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Effects of hydrogen peroxide on pig coronary artery endothelium

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Abstract

Peroxides and other reactive oxygen species damage arteries during ischemia-reperfusion. Here, we report on the effects of H_2O_2 on contractility of pig coronary artery. We either treated 3-mm coronary artery rings with 0 to 0.5 mM H_2O_2 in organ baths or we perfused the arteries with H_2O_2 and then cut them into rings. In each instance, we monitored the force of contraction of 3-mm rings in H_2O_2-free solution with 30 mM KCl and then we determined the A23187 induced endothelium dependent relaxation as a percent of this contraction. Treatment with H_2O_2 in the organ bath caused a decrease in the contraction but it did not affect the percent relaxation. Treating arteries with H_2O_2 by luminal perfusion did not affect the contraction but it decreased the percent relaxation. Perfusion alone decreased the amount of endothelium remaining in the arteries and perfusing with H_2O_2 decreased it further. The percent relaxation with A23187 correlated well with the endothelium remaining in the arteries. We propose that H_2O_2 and shear stress can cause a loss of endothelium and that endothelium can also protect the underlying smooth muscle against luminal H_2O_2. © 2000 Published by Elsevier Science B.V.

Keywords: Flow; Nitric oxide (NO); Oxidative stress; Ischemia-reperfusion; Vascular

1. Introduction

Reactive oxygen is the main source of cardiovascular damage in an ischemia-reperfusion injury (Cosentino and Luscher, 1997; Darley-Usmar and Halliwell, 1996; Halliwell, 1996; Singal et al., 1998). In particular, the reperfused ischemic tissues contain high concentrations of reactive oxygen species such as peroxide, superoxide, peroxynitrite and perhydroxyl radicals. The high concentrations of these species can produce substantial insult to cells. For example, they can cause lipid peroxidation, oxidation of protein sulfhydryl groups, elevated intracellular Ca^{2+} levels, and DNA breakage. In turn, the lipid peroxidation can increase membrane damage thereby causing transmembrane ion gradients to collapse and altering cellular metabolism. For example, pyruvate dehydrogenase activity in the mitochondria and organellar Ca^{2+}-pump are very sensitive to such damage (Grover et al., 1992, 1995; Vlessis et al., 1991).

Endothelium is involved in several processes including inflammation, platelet aggregation and thrombosis, fibrinolysis, angiogenesis, and regulation of vascular tone (Beny and von der, 1991; Hoover et al., 1987; Lewis et al., 1988; Luscher and Barton, 1997; Van Breemen et al., 1997; Vequaud et al., 1999; Zhang et al., 1998). There are very few studies on the effects of reactive oxygen on the function of endothelium. The compound *t*-butyl-hydroxy-peroxide inhibits bradykinin-stimulated Ca^{2+} influx from extracellular space but not the agonist-induced release of Ca^{2+} from intracellular stores (Elliott et al., 1989). Prolonged exposure to reactive oxygen species generated by *t*-butyl-hydroxy-peroxide also affects the Ca^{2+} release induced by the organellar Ca^{2+} pump inhibitor. Reactive oxygen species also increase the adherence of neutrophils to endothelium (Lewis et al., 1988). Recently, we suggested that endothelium may protect smooth muscle against luminal H_2O_2 (Grover et al., 2000). However, the effects of H_2O_2 on endothelium itself remain to be determined. Here, we examine the effects of H_2O_2 on endothelium in three different experiments — the effect of pretreating 3-mm artery rings with H_2O_2 in an organ bath on the subsequent ability of endothelium to relax the smooth muscle with A23187, the effect of luminally perfused H_2O_2 on the subsequent ability of endothelium to relax the smooth muscle, and the amount of endothelium remaining after perfusion with different concentrations of H_2O_2.

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2. Experimental methods

2.1. Dissection and perfusion of coronary arteries

Fresh pig hearts were obtained from the slaughter house Maple Leaf Meats (Burlington, Ontario, Canada) and placed immediately in an ice-cold physiological saline solution at pH 6.4 (Grover and Samson, 1997). The left anterior descending coronary arteries were dissected and placed in a Krebs' solution bubbled with 95% O_2–5% CO_2. The Krebs' solution had the following composition in millimolar: 115 NaCl, 5 KCl, 22 $NaHCO_3$, 1.7 $CaCl_2$, 1.1 $MgCl_2$, 1.1 KH_2PO_4, 0.03 EDTA, and 7.7 glucose. The cardiac muscle, fat and connective tissue were removed leaving origins of the branch arteries intact, which were subsequently tied off to allow perfusion through the length of the artery, as described previously (Grover et al., 2000). Tygon tubing was then inserted into the origin of the arteries for perfusion at 37–38°C using a peristaltic pump while keeping the arteries moist on the outside with Krebs' solution at the same temperature as described previously. The arteries were perfused with Krebs' solution for 30 min, then with Krebs' solution containing 0 or a specified concentration of H_2O_2 for 30 min and then with Krebs' solution without H_2O_2 for 60 min. The perfusion pressure was kept constant so as to yield a flow of 5.88 ml/min per artery.

