Hydrogen Peroxide Pretreatment of Perfused Canine Vessels Induces ICAM-1 and CD18-Dependent Neutrophil Adherence

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**Background.** Cytotoxic products of neutrophils (polymorphonuclear leukocytes, PMNs) contribute to ischemia-reperfusion injury of several tissues. Hydrogen peroxide (H$_2$O$_2$), one of the cytotoxic products of PMNs, also promotes the adherence of PMNs to cultured vascular endothelial cells in vitro. The present study was undertaken to determine if H$_2$O$_2$ also augmented adhesion of PMNs to intact vessels perfused ex vivo and to determine if H$_2$O$_2$-induced PMN adherence to intact canine carotid arteries and external jugular veins or to cultured canine venous endothelium is mediated by specific adherence ligands on the neutrophil and/or the endothelium.

**Methods and Results.** Vessels were perfused for 20 minutes with oxygenated Krebs-Henseleit bicarbonate buffer with and without H$_2$O$_2$, washed with buffer alone, and then exposed to $^{111}$In-labeled isolated PMNs (10$^7$ cells/vessel) under static conditions for up to 20 minutes before being washed again. Residual radioactivity retained by the washed vessel was counted as an index of PMN retention. The adherence of unlabeled PMNs to cultured endothelial cells was determined by a visual assay method after pretreatment of the endothelium with H$_2$O$_2$ for brief periods followed by washing. Perfusion of vessels with H$_2$O$_2$ produced a transient, concentration-dependent increase in PMN adherence to both canine carotid arteries and external jugular veins that was two to four times that of control values at 1 mmol/l and declined at higher H$_2$O$_2$ concentrations. Peak retention of PMNs by canine carotid arteries occurred 10 minutes after exposure to 1 mmol/l H$_2$O$_2$ and then rapidly declined to control values; this effect was replicated by a second 20-minute exposure of canine carotid arteries to 1 mmol/l H$_2$O$_2$ 60 minutes after the first exposure. Scanning and transmission electron microscopy revealed not only adherence of PMNs to but migration through the vascular endothelium of the carotid artery after H$_2$O$_2$ perfusion. The endothelium was intact in H$_2$O$_2$-treated arteries not exposed to PMNs. H$_2$O$_2$-induced PMN retention was completely inhibited by addition of catalase or the hydroxyl radical scavenger dimethylthiourea to the perfusate by incubation of the PMN with a monoclonal antibody (Mab) against CD18 (R15.7) or by perfusion of the H$_2$O$_2$-treated vessel with CL18/6, a Mab against canine ICAM-1 (intercellular adhesion molecule-1). Similar effects of Mabs on PMN adhesion to H$_2$O$_2$-pretreated cultured endothelium were noted. The retention of PMNs by vessels mechanically denuded of endothelial cells was markedly increased. H$_2$O$_2$ pretreatment of these vessels did not further augment PMN adherence, and no inhibitory effect of R15.7 was noted. Incubation of carotid arteries and PMNs with a specific platelet-activating factor antagonist, WEB2086, completely inhibited the H$_2$O$_2$-induced increased PMN retention by these vessels.

**Conclusions.** These results indicate that H$_2$O$_2$, in the absence of evidence for permanent endothelial cell injury, can induce a transient, reversible, platelet-activating factor-dependent adherence of PMNs to vessels by mechanisms that depend on an intact endothelium and involve CD18 on the PMN and ICAM-1 on the endothelium. *(Circulation 1991;84:2154–2166)*

Ischemia and reperfusion of various tissues is accompanied by accumulation of neutrophils in the reperfused tissue.$^{1–8}$ Several studies indicate that the localization of neutrophils under these conditions depends in part on intracellular adhesion as shown by reduction of polymorphonuclear leukocyte (PMN) accumulation or tissue injury after administration of monoclonal antibodies against the CD18 family of integrins.$^{9–14}$ Leukocyte accumulation also appears to depend on reactive oxygen...
species as shown by the inhibitory effects of catalase and superoxide dismutase.\textsuperscript{15,16} Mechanisms for a possible interplay between reactive oxygen and leukocyte adhesion may be drawn from two published studies. The first study presents evidence that reactive oxygen induces generation of a chemotactic factor from serum.\textsuperscript{17} Although the identity of this factor is not known, it is clear that a variety of chemotactic factors stimulate increases in PMN adhesiveness dependent on the CD18 integrins.\textsuperscript{18-24}

The second study presents evidence that hydrogen peroxide (H$_2$O$_2$) rapidly alters endothelial cells to increase the adhesion of neutrophils in vitro.\textsuperscript{25} This effect appears to be linked to endothelial cell production of platelet-activating factor (PAF), a known chemotactic stimulus for PMNs capable of stimulating increased CD18-dependent adhesion.\textsuperscript{18,22} Although these observations form the basis for a hypothetical link between the effects of reactive oxygen species and leukocyte adhesion, no published experiments provide direct evidence.

