Halothane does not protect against vascular injury in isolated cerebral and mesenteric arteries

[L'halothane ne protège pas contre les lésions vasculaires dans des artères cérébrales et mésentériques isolées]

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Purpose: This study was designed to examine regional differences in the vascular injury induced by hydrogen peroxide (H_2O_2), and to determine its modulation by halothane in canine basilar and mesenteric arteries.

Methods: Rings of canine basilar and mesenteric arteries with intact endothelium were mounted in Krebs bicarbonate solution for isometric tension recording. The relaxation responses to substance P (10^{-8} M) and sodium nitroprusside (SNP; 10^{-8} to 10^{-5} M) were examined before and after exposure to H_2O_2 (1 mM) for eight minutes in the presence or absence of halothane (2%), to evaluate the effects of oxidative injury on the endothelium-dependent and -independent relaxation. The contractile responses to KCl (30 mM) and prostaglandin (PG) $F_{2\alpha}$ (3×10^{-6} M) were also compared in rings with and without exposure to H_2O_2 .

Results: After exposure to H_2O_2 , the relaxant responses to substance P were significantly inhibited in basilar arteries ($P < 0.01$), but not in mesenteric arteries. Exposure to H_2O_2 also attenuated SNP-induced relaxation in basilar ($P < 0.05$), but not in mesenteric arteries. The attenuation of the contractile responses to KCl and $PGF_{2\alpha}$ after H_2O_2 exposure was observed only in basilar arteries ($P < 0.01$). Simultaneous exposure to halothane did not affect the attenuation of these relaxant and contractile responses. Scanning electron microscopy revealed that H_2O_2 resulted in marked disruption of the endothelial layer in basilar arteries, compared to almost no morphological changes in mesenteric arteries.

Conclusion: These results indicate that the endothelium and vascular smooth muscle of the basilar artery are more susceptible to oxidative stress than those of the mesenteric artery. Halothane, at clinically relevant concentrations, exerts no significant influence on this vascular injury.

Objectif : Vérifier les différences régionales des lésions vasculaires induites par le peroxyde d'hydrogène (H_2O_2) et déterminer leur modulation par l'halothane dans des artères basilaires et mésentériques canines.

Méthode : Des anneaux d'artères basilaires et mésentériques canines, comportant un endothélium intact, ont été montés dans une solution bicarbonate de Krebs pour l'enregistrement de la tension isométrique. La réaction de relâchement à la substance P (10^{-8} M) et au nitroprussiate de sodium (NPS ; 10^{-8} à 10^{-5} M) a été examinée avant et après l'exposition à H_2O_2 (1 mM) pendant huit minutes en présence ou non d'halothane à 2 % pour évaluer les effets de la lésion oxydative sur le relâchement dépendant ou non de l'endothélium. Les réactions contractiles au KCl (30 mM) et à la prostaglandine (PG) $F_{2\alpha}$ (3×10^{-6} M) ont été aussi comparées dans des anneaux exposés ou non à H_2O_2 .

Résultats : Après l'exposition à H_2O_2 , les réactions de relâchement à la substance P ont été significativement inhibées dans les artères basilaires ($P < 0,01$), non dans les mésentériques. L'exposition à H_2O_2 a aussi diminué le relâchement induit par le NPS dans les artères basilaires ($P < 0,05$) seulement. L'atténuation des réactions contractiles au KCl et à la $PGF_{2\alpha}$ après l'exposition à H_2O_2 a été observée seulement dans les artères basilaires ($P < 0,01$). L'exposition simultanée à l'halothane n'a pas modifié la baisse de ces réactions de relâchement et de contraction. L'examen au microscope électronique à balayage a révélé que H_2O_2 a provoqué une rupture marquée de la couche endothéliale des artères basilaires, comparativement à la quasi-absence de changements morphologiques dans les artères mésentériques.