2.2. Contractility measurements

The length-tension relationship for the 3-mm long coronary rings was studied previously and the optimum tension was found to be 3 g — corrected after another 30 min to 3 g (Grover and Samson, 1997). The artery rings were always taken from the middle section of the arteries thus avoiding the sections, which could have been disturbed by being very close to the Tygon tubing. This level of force was applied to the arteries in all subsequent experiments. After mounting, the tissues were equilibrated in Krebs' solution under the specified tension for 60 min. Contractions of all arteries were monitored by adding 3 M KCl to obtain a final concentration of 60 mM. Tach response was monitored for 10 min. The rings were then washed five times with 5-ml Krebs' solution over a period of 5 min and then allowed to equilibrate for 10 min. The arteries were contracted with 30 mM KCl and washed again. The rings were again depolarized by Krebs' solution containing 30 mM KCl. A23187 gives an endothelium-dependent relaxation in precontracted arteries (Furchgott, 1983; Rapoport and Murad, 1983). When the contraction reached a steady level, relaxation with 10 μM A23187 was examined (Grover and Samson, 1997). The tissues were then blotted and weighed at the end of the experiment.

In another set of experiments, the artery rings were treated with H_2O_2 without perfusion in organ baths. The rings were cut and placed under optimum tension in the organ baths immediately after the dissection. After 60 min of equilibration, the arteries were contracted with 60 mM KCl, washed and then contraction with 30 mM KCl was monitored. The arteries were washed and then treated with 0 or specified concentrations of H_2O_2 for 30 min, washed and allowed to equilibrate for 60 min and then contracted again with 30 mM KCl. When the contraction reached a steady level, relaxation with 10 μm A23187 was examined.

2.3. Quantification of endothelium remaining in the artery after perfusion

The arteries were perfused as described above, placed in a dissecting dish containing Krebs' solution and 10 μM isoproterenol, pinned at the ends and cut open longitudinally. The moist, opened artery was placed onto a plastic lid or dish with the endothelium side up. A cellulose acetate paper ($\sim 1 \times 4$ cm^2) was placed on top to cover the cells and positioned in place by lightly pressing down uniformly (Hatton et al., 1980). The cellulose acetate strip with the adhering endothelium was peeled away and fixed in 4% paraformaldehyde in 100 mM sodium cacodylate buffer, pH 7.4. Using a scanning microscope, it was determined that no endothelium remained on the tissue after the peeling. The strip was stained using toluidine blue (or hematoxylin) for 10–15 s and rinsed in water. As the edges may have been disturbed during the dissection, only the middle of the artery was used for determining the amount of endothelium remaining in the artery. A 2-cm-long section was cut from the middle of the cellulose acetate strip, placed on a glass slide and mounted using two drops of the aqueous mounting agent gelatin/water/glycerine (prepared by heating 10 g gelatin + 60 ml water and 70 ml glycerol and a few crystals of thymol). Fractional area occupied by cells on the cellulose strip was determined using a digitizing board.

2.4. Data analysis

All the values reported are mean ± SEM of the specified number of replicates. A one way analysis of variance (ANOVA) test was performed to test levels of significance and p values < 0.05 were considered to be significant.

3. Results

3.1. Effect of treating artery rings with H_2O_2 in an organ bath

A23187 is known to cause an enothelium-dependent relaxation of blood vessels (Furchgott, 1983; Rapoport and Murad, 1983). We have previously examined this endothelium-dependent relaxation to A23187 in pig coronary artery rings (Grover and Samson, 1997). Here, we treated the