In an effort to directly assess whether reactive oxygen can induce CD18-dependent adhesion of neutrophils, we chose to focus attention on the effects of H$_2$O$_2$ on the endothelial cell by using a canine model. Experiments were performed in vitro by using monolayers of canine jugular vein endothelial cells and isolated canine neutrophils and in vivo by using isolated perfused canine jugular veins or carotid arteries and canine neutrophils. The results demonstrate that in both settings, the enhanced adhesion resulting from exposing endothelial cells in culture or on intact vessel walls to H$_2$O$_2$ is CD18 dependent. This conclusion is drawn from our findings that monoclonal antibodies (Mabs) against CD18 completely block the adhesion. In addition, the results indicate that the ligand on the endothelial cells interacting with CD18 is intercellular adhesion molecule-1 (ICAM-1). Furthermore, PAF plays an important role in this H$_2$O$_2$-induced CD18–ICAM-1 adhesive interaction.

**Methods**

**Materials**

All reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise specified. $^{111}$In oxine was obtained from Amersham Corp. (Arlington Heights, Ill.). Vessels were perfused with Krebs-Henseleit bicarbonate buffer (pH 7.4) containing (in mmol/l) NaCl 118, KCl 4.0, MgCl$_2$·H$_2$O 1.2, KH$_2$PO$_4$ 1.1, CaCl$_2$·H$_2$O 2.4, NaHCO$_3$ 25, glucose 5, and L-glutamate 5 that was filter sterilized by passage through 0.22-µm disposable filterware obtained from Nalgene (Rochester, N.Y.). Electron microscopy grade glutaraldehyde (50%) was obtained from Electron Microscopy Sciences (Fort Washington, Pa.) and H$_2$O$_2$ (30%) from Sigma.

**Neutrophil Isolation**

Citrate anticoagulated venous blood was collected from mongrel dogs, and neutrophils were isolated on Ficol-Paque cushions by a method previously described for the isolation of human neutrophils.\textsuperscript{26} This yielded a cell preparation of greater than 95% neutrophils with a viability, as assessed by trypan blue exclusion, of greater than 98%. Cells (10$^7$ cells/ml) were suspended in Dulbecco’s Ca$^{2+}$/Mg$^{2+}$-added phosphate-buffered saline (PBS) and stored up to 4 hours at 4°C. Hypotonic lysis of red blood cells was omitted to minimize neutrophil activation. An aliquot of the PMN cell suspension fixed in 1% glutaraldehyde was examined under a microscope for evidence of shape change\textsuperscript{26} as an index of neutrophil activation. Bipolar cells represented (mean±SEM; n=23) 10±2.1% and 6.5±1.6% of the neutrophils before and after labeling with $^{111}$In, respectively.

**Neutrophil Labeling With $^{111}$In**

Neutrophils were labeled with 0.5 µCi $^{111}$In per 10$^6$ cells by using the method of Thakur et al.\textsuperscript{27} Labeling efficiency was 65±3.9% (mean±SEM) with a range of 50–90%. Labeled neutrophils were stored in PBS for no more than 45 minutes before their installation in the vessels.

$^{111}$In-labeled PMN Adhesion to Ex Vivo Perfused Vessel

Canine carotid arteries and external jugular veins were freshly harvested from mongrel dogs anesthetized with sodium pentobarbital (30 mg/kg i.v.). Polypropylene connectors were attached to each end of a 5–7-cm vessel segment, nonvascular tissue adherent to the adventitial surface of the vessel was carefully removed, and any side branches were ligated with silk suture. The vessels were kept in cold (4°C) perfusion buffer until mounted in the perfusion chamber (4 hours or less). Vessels were placed in a manifold in an organ chamber bathed and perfused with warmed (37°C) Krebs-Henseleit bicarbonate buffer gassed with 95% O$_2$–5% CO$_2$ (Figure 1). The vessels were mounted vertically and perfused at a rate of 1 ml/min throughout the entire experiment (except during the neutrophil dwell period) by using a peristaltic pump (Watson-Marlow 502S; Falmouth Cronwell, England). Vessels were perfused for 20 minutes with Krebs-Henseleit buffer (with and without H$_2$O$_2$), washed with buffer alone for 10 minutes, and exposed to 10$^{-7}$ (1 ml) $^{111}$In-labeled PMNs (with and without Mabs) under static conditions for 20 minutes (arteries) or 400 seconds (veins). After the