Conclusion : L'endothélium et les muscles lisses de l'artère basilaire sont plus sensibles au stress oxydatif que ceux de l'artère mésentérique. L'halothane, en concentrations cliniquement significatives, n'exerce pas d'influence sensible sur cette lésion vasculaire.

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DETERMINATION of the effects of anesthetics on oxygen radical-induced vascular injury may provide information to better understand ischemia-reperfusion injury during anesthesia. Volatile anesthetics, including halothane, have been reported to modify the endothelial cell injury induced by hydrogen peroxide (H₂O₂).^{1,2} H₂O₂ is one of the major reactive oxygen species involved in oxidative stress-induced vascular injuries.^{3,4} H₂O₂, administered exogenously⁵⁻⁹ and released from activated neutrophils,^{10,11} has been demonstrated experimentally to induce functional and morphological alterations in both endothelial and vascular smooth muscle cells.

Reactive oxygen species have been implicated in inducing vascular damage during post-ischemic reperfusion.^{12,13} Although these radicals are well documented as causing vascular endothelial cell injury that results in organ dysfunction and damage, the endothelial dysfunction and injury seem to exhibit large variability.¹⁴⁻¹⁷ Some vessels were relatively unaffected, whereas others were damaged irreversibly. The endothelium and underlying vascular smooth muscle of the cerebral artery are recognized to exhibit heterogeneous functional and histological properties compared with those of other extracerebral arteries.¹⁸⁻²⁰ However, little information is available regarding the differences in response to oxidative injury between cerebral and extracerebral arteries, and how inhaled anesthetics might differentially modify these responses.

The first goal of the present *in vitro* study was to investigate and compare the pharmacological and morphological differences in the endothelial and vascular smooth muscle injuries induced by H₂O₂ in isolated dog basilar and mesenteric arteries. The second goal was to determine whether halothane can modulate H₂O₂-induced vascular injury in both cerebral and extracerebral arteries.

Materials and methods

All experimental procedures and protocols were approved by the Animal Use Committee at Wakayama Medical University.

Preparation of basilar and mesenteric arteries

Ten adult male ($n = 7$) and female ($n = 3$) mongrel dogs weighing 10 to 18 kg (12–24 months old) were anesthetized with *ip* ketamine (10 mg·kg⁻¹), and then killed by bleeding from the common carotid arteries. After craniotomy and laparotomy, the brain and mesentery were rapidly removed. Basilar and mesenteric arteries of similar external diameter (0.8–1.0 mm) were carefully dissected and immersed in cold Krebs

bicarbonate solution (composed of 118.2 mM NaCl, 4.6 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 24.8 mM NaHCO₃ and 10 mM dextrose). The arteries were cleaned of surrounding tissues and cut into ring segments 3 mm in length, with care taken not to damage the endothelium. The integrity of the endothelium was later confirmed by assessing the vasorelaxant responses to substance P (10⁻⁸ M).²¹

Isometric tension experiment

Arterial rings with an intact endothelium were mounted vertically between two stainless steel hooks in organ baths (10 mL capacity) filled with Krebs bicarbonate solution (37°C), which was aerated with a mixture of 5% carbon dioxide and 95% oxygen. One of the hooks was anchored and the other was connected to a force transducer (Nihondenki San-Ei Co., Tokyo, Japan). Changes in isometric force were amplified and displayed on a chart recorder (Nihondenki San-Ei Co., Tokyo, Japan). Optimal resting tension was adjusted to 2.0 g in both arteries, which was the minimum amount of stretch necessary to achieve the maximal contractile response to 30 mM KCl, as determined in a preliminary investigation. Before the start of the experiment, the arterial rings were allowed to equilibrate for 60 min, during which time the bathing solution was replaced every 15 min.

Experimental protocols

Submaximal contraction of both arteries in response to 30 mM KCl was evaluated initially, and the preparations were washed with fresh bathing solution at least three times.