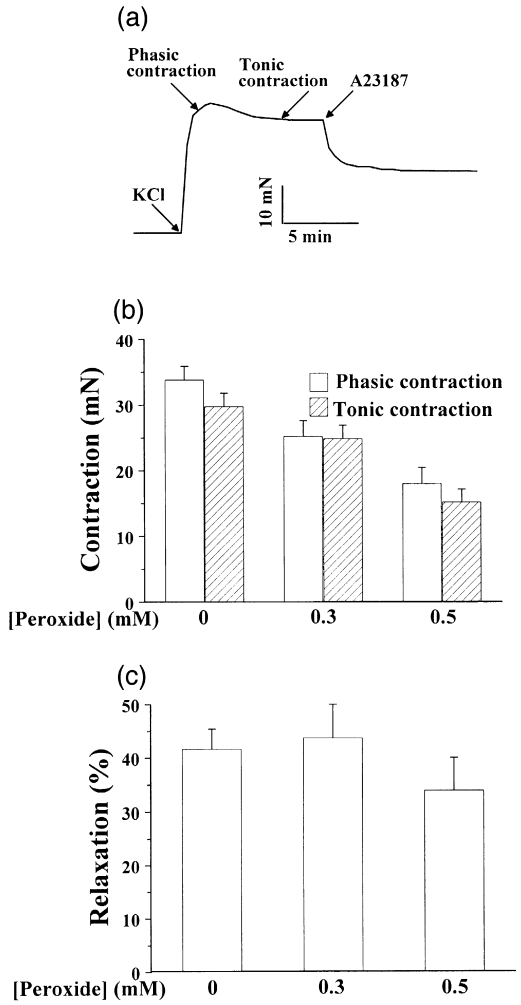

Fig. 1. Contractility of rings treated with H_2O_2 in organ baths. (a) A tracing showing KCl contraction and A23187 relaxation. A 3-mm ring cut from the middle of the coronary artery was hung and treated without H_2O_2 as described in Section 2. Thirty millimolar KCl produced a phasic and then a tonic contraction. When the tonic contraction reached a steady level, 10 μM A23187 was added and the resulting relaxation was monitored as percent of the steady level of the contraction. Rings (3-mm long) from the middle of the artery were hung in an organ bath and treated as described in Section 2. The values shown are mean ± SEM of 31, 15 and 13 animals for 0, 0.3 and 0.5 mM H_2O_2, respectively. Note that all the contraction and relaxation experiments were conducted in H_2O_2 free solutions. (b) Contraction with 30 mM KCl. Values for 0.5 mM H_2O_2 differed significantly ($P < 0.05$) from those of 0 mM but those for 0.3 mM did not. (c) During tonic phase of the contraction, A23187 was added and percent relaxation for each artery was determined. Values for 0.3 or 0.5 mM H_2O_2 did not differ significantly.

3-mm artery rings with H_2O_2 in an organ bath, washed the tissues and then determined their contraction in response to membrane depolarization by 30 mM KCl and the endothelium-dependent relaxation of this contraction. With 30 mM KCl, the artery rings gave a phasic response followed by a tonic response (Fig. 1a). Treatment with 0.5 mM H_2O_2 significantly ($P < 0.05$) decreased both components of the contraction (Fig. 1b) but 0.3 mM H_2O_2 did not produce a significant ($P > 0.05$) change. Challenging the control (no H_2O_2) tissues with 10 μM A23187 during the tonic contraction produced a relaxation which was 42 ± 4% of the contraction. Treating the tissues with 0.3 or 0.5 mM H_2O_2 did not alter this value significantly ($P > 0.05$) (Fig. 1c). Thus, bathing the arteries in 0.5 mM H_2O_2 lowered the force of contraction but not the percent relaxation.

3.2. Effect of H_2O_2 on contractility with perfusion

In this experiment, the H_2O_2 treatment occurred during luminal perfusion with a Krebs' solution and then the rings from these tissues were placed in an organ bath. It is emphasized that whether the H_2O_2 treatment occurred by immersing artery rings in H_2O_2 or by perfusing them, the contractility experiments were always conducted in organ baths after washing the tissues to remove the H_2O_2. Tissue perfusion alone (no H_2O_2) did not significantly change ($P > 0.05$) the force of contraction produced in response to KCl ($P > 0.05$). Including 0.3 or 0.5 mM H_2O_2 during perfusion also had no significant effect on the force of contraction ($P > 0.05$; Fig. 2a). The relaxation produced