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PMN dwell period, vessels were again perfused at 1 ml/min for 5 minutes to remove unadhered PMNs. The vessels were then carefully removed from the chamber and weighed without the connectors, and radioactivity was counted in a gamma counter (Gamma-trac 1290; TM Analytic, Elk Grove Village, Ill.) as an index of PMN retention. Dose response to H$_2$O$_2$ was assessed using concentrations of 100 μmol/l to 10 mmol/l. Time dependency of H$_2$O$_2$-mediated PMN adhesion was investigated by varying the wash period after H$_2$O$_2$ stimulation from 5 to 60 minutes.

**Mabs Against PMN and Endothelial Cell Determinants**

R15.7 (IgG1) and CL18/6 (IgG1) are Mabs found previously to bind to and block the functions of canine CD18 and ICAM-1 (unpublished observations, C.W. Smith, D. Anderson, and M.L. Entman), respectively. Antibodies were used as IgG preparations. A Mab without binding specificity for canine cells was obtained as purified IgG1 from Damon Biotech (Needham Heights, Mass.) and served as an isotype-matched nonbinding control.

R15.7 and IgG1 were added to PMNs 5 minutes before instillation. CL18/6 was infused through a proximal port of the perfusion apparatus (Figure 1) 5 minutes before the instillation of PMNs. Each was used at a saturating final concentration of 10 μg/ml.

**PMN Retention by Carotid Artery Mechanically Denuded of Endothelium**

The role of endothelial cells in H$_2$O$_2$-induced PMN adhesion was assessed in endothelium-denuded carotid arteries. Denudation of endothelium was achieved by inserting a metal or glass rod into the lumen and gently rolling the vessel over a paper towel impregnated with perfusion buffer. Loss of endothelium-dependent vasodilatory responses to acetylcholine during phenylephrine contraction provided a functional assessment of endothelial denudation of the artery. The effect of R15.7 on denuded artery (with and without H$_2$O$_2$) was studied as described above for intact vessels.

**Calculation of PMN Retention In Ex Vivo Perfused Vessels**

Total vessel radioactivity (in counts per minute) was normalized by vessel wet weight (g), and PMN/gram vessel was calculated using the formula

$$\text{PMN/g vessel} = \frac{[\text{Total vessel cpm}][\text{wt wt in g}]}{[\text{cpm/PMN}]}$$

In some experiments, we estimated the number of PMNs per luminal surface area of ex vivo perfused artery before and after H$_2$O$_2$ exposure for comparison with values for PMN adhesion to rat colon venular endothelium in vivo as determined by intravital microscopy. After the number of PMNs retained by the artery was determined by the above formula, the vessel was opened longitudinally, the length and width of the vessel were measured with a ruler, and the surface area was calculated. For purposes of comparison with values reported under in vivo conditions, the values were expressed as number of retained PMNs/10,000 μm$^2$ luminal surface area.

**Electron Microscopic Studies of Ex Vivo Perfused Vessels**

Small rings of carotid arteries from controls (n=3) and H$_2$O$_2$ exposed with (n=2) and without (n=2)
R15.7 coincubated PMNs were studied with scanning electron microscopy (SEM) and transmission electron microscopy (TEM) by using the method of Goldstein et al. Vessels were perfused at 1 ml/min for several minutes with perfusion buffer containing 2% glutaraldehyde at 37°C after the final wash after PMN instillation. After careful removal from the organ chamber apparatus, each vessel was immersed in the same buffered fixative for an additional 30 minutes and was then slit longitudinally into two halves. One half was processed for TEM and oriented to view the endothelial surface. The other half was prepared for SEM by critical-point drying, and the entire half was mounted on a copper stub. Vessels were examined in a JEOL 200CX at 80 kV (TEM) and 40 kV (SEM). For SEM, random fields (three experimental and four control) of 333,216 μm² each) were photographed and studied. Selected regions were rephotographed at higher magnifications to show surface details. For TEM, contiguous fields of endothelial cell surface were photographed and examined for morphological changes. The perimeter of one control vessel was measured by summing the perimeters of adjacent endothelial cells in a series of 27 electron micrograph prints (×13,500) for a total of 282.68 μm. A comparable number of electron micrograph prints were examined for neutrophil interaction with the endothelial cell surface. Sixty-one random fields at plate magnification of ×5,000 or ×7,300 were counted.