First, the maximal substance P-induced relaxation was examined, before and after exposure to H₂O₂, to evaluate the effects of the oxidative insult on the endothelium-dependent relaxation. Prostaglandin (PG) F_{2α} (3 × 10⁻⁶ M) was used to attain submaximal contraction of the basilar and mesenteric arteries. After a plateau was reached, substance P (10⁻⁸ M) was added to ascertain the maximal relaxation. Each ring, except for the control ring, was exposed to H₂O₂ (1 mM) for eight minutes in the presence or absence of halothane (2%), and then washed repeatedly with fresh bathing solution for 30 min to wash H₂O₂ and/or halothane from the organ bath. Thirty minutes after exposure to H₂O₂, the relaxant responses of PGF_{2α}-precontracted rings to substance P were again obtained. The relaxation responses to substance P in rings exposed to H₂O₂ were compared to those in unexposed (control) basilar and mesenteric arteries. Functional endothelial injury caused by H₂O₂ was evaluated by attenuation of the relaxant responses to substance P.

Secondly, the relaxation responses to sodium nitroprusside (SNP; 10^{-8} to 10^{-5} M) were investigated to evaluate the effects of oxidative stress on endothelium-independent relaxation. Similar to the first experiment, the relaxation responses to SNP in rings with and without eight-minute exposure to H_2O_2 were compared in both arteries. The relaxation responses to substance P and SNP were expressed as the percentage relaxation of the precontraction induced by $PGF_{2\alpha}$. One hundred percent relaxation indicates complete reversal of the precontraction.

Thirdly, the contractile responses to KCl (30 mM) and $PGF_{2\alpha}$ (3×10^{-6} M) were also examined. The contractile responses to each vasoconstrictor in rings with and without exposure to H_2O_2 were compared and expressed as a percent relative to those obtained before exposure.

Scanning electron microscopy

In order to evaluate the endothelial cell injury morphologically, basilar and mesenteric arteries cut lengthwise with a razor blade were exposed to 1 mM H_2O_2 for eight minutes in the presence or absence of 2% halothane. Some rings were exposed to Krebs bicarbonate solution, without H_2O_2 or halothane, as controls. After vigorous washing with fresh bathing solution for 30 min, the arterial preparations were fixed overnight in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). After washing in phosphate buffer, the tissues were dehydrated in a graded ethanol series, critical-point-dried, and coated with gold in an ion sputter-coating system (JFC-1100, JEOL, Tokyo, Japan). All specimens were examined using a JSM-T220 scanning electron microscope (JEOL, Tokyo, Japan) at a magnification ratio of 2000.

Drugs and solutions

All drugs were of highest purity commercially available: hydrogen peroxide, SNP (Sigma Chemical, St. Louis, MO, USA), substance P (Protein Research Foundation, Osaka, Japan) and halothane (Takeda Pharmaceutical Co., Tokyo, Japan). Halothane was delivered by a calibrated vaporizer (Fluotec 3, Ohmeda, Steeton, UK) to provide the appropriate concentrations in the gas mixture aerating the bathing solution. The concentrations in the resulting gas mixture were measured and adjusted using an Atom 303 anesthetic gas monitor (Atom, Tokyo, Japan). Drug concentrations are expressed as the final concentration in the organ bath. All drugs except halothane were dissolved in distilled water and added directly to the organ bath in a volume of 100 μ L or less.

Statistical analysis

All data were expressed as mean $\pm$ SEM; n refers to the number of the dogs from which basilar and mesenteric arterial rings were taken. Statistical analyses were performed using Student t test for unpaired comparisons. When more than two means were compared, one-way analysis of variance with Scheffe's post-hoc F test was used. P -values less than 0.05 were considered significant.

Results

Effects of H_2O_2 exposure on substance P-induced endothelium-dependent vasorelaxation

The relaxations induced by substance P (10^{-8} M) in basilar and mesenteric arterial rings that were not exposed to H_2O_2 were $69 \pm 5\%$ ($n = 6$) and $74 \pm 4\%$ ($n = 5$) of the precontraction tension, respectively. After exposure to H_2O_2 , the relaxant responses to substance P were significantly reduced in basilar arteries ($29 \pm 7\%$, $n = 6$, $P < 0.01$), but unaffected in mesenteric arteries ($70 \pm 4\%$, $n = 5$). Simultaneous exposure to 2% halothane resulted in no significant modulation of the attenuation of the relaxant responses to substance P in basilar arteries ($n = 6$), (Figure 1).