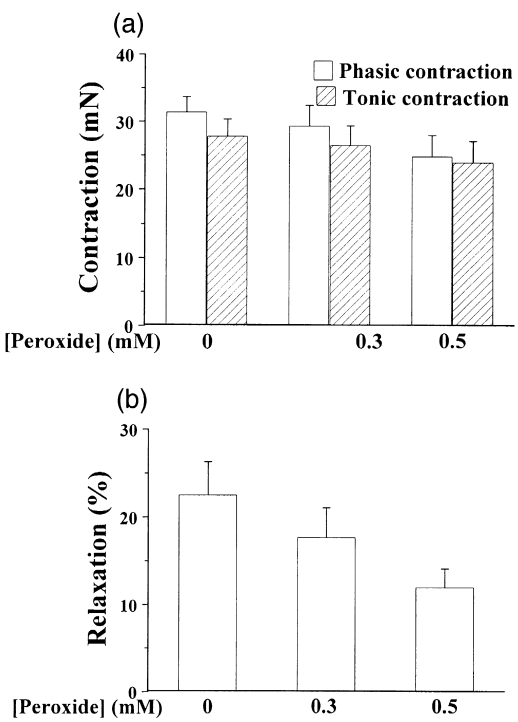

Fig. 2. Contractility of rings cut from arteries treated with H_2O_2 by perfusion. Arteries were treated with H_2O_2 by perfusion, washed to remove H_2O_2, rings (3-mm long) from the middle of the artery were hung in an organ bath and treated as described in Section 2. The values shown are mean ± SEM of 19, 18 and 17 animals for 0, 0.3 and 0.5 mM H_2O_2, respectively. Note that all the contraction and relaxation experiments were conducted in H_2O_2 free solutions. (a) Contraction with 30 mM KCl. Values for 0.3 or 0.5 mM H_2O_2 did not differ significantly. (b) During tonic phase of the contraction, A23187 was added and percent relaxation for each artery was determined. Values for 0.5 mM H_2O_2 differed significantly ($P < 0.05$) from those of 0 mM but those for 0.3 mM did not.

by A23187 in perfused control (no H_2O_2) tissues was only $23 \pm 4\%$ — a value significantly lower than in the control (no H_2O_2) unperfused tissues. Treating the arteries with 0.5 mM H_2O_2 further lowered this value significantly ($P < 0.05$) to $12 \pm 2\%$ (Fig. 2b). Thus, treating arteries luminally with H_2O_2 in the perfusion experiments lowered the percent relaxation but not the force of contraction.

3.3. Amount of endothelium remaining after perfusion with H_2O_2

The unperfused arteries had $68 \pm 9\%$ of the surface covered with endothelium indicating that some endothelium had been lost during transport of the arteries from the slaughter house and during dissection. Perfused arteries contained only $45 \pm 12\%$ of the surface area covered with endothelium. Including H_2O_2 in the perfusion medium led to a further reduction in the amount of endothelium remaining in the arteries (Fig. 3a). The amount of endothelium remaining correlated negatively ($r^2 = 0.9936$) with the H_2O_2 concentration with this value being only $14 \pm 9\%$ after perfusion with 0.5 mM H_2O_2.

We further analyzed the data from unperfused arteries and those perfused with 0, 0.3 and 0.5 mM H_2O_2 to determine if the percent relaxation correlated with the endothelium remaining in the arteries. There was a significant ($P < 0.05$) correlation ($r^2 = 0.8445$) between the two parameters (Fig. 3b).