**Immunohistochemistry of Intact Canine Artery and Cultured Venous Endothelium**

The tissue samples were snap frozen in liquid nitrogen and stored at −70°C. Cryostat sections (5 μm) were air-dried for 30 minutes, fixed in reagent-grade acetone for 10 minutes at room temperature, and air-dried. The sections were stained by using the immunoalkaline phosphatase method. Briefly, the fixed sections were incubated with the Mab, CL18/6, unlabeled sheep anti-mouse Ig (Dako Corp., Carpinteria, Calif.), and finally alkaline phosphatase-anti-alkaline phosphatase (APAAP) complexes (Boehringer Mannheim, Indianapolis, Ind.). Each incubation step lasted 30 minutes with additional 10-minute incubations with the anti-mouse Ig and the APAAP complexes after the first three steps. Slides were washed briefly in Tris-buffered saline, pH 7.6, between each incubation step. The alkaline phosphatase reaction was developed by using Substrate Kit 1 (Vector Laboratories Inc., Burlingame, Calif.) with 1 mmol/l levamisole added to inhibit endogenous alkaline phosphatase activity.

**Cytotoxicity Studies**

The potential cytotoxic effect of H₂O₂ on ex vivo perfused vessels was studied both functionally and histologically. In addition to measuring endothelial cell–dependent vascular responses and examining vessels for ultrastructural change, specific release of In-label from carotid arteries prelabeled with this isotope was measured after their exposure to varying concentrations of H₂O₂. Vessels were mounted in the organ chamber apparatus as previously described, and each vessel was incubated with approximately 80 μCi In for 20 minutes. The vessels were perfused with buffer (1 ml/min) for 10 minutes to wash out unincorporated isotope. After collection of a 15-minute baseline fraction, the vessels were perfused with 100 μmol/l, 1 mmol/l, or 10 mmol/l H₂O₂ for 20 minutes. Fractions were collected every 15 minutes for 4 hours. An aliquot was taken from each fraction for measurement of radioactivity. Specific release of In from H₂O₂-exposed vessels was determined using the method described by Andreoli.

Dog jugular venous endothelial cell (DJVEC) viability was assessed by measuring the cell’s capacity to synthesize the adherence ligand ICAM-1 after a 20-minute exposure to H₂O₂ followed by a 3-hour stimulation with lipopolysaccharide (LPS).

**In Vitro Studies on PMN Adhesion to Venous Endothelium**

DJVEC monolayers were grown to confluency on coverslips as described previously. DJVECs were placed in Dulbecco’s balanced salt solution in the presence or absence of H₂O₂ and incubated for varying times. DJVECs were washed after stimulation by dipping five times in two changes of PBS. The coverslip was placed in specially designed adhesion chambers. PMN suspensions (10⁶/ml) were injected and allowed to settle onto the monolayer for 500 seconds, and the chamber then was inverted for an additional 500 seconds. The percent adherence (percentage of cells that remain attached when the chamber is initially inverted) was determined.

In some experiments, R15.7, IgG1, and CL18/6 were added to the neutrophil suspension 5 minutes before the adhesion assay. Each antibody was used at a final concentration of 10 μg/ml. In other experiments, WEB2086 (PAF antagonist, Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, Conn.) or WEB2170, a more water-soluble analogue of WEB2086 with similar PAF receptor antagonist affinity, was added to the neutrophil suspension 5 minutes before adhesion assay. The effects of WEB2086 or WEB2170 on adherence of neutrophils activated by either PAF or zymosan-activated dog serum (ZADS) to cultured endothelium were also examined.

**Effects of Catalase, Dimethylthiourea, or WEB2086 on PMN Retention by Carotid Arteries**

Canine carotid arteries were perfused with buffer alone or buffer containing 1 mmol/l H₂O₂ in the presence or absence of 10 units/ml catalase, 5 mmol/l dimethylthiourea, or 5 μmol/l WEB2086. Arteries were perfused according to the protocol outlined in Figure 1. In some experiments, WEB2086 (5 μmol/l in PBS) was also added to ¹¹¹In-labeled canine neutrophils before their instillation in the vessel.
Statistics

The retention of neutrophils by vessels or adherence of neutrophils to cultured endothelium subjected to the same intervention was compared with control (buffer-perfused vessels or unstimulated endothelium) or with H2O2-treated vessels or endothelium using Student's t test for unpaired values with Bonferroni's correction.

