, 21, 30, 37). A similar lack of smooth muscle dysfunction after ischemia-reperfusion was also observed by our group, after stimulation with methacholine, in isolated pig coronary arteries previously exposed to 2 h of coronary occlusion followed by reperfusion (34). In contrast, others have found an attenuated vasodilation after in vivo exposure to papaverine, an endothelial-independent vasodilator (3). In fact, it has been described that endothelium is more vulnerable to ischemia-reperfusion injury than smooth muscle cells, which may explain the general lack of dysfunction in those studies (26). Our present results show that contractile responses to norepinephrine are highly affected by hypoxia/reoxygenation, whereas vasodilation to NO donors are more in the line of those studies. The reasons for the different effects of hypoxia/reoxygenation on contractile and vasodilator smooth muscle responses are unknown. Moreover, and in contrast with our findings on endothelial function, acidosis, either during hypoxia or reoxygenation, did not improve the contractile dysfunction to norepinephrine observed during reoxygenation.

**Effect of Hypoxia/Reoxygenation on cGMP Content**

Previous studies have demonstrated that both hypoxia and acidosis acutely reduce cGMP synthesis in endothelial cells (1) but not in myocardial cells (2). Similar to cardiomyocytes, the present study shows that cGMP content in aortic rings was insensitive to hypoxia and/or acidosis. With the consideration of the effects of hypoxia and acidosis on endothelial cGMP synthesis (1), and the high proportion of smooth muscle cells present in aortic rings, it is reasonable to think that most of the cGMP content in this tissue would originate in the media layer. If this is the case, our results suggest that cGMP synthesis remains basically unaltered during transient-simulated ischemia in smooth muscle cells. This fact would explain the lack of effect of hypoxia/reoxygenation on sodium nitroprusside-induced relaxations. However, when normoxic aortic rings were stimulated with acetylcholine, there was an increased synthesis of cGMP, which was markedly impaired after hypoxia and reoxygenation at pH 7.4 and restored when reoxygenation was performed under acid conditions. The decrease in cGMP synthesis seen after hypoxia/reoxygenation at pH 7.4, and the impairment in acetylcholine-induced relaxations, appears to be due to endothelial injury. The effects of acetylcholine on aortic rings have been clearly demonstrated to be dependent on intact endothelium (11), through stimulation of muscarinic receptors located in endothelial cells and induction of cGMP synthesis in smooth muscle cells (36). From these data, it is clear that hypoxia/reoxygenation alters endothelial function, attenuating acetylcholine-induced relaxations and cGMP synthesis, and that acid reoxygenation has a protective action against endothelial dysfunction.

The fact that acidosis was previously shown to reduce cGMP synthesis in microvascular endothelial cells (1) is not contradictory with protective actions on endothelium when applied during reoxygenation. Whereas the reduction in cGMP synthesis seen after hypoxia/reoxygenation at pH 7.4, and the impairment in acetylcholine-induced relaxations, appears to be due to endothelial injury. The effects of acetylcholine on aortic rings have been clearly demonstrated to be dependent on intact endothelium (11), through stimulation of muscarinic receptors located in endothelial cells and induction of cGMP synthesis in smooth muscle cells (36). From these data, it is clear that hypoxia/reoxygenation alters endothelial function, attenuating acetylcholine-induced relaxations and cGMP synthesis, and that acid reoxygenation has a protective action against endothelial dysfunction.

**Conclusions**

The protective effects of acidic reperfusion on infarct size may depend on multiple factors, including direct effects on cardiomyocytes or microvasculature. The present results identify the protection of endothelial function as an additional beneficial effect and further support the translation of this therapeutic approach to patients with myocardial ischemia receiving reperfusion therapy.

**GRANTS**

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ACIDIC REOXYGENATION AND AORTIC FUNCTION