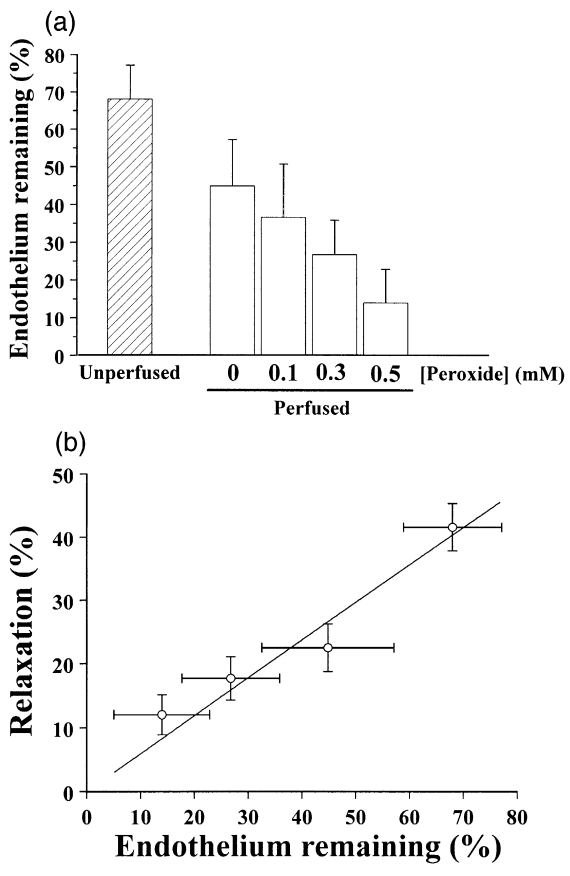

Fig. 3. Effect of perfusion and H_2O_2 on endothelium remaining in the arteries. (a) Determination of endothelium remaining. Arteries were perfused immediately or perfused with the specified concentrations of H_2O_2 as for Fig. 2. The arteries were then used for monitoring the amount of endothelium remaining as described in Section 2. The values shown are mean ± SEM of seven unperfused arteries and five arteries for perfusion at each concentration. Perfusion without H_2O_2 caused a marginal but not statistically significant ($0.1 < P < 0.2$) decrease in the amount of endothelium remaining. The amount of endothelium remaining correlated negatively ($r^2 = 0.9936$, $P < 0.05$) with the H_2O_2 concentration. (b) Correlation between percent relaxation and endothelium remaining. Data for relaxation of unperfused arteries from Fig. 1 and arteries perfused with 0, 0.3 and 0.5 mM H_2O_2 from Fig. 2 were plotted against corresponding endothelium remaining data from A. There was a significant positive correlation ($r^2 = 0.8445$, $P < 0.05$) between the two parameters.

4. Discussion

The effects of H_2O_2 on coronary artery contractility differed in the experiment where arteries were perfused with H_2O_2 from those where 3-mm artery rings were bathed in it. The discussion will focus on the effects of reactive oxygen on arterial smooth muscle and endothelium functions.

The direct effects of H_2O_2 on contractility have been reported previously. In the de-endothelialized pig coronary artery rings, H_2O_2 added during measurement produced a transient contraction (Grover et al., 1999). However, in precontracted rat and rabbit aortic rings with intact endothelium, H_2O_2 produced an endothelium-dependent relaxation by possibly stimulating NO synthase (Zembowicz et al., 1993). Pretreatment experiments with reactive oxygen show irreversible loss of contractility but at lower concentrations of reactive oxygen the damage is restricted to contraction with agents, which depend on mobilization of SR Ca^{2+} pools, e.g., angiotensin II and cyclopiazonic acid (Grover et al., 1995). This is consistent with the reports that H_2O_2 and superoxide damage the organellar Ca^{2+} pump in smooth muscle (Grover et al., 1992, 1995; Suzuki and Ford, 1991). The damage to the endothelial organellar Ca^{2+} pump requires considerably higher concentrations of H_2O_2 than in smooth muscle (Grover and Samson, 1997; Grover et al., 1997). It is not known if and how pretreating endothelium with reactive oxygen species such as H_2O_2 affects its ability to subsequently relax smooth muscle.

In our experiments, we found that incubating the artery rings in Krebs' solution containing H_2O_2 for 30 min in the organ bath decreased the contractile response of the arteries to KCl, probably due to the damage to the organellar Ca^{2+} pump in smooth muscle as discussed above. In contrast, damage to the endothelium was minimal because endothelium-dependent relaxation due to stimulation with A23187 was not affected by H_2O_2 treatment in the organ

bath. However, when H_2O_2 treatment was given by perfusion, we found that it caused considerable damage to the endothelium based on morphological and functional results. However, at present, we cannot distinguish between the damage caused by handling of the arteries for the perfusion experiments from the effect of perfusion per se. Thus, our data only suggest that the combination of shear stress and H_2O_2 leads to loss of endothelium and that the decrease in relaxation correlates well with this loss. During the long-term course after coronary artery bypass grafting, vascular regions with grafts loose their endothelium-dependent relaxation although regions grafted with arteries survive better than those grafted with veins (Hartmann et al., 1997, 1998). However, it is not known how H_2O_2 affects the adhesion of endothelium to smooth muscle during flow. We have recently shown that endothelium contains a high level of catalase that allows it to protect the underlying smooth muscle (Grover et al., 2000). However, the protection at the higher concentrations of H_2O_2 is only partial. The damage to KCl contractions by 0.5 mM H_2O_2 in organ bath experiments but not in the perfusion experiments confirms that endothelium can breakdown the luminally applied H_2O_2 but there is a limit to this protection as H_2O_2 can also cause a gradual loss of the endothelium.

We conclude that H_2O_2 and shear stress can cause a loss of endothelium. Endothelium can protect the underlying smooth muscle against luminal H_2O_2 and this loss may be a factor in limiting the level of protection.

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