Results

H2O2 Induces PMN Adhesion to Vascular Endothelium

H2O2 produced a dose-dependent increase in PMN adhesion to canine carotid arteries and jugular veins. Maximal PMN retention was observed in vessels exposed to 1 mmol/l H2O2 (Figure 2A). PMN adhesion to vascular endothelium preexposed to H2O2 was

![Graph showing the effect of H2O2 concentration on neutrophil retention](image)

**Figure 2.** Panel A: Graph shows that pretreatment of canine carotid arteries for 20 minutes with increasing concentrations of H2O2 produced a dose-dependent increase in neutrophil retention that was maximal at 1 mmol/l (mM); n=4, values are mean±SEM. Panel B: Graph shows that polymorphonuclear leukocyte retention by H2O2 (1 mmol/l) preexposed arteries was both time dependent and transient. Maximal effect was observed 10 minutes after H2O2 exposure and declined to control values by 60 minutes; n=4. *p<0.05 compared with control values.

![Bar graph showing PMN adhesion](image)

**Figure 3.** Bar graph of canine carotid arteries perfused at 1 ml/min with buffer alone (control) or buffer containing 1 mmol/l (mM) H2O2 for 20 minutes and washed with buffer alone for 10 minutes before instillation of 111In-labeled isolated neutrophils with and without R15.7, anti-CD18 monoclonal antibody, as described in Figure 1. Some arteries were perfused with buffer for another 60 minutes after their initial exposure to H2O2, and were then perfused with buffer (control, 20±80 minutes) or 1 mmol/l H2O2 for an additional 20 minutes before the 10-minute wash and neutrophil instillation. n=4 arteries in each group. *p<0.05 compared with control. **p<0.05 compared with corresponding 1 mmol/l H2O2 treatment.
time dependent and transient (Figure 2B). PMN adhesion was maximal 10 minutes after H$_2$O$_2$ exposure. By 20 minutes of buffer perfusion after H$_2$O$_2$ exposure, PMN adhesion had decreased to values indistinguishable from control levels (Figure 2B). Repeat exposure to 1 mmol/l H$_2$O$_2$ for 20 minutes again caused PMN retention by the arteries of a magnitude similar to that observed after the first exposure to H$_2$O$_2$ (Figure 3).

The number of PMNs retained by arteries after perfusion with buffer alone was estimated to be 2–3/10,000 $\mu$m$^2$ luminal surface area. This value increased to 7–10/10,000 $\mu$m$^2$ after perfusion of the artery with buffer containing 1 mmol/l H$_2$O$_2$ for 20 minutes. This value is strikingly similar to the number of leukocytes adherent to rat colonic venules determined by intravital microscopy after addition of either formyl-methionyl-leucyl-phenylalanine or leukotriene B$_4$ to the suffusate.

SEM and TEM studies confirmed the findings of H$_2$O$_2$-induced PMN retention as measured by $^{111}$In-labeled neutrophils. In control vessels not exposed to H$_2$O$_2$, an occasional PMN was rarely adherent to the endothelium (Figure 4A). In the H$_2$O$_2$-exposed carotid artery, numerous clusters of adherent PMNs were observed by SEM (Figure 4B). These PMNs had the morphology typical of activated cells (Figures 4C and 4D). TEM revealed PMN transendothelial migration only in H$_2$O$_2$ preexposed vessels. Twenty-nine interactions of neutrophils with endothelium were observed (four PMNs on top, three partially under, and 22 completely under the endothelium). Although the endothelium of arteries exposed to H$_2$O$_2$ appeared intact, endothelial cells underlying adherent PMNs demonstrated swelling and loss of plasma membrane integrity (Figure 5A). In areas where PMNs were observed to be emigrating through the vessel wall, the endothelial cell monolayer was frequently disrupted (Figure 5B). In this study, there were no significant differences in specific $^{111}$In release among vessels exposed to H$_2$O$_2$ in concentrations of 100–10,000 $\mu$mol/l and controls not exposed to H$_2$O$_2$ (Figure 6), providing further evidence for the lack of cytotoxic effect of H$_2$O$_2$ on the endothelium in the absence of PMNs in this model.