Effects of H_2O_2 exposure on SNP-induced endothelium-independent vasorelaxation

SNP induced a concentration-dependent relaxation of basilar and mesenteric arterial rings that was maximal

FIGURE 1 Effects of hydrogen peroxide (H_2O_2 , 1 mM) exposure on substance P (10^{-8} M)-induced endothelium-dependent relaxation in canine basilar (left) and mesenteric (right) arteries. Arterial rings were precontracted with prostaglandin (PG) $F_{2\alpha}$ (3×10^{-6} M) and the relaxation responses are expressed as a percentage of the $PGF_{2\alpha}$ precontraction. H_2O_2 exposure significantly inhibited the relaxant responses to substance P in basilar arteries, but not in mesenteric arteries ($P < 0.01$). Simultaneous exposure to halothane (2%) produced no significant influence on the attenuation of substance P-induced relaxation. $^{**}P < 0.01$ vs control.

FIGURE 2 Effects of hydrogen peroxide (H_2O_2), (1 Mm) exposure on the relaxation response to sodium nitroprusside (SNP; 10^{-5} to 10^{-8} M) in canine basilar (left) and mesenteric (right) arteries. Arterial rings were precontracted with prostaglandin (PG) $F_{2\alpha}$ (3×10^{-6} M) and relaxation responses are expressed as a percentage of the $PGF_{2\alpha}$ pre-contraction. Exposure to H_2O_2 significantly attenuated the relaxant responses to SNP at concentrations of 10^{-6} M and higher in basilar arteries, but not in mesenteric arteries. Simultaneous exposure to halothane (2%) produced no further modulation of the attenuation of SNP-induced relaxation. * $P < 0.05$, ** $P < 0.01$ vs control.

at a concentration of 10^{-5} M in both arteries [$88 \pm 3\%$ ($n = 7$) and $89 \pm 2\%$ ($n = 6$) in basilar and mesenteric arteries, respectively]. The relaxant responses to SNP were also attenuated after H_2O_2 exposure in basilar arteries ($n = 7$). The relaxation induced by SNP at concentrations of 10^{-6} M ($P < 0.05$) and higher ($P < 0.01$) was significantly inhibited in basilar arteries exposed to H_2O_2 . By contrast, in mesenteric arteries, the concentration-response curve for SNP was not affected by exposure to H_2O_2 ($n = 5$). Halothane did not affect the response to SNP in either artery ($n = 4$, each), (Figure 2).

Effects of H_2O_2 on the contractile responses to KCl, phenylephrine or $PGF_{2\alpha}$

Figure 3 indicates the effects of H_2O_2 exposure on the contractile responses to KCl (30 mM) and $PGF_{2\alpha}$ (3×10^{-6} M) in basilar and mesenteric arteries. The contractile responses to KCl in basilar rings with and without exposure to H_2O_2 were $59 \pm 6\%$ and $114 \pm 7\%$ of the prior control exposures, respectively ($n = 7$, each). H_2O_2 exposure significantly attenuated the contractile responses to KCl ($n = 7$, $P < 0.01$). The $PGF_{2\alpha}$ -induced contraction was also attenuated in basilar rings after exposure to H_2O_2 ($65 \pm 9\%$, $n = 7$) compared to those without exposure ($100 \pm 4\%$, $n =$

FIGURE 3 Effects of hydrogen peroxide (H_2O_2), (1 Mm) exposure on the contractile responses to KCl (30 mM) and prostaglandin (PG) $F_{2\alpha}$ (3×10^{-6} M) in canine basilar (left) and mesenteric (right) arteries. The contractile response to each vasoconstrictor is expressed as a percentage of that obtained before H_2O_2 exposure. H_2O_2 exposure significantly inhibited the contractile responses to KCl and $PGF_{2\alpha}$ in basilar arteries, but not in mesenteric arteries ($P < 0.01$). Simultaneous exposure to halothane (2%) did not affect H_2O_2 -induced attenuation of the contractile responses to these constrictors.