**Effects of H$_2$O$_2$ on CD18-ICAM-Dependent Adhesion of PMN to DIVEC In Vitro**

To determine if the effects of H$_2$O$_2$ on neutrophil retention in the isolated perfused vessel could be due
to changes in endothelial cell adhesiveness, DJVEC monolayers were exposed to various concentrations of H$_2$O$_2$ in vitro. Concentrations of H$_2$O$_2$ of 0.5, 1, and 10 mmol/l were without effect on PMN adhesion. However, when monolayers were exposed to 20 mmol/l H$_2$O$_2$ for 10 minutes and rinsed in two changes of PBS, significant increases in PMN adhesion were seen (Table 1). Monolayers exposed to 20 mmol/l H$_2$O$_2$ for 10 minutes exhibited no visual evidence of toxicity for up to 3 hours and remained fully capable of responding to LPS stimulation as evidenced by the level of PMN adhesion (Table 1).

Previous studies have shown that CD18 and ICAM-1 participate in the adhesion of canine PMNs to endothelium as they do in human cells. A possible role for these adhesion molecules in H$_2$O$_2$-stimulated adhesion is indicated by the results in Table 1. When R15.7 and/or CL18/6 were present, H$_2$O$_2$-treated DJVEC monolayers were no more adhesive than untreated monolayers.

Using an enzyme-linked immunosorbent assay (ELISA) procedure previously used to assess upregulation of ICAM-1 on human endothelial monolayers after stimulation with cytokines, we attempted to determine if exposure to H$_2$O$_2$ induced increased expression of ICAM-1 on DJVEC monolayers. Whereas LPS (10 ng/ml, 3 hours) resulted in a sevenfold increase in surface ICAM-1 (i.e., binding of Mab CL18/6), exposure of DJVEC to 20 mmol/l H$_2$O$_2$ for 10 minutes did not alter the binding of CL18/6 at observation times of 10, 30, 60, and 120 minutes after H$_2$O$_2$ pretreatment, and it did not inhibit the upregulation of ICAM-1 caused by LPS.

**Effect of Anti-CD18 and Anti-ICAM-1 Antibodies on H$_2$O$_2$-Induced PMN Retention in Perfused Vessels**

When PMNs were incubated with a Mab against canine PMN CD18 (R15.7), H$_2$O$_2$-induced adhesion was inhibited (Figure 7). The isotype-matched non-binding IgG1 control was without effect (Figure 7).
Residual radioactivity from vessels treated with R15.7 was comparable with control values. Mab against canine ICAM-1 inhibited H₂O₂-induced PMN adhesion to the same degree as R15.7 (Figure 7). No additive effect was observed by addition of both R15.7 and CL18/6 Mabs to PMN cell suspensions and vessel endothelium. ICAM-1 was present on endothelium of DJVEC as determined by immunohistochemical staining with CL18/6 and was not qualitatively altered by H₂O₂ treatment (data not shown).

CD18-Dependent H₂O₂-Induced PMN Retention by Perfused Arteries Is Dependent on Endothelial Cells

Retention of PMNs by arteries mechanically denuded of endothelial cells was markedly increased compared with carotid arteries with intact endothelium. H₂O₂ pretreatment of endothelium-denuded arteries did not enhance PMN retention, nor did the anti-CD18 antibody R15.7 inhibit retention in these arteries (Figure 8). Collectively, these observations indicate that H₂O₂-induced retention of neutrophils by canine carotid arteries requires an intact endothelium and that binding to denuded arteries is CD18 independent.

Effect of Catalase and Dimethylthiourea on H₂O₂-Induced PMN Retention

Addition of 10 units/ml catalase or 5 mmol/l dimethylthiourea to the buffer perfusate had no effect on basal PMN retention but completely inhibited the augmented PMN retention induced by exposure of the vessel to 1 mmol/l H₂O₂ for 20 minutes (Figure 9).

Effect of WEB2086 on H₂O₂-Induced PMN Retention

Addition of 5 mmol/l of the PAF antagonist WEB2086 to the buffer perfusate (and to the ¹¹¹In-labeled PMNs) had no effect on basal PMN retention but completely inhibited the augmented PMN retention induced by exposure of the vessel to 1 mmol/l H₂O₂ for 20 minutes (Figure 10). This inhibitor was also effective in blocking the increased adhesion in vitro when DJVEC were stimulated with PAF (Tables 2 and 3) but was without effect on adherence of neutrophils stimulated with ZADS (Table 3).

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**TABLE 1. Effect of H₂O₂ on Canine Endothelial Adhesiveness In Vitro**

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<th>PMN</th>
<th>DJVEC</th>
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<th>Percent adherence (±SEM)</th>
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<tr>
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PMN, polymorphonuclear leukocytes; DJVEC, canine jugular vein endothelial cells; LPS, lipopolysaccharide; PBS, phosphate-buffered saline.

*Monoclonal antibodies were used at a final concentration of 10 μg/ml. IgG1, nonbinding isotype-matched control; R15.7, anti-CD18; CL18/6, anti–canine intercellular adhesion molecule-1.

†In the cultures stimulated with LPS, LPS was added at 10 ng/ml directly to the culture medium, or the monolayers were exposed first to PBS or H₂O₂ (20 mmol/l) for 10 minutes, 37°C, rinsed twice in PBS and then exposed to medium containing 10 ng/ml LPS for 3 hours.

‡Monolayers were exposed to H₂O₂ (20 mmol/l) or PBS for 10 minutes, rinsed in PBS, and then incubated in complete medium for an additional 3 hours.

§p<0.05 compared with PMN and DJVEC control.

||p<0.05 compared with PMN control and DJVEC treated with H₂O₂.
we mmol/l H2O2 (intercellular ICAM-1 dependent relaxation with precontracted vessels. prelabeled indomethacin still retained vessels or as treated vessels inhibited by conditions morphological reveal cell death 1) observations: injury in experiments that result in increased adhesiveness for neutrophils. The results of this report confirm and extend the results of Lewis et a125 and demonstrate that brief exposure of canine endothelial cells to high concentrations of H2O2 either in situ in isolated perfused vessels or as isolated cell monolayers in vitro does not cause cell death but induces a transient increase in adhesion of isolated canine neutrophils.

The conclusion that H2O2 was not toxic to the endothelial cells in the perfused vessels under the conditions of these experiments is supported by three observations: 1) SEM and TEM studies did not reveal morphological evidence of endothelial cell injury in areas without adherent PMNs or in H2O2-treated vessels in which adherence of PMNs was inhibited by the anti-CD18 Mab R15.7. 2) H2O2 treatment did not increase the loss of 111In from prelabeled vessels. 3) In studies reported elsewhere, we have demonstrated that canine carotid arteries precontracted with phenylephrine in the presence of indomethacin still exhibit normal endothelium-dependent relaxation to acetylcholine after perfusion with 1 mmol/l H2O2 for 20 minutes35 but only contraction after perfusion with either 10 mmol/l or 100 mmol/l H2O2 for 20 minutes (A.A. Taylor and G.M. McGuire, unpublished observations). This last observation provides evidence for functional endothelial viability after perfusion with 1 mmol/l H2O2 but not 10 or 100 mmol/l in light of the findings first reported by Furchgott and Zawadzki29 that acetylcholine-induced vascular relaxation is dependent on a functionally intact endothelium. In addition, the conclusion that H2O2 was not toxic to the endothelial cell monolayers in vitro is supported by the observations that the monolayers remained visually intact for up to 3 hours after H2O2 exposure (20 mmol/l, 10 minutes) and that they responded normally to stimulation with LPS by increasing their adhesiveness for isolated canine neutrophils and expressing ICAM-1 normally.

The conclusion that H2O2 increased PMN–endothelial cell adhesion is supported by results from both the isolated vessels and cultured endothelial cells. The visual assay performed in vitro revealed an approximately threefold increase in cells remaining associated with the monolayer. The monolayer remained intact throughout the assay procedure, and PMNs were attached to the apical surface of the endothelial cells. The increase in adhesiveness of cultured canine venous endothelium for PMNs after H2O2 exposure in these studies is quantitatively and qualitatively similar to that reported previously by Lewis and colleagues for H2O2–treated human umbilical vein endothelium.25 A similar level of increase was seen using radiolabeled neutrophils in the per-