7). In contrast to the basilar artery, H_2O_2 exposure did not affect the contractile responses to KCl and $PGF_{2\alpha}$ in mesenteric arteries ($n = 7$). Simultaneous exposure to halothane (2%) did not affect the responsiveness to these vasoconstrictors in either artery ($n = 4$, each).

Histological examination of the endothelial cell layer by scanning electron microscopy

The normal endothelial surface in control basilar artery demonstrated endothelial cells that were flat or spindle shaped, with a slight elevation in their central portions (Figure 4A). Exposure to H_2O_2 resulted in marked disruption of the endothelial layers. Endothelial cells were fractured and fragments of cellular debris appeared on the partially exposed basal lamina (Figure 4B). Simultaneous exposure to halothane (2%) resulted in cellular appearances similar to that seen with H_2O_2 alone (Figure 4C). The endothelial surface of control mesenteric arteries demonstrated a characteristic "cobblestone" appearance (Figure 4D). Exposure to H_2O_2 exhibited no substantial changes on the endothelial surface (Figure 4E). Similar findings were observed with simultaneous exposure to halothane (Figure 4F).

FIGURE 4 Photographs of the endothelium of canine basilar (A, B, C) and mesenteric (D, E, F) arteries. Scanning electron microscopic image showing the endothelial surface of normal basilar (A) and mesenteric (D) arteries, the endothelial surface of basilar (B) and mesenteric (E) arteries exposed to hydrogen peroxide (H_2O_2), (1 mM), and the endothelial surface of basilar (C) and mesenteric (F) arteries simultaneously exposed to H_2O_2 (1 mM) and halothane (2%).

Discussion

The main findings of the study were as follows. Exposure to H₂O₂ markedly inhibited the endothelium-dependent vasorelaxant response to substance P in canine basilar arteries, but not in mesenteric arteries. Histological examination using scanning electron microscopy revealed disruption of the endothelial cell layer of basilar arteries. Exposure to H₂O₂ also attenuated the responsiveness to an endothelium-independent vasodilator, SNP, as well as the contractile response to KCl and PGF_{2α} in basilar arteries. Halothane (2%) exhibited no significant effects on the functional and morphological changes in endothelial cells in response to H₂O₂.

Reactive oxygen species generated during post-ischemic reperfusion mediate vascular damage, resulting in organ dysfunction.^{12,13} H₂O₂, generated from superoxide anion with enzymatic or nonenzymatic dismutation, induces endothelial and vascular smooth muscle cell damage.^{5–11} This molecule is more stable and diffusible across the cell membrane than superoxide anion, and may therefore cause oxidative damage.⁴ H₂O₂ also reacts with superoxide anion to generate highly reactive hydroxyl radicals in the presence of ferrous ion.³ In the present study, exposure of basilar arteries to H₂O₂ resulted in loss of endothelium-dependent relaxation in response to substance P and marked disruption of the endothelial layer, supporting the cytotoxicity of H₂O₂ in endothelial cells.^{1,2,7,9,11} Exogenous H₂O₂ increases intracellular calcium and depletes cellular adenosine triphosphate,^{2,7,22} which may result in morphological changes in endothelial cells.⁷ Exposure to H₂O₂ also inhibited the endothelium-independent relaxation response to SNP and the contractile response to KCl and PGF_{2α} in basilar arteries, suggesting that vascular smooth muscle cells are also involved in H₂O₂-induced vascular injury. Similar to the present findings, H₂O₂-induced attenuation of the relaxation response to SNP has been reported in cat cerebral artery.⁵

The extent of the injury depends upon the concentration of H₂O₂ and duration of exposure.⁹ Eight minutes of exposure to H₂O₂ at a concentration of 1 mM that was used in the present investigation appeared sufficient to cause endothelial injury in basilar, but not mesenteric arteries. In our preliminary study, more than ten minutes exposure suppressed the contractility of basilar artery too strongly to allow for pharmacological assessment of the endothelium-dependent relaxation, and less than 1 mM H₂O₂ did not induce any endothelial damage.

Heterogeneous susceptibility to oxidative stress has been reported in different types and sizes of arter-

ies.^{14–17} From the present findings, the mesenteric artery was more resistant to oxidant stress than the basilar artery. There is extreme diversity in endothelial cell structure, architecture and physiology.²³ The glutathione redox cycle is one of the most important endogenous antioxidant defense systems in endothelial cell,⁴ and has been shown to serve as a protecting system against H₂O₂-induced endothelial cell lysis.¹⁵ Differences in cellular stores of reduced glutathione may contribute to variations of susceptibility to oxidative injury in experimental models.¹⁵ Further, the endothelium of the cerebral artery has been recognized to exhibit heterogeneous pharmacological and histological properties, compared with that of other extracerebral arteries.¹⁹ For example, the endothelial layer of the cerebral artery forms tight junctions that act as a strong permeability barrier against some substances and plays an important role in maintaining the blood brain barrier.²⁴ This unique property of cerebral arterial endothelium may be a target of reactive oxygen species.²⁵

In addition to the endothelium, vascular smooth muscle cells of the basilar artery have been demonstrated to be more sensitive to H₂O₂ than those of the mesenteric artery in the present study. The muscular layer of the canine mesenteric artery is approximately twofold thicker than that of the cerebral artery, when comparing vessels with the same outer diameter size.¹⁸ It is possible that exogenously administered H₂O₂ could easily penetrate the thin muscular layer of the basilar artery from the luminal and adventitial surfaces, and result in extensive vascular smooth muscle dysfunction and damage. By contrast, certain portions of vascular smooth muscle of the mesenteric artery may remain intact against H₂O₂ injury because of the thick muscular layer. Susceptibility of the endothelium and the vascular smooth muscle of the cerebral artery to the oxidative stress may contribute to the unusual sensitivity to post-ischemic reperfusion injury of the brain.²⁶

The effects of volatile anesthetics on oxidant-induced endothelial cell injury appear to be controversial.^{1,2} Shayevitz *et al.* demonstrated that halothane and isoflurane potentiated the cytotoxic effects of H₂O₂ on rat pulmonary endothelial cells in a concentration-dependent manner.¹ Potentiation of oxidant-induced increases in permeability of pulmonary capillaries by halothane has also been reported *ex vivo* in a perfused rabbit lung model.²⁷ However, Johnson *et al.* demonstrated that halothane, and to a lesser extent, isoflurane, protected against H₂O₂-induced injury in cultured human aortic endothelial cells by slowing the rate of cytosolic Ca²⁺ increase after H₂O₂ exposure.² They also found that this protecting effect of volatile

anesthetics was absent in pulmonary artery endothelial cells and concluded that the effects of anesthetics depended upon the vascular bed investigated and the concentration of anesthetics tested.² Neither deteriorative nor ameliorative effects of halothane were observed in the present study. Since only 2% halothane was investigated in basilar and mesenteric arteries, we cannot exclude the possibility that higher or lower concentrations of halothane would have substantial effects on H₂O₂-induced vascular injury. Further, the results from the present study with halothane may not necessarily be extrapolated to other new volatile anesthetics, such as sevoflurane and desflurane.

In summary, exposure to H₂O₂ induced both endothelial and vascular smooth muscle cell damage in basilar arteries, but not in mesenteric arteries. These findings suggest that the basilar artery is more susceptible to oxidative stress than the mesenteric artery. Halothane, at least at a concentration of 2%, exhibits no significant influence on this injury.

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