**FIGURE 7.** Graphs show H2O2-induced polymorphonuclear leukocyte (PMN) retention by ex vivo perfused arteries (panel A) and veins (panel B) is completely inhibited by incubation of the PMNs with monoclonal antibody against CD18 (R15.7) but not with an isotype-matched nonbinding control (IgG1). Similarly, vessels perfused with monoclonal antibody against canine endothelial ICAM-1 (intercellular adhesion molecule-1) (CL18/6) 5 minutes before PMN instillation completely inhibited the increased PMN retention produced by H2O2 pretreatment. Values are mean ± SEM. Number of vessels are shown in parentheses. *p<0.05 compared with control. **p<0.05 compared with H2O2-treated vessels.
fused vessels, and morphological studies indicated that the PMNs retained in the vessels were associated with the endothelium, either attached to or beneath the endothelial cells along the vessel lumen. Catalase, when added to the perfusion buffer in these experiments, prevented the increased adhesiveness of H$_2$O$_2$-treated intact canine carotid arteries for unstimulated canine neutrophils as it did the adhesion of human neutrophils to H$_2$O$_2$-treated cultured human umbilical vein endothelial cells. The observation reported here that dimethylthiourea, a hydroxyl radical scavenger that freely diffuses across plasma membranes, is as effective as catalase in inhibiting H$_2$O$_2$-induced PMN adhesion to carotid arteries, is consistent with the notion that a reduced metabolite of H$_2$O$_2$ rather than H$_2$O$_2$ itself is the reactive oxygen species responsible for initiating the biochemical changes in the endothelium that lead to this increased adhesion. Other studies reported in preliminary form also implicate iron in this process, possibly via the iron-catalyzed Fenton reaction by which hydroxyl radical is produced from H$_2$O$_2$.

The results of the present investigation and that reported by Lewis et al. demonstrate a concentration-dependent effect of H$_2$O$_2$ on the adhesiveness of cultured endothelium for PMNs. In both studies, PMN adherence was maximal after exposure of endothelial cells to H$_2$O$_2$ concentrations of 10–20 mmol/l but was reduced at H$_2$O$_2$ concentrations of 100 mmol/l. A similar concentration-dependent effect of H$_2$O$_2$ on PMN retention by intact canine carotid arteries was noted in the present study, although the concentration of H$_2$O$_2$ in the perfusion buffer that produced a maximal effect was only 1 mmol/l. These two models are not strictly comparable because cultured endothelial cells are exposed to H$_2$O$_2$ under static conditions, whereas a fresh supply of H$_2$O$_2$ is being perfused through the intact vessel throughout the time of exposure. It is likely that the rates at which H$_2$O$_2$ is metabolized by intracellular antioxidant enzymes such as catalase and glutathione peroxidase are different in the static versus continuous perfusion models. The findings of Lewis et al. that high concentrations of H$_2$O$_2$ are associated with a reduction in the apparent PAF production provide one possible explanation for why PMN adhesion is reduced after either cultured endothelial cells or perfused arteries are exposed to high H$_2$O$_2$ concentrations. It is also possible that the antioxidant capacity of endothelial cells is overwhelmed by these high concentrations of H$_2$O$_2$ and leads to derangement of biochemical processes that result in increased endothelial cell adhesiveness for PMNs after H$_2$O$_2$ exposure. Additional studies will be required to identify the precise biochemical mechanisms by which these high concentrations of H$_2$O$_2$ impair the adhesive capacity of the endothelium.

Although the concentrations of H$_2$O$_2$ used in these and previous studies appear high, several lines of evidence suggest that they may have physiological relevance to conditions that may exist in the intact organism. Studies carried out by several laboratories, including ours, have demonstrated that PMNs adherent to cultured endothelium or certain extracellular matrix proteins are capable of undergoing a sustained
respiratory burst that results in the production of micromolar quantities of \( \text{H}_2\text{O}_2 \). The total amount of \( \text{H}_2\text{O}_2 \) produced by these adherent PMNs is comparable with the lower concentrations of \( \text{H}_2\text{O}_2 \) used in these studies and far exceeds that produced by PMNs in suspension when stimulated by cytokines or chemotactic factors. Furthermore, our studies of the adherence of PMNs stimulated with chemotactic factors to isolated cardiac myocytes stimulated with cytokines indicate that the reactive oxygen species produced by PMNs under these conditions may escape the action of antioxidant enzymes that are present in the extracellular fluid. The addition of superoxide dismutase and catalase to the incubation medium containing dichlorofluorescein-loaded cardiac myocytes did not prevent the intracellular oxidation of dichlorofluorescein in the myocyte produced by adherent PMNs, whereas pretreatment of the myocytes with either the lipid-permeant hydroxyl radical scavenger dimethylthiourea or the iron chelator desferrioxamine prevented this oxidation without affecting PMN adhesion. Thus, it is plausible to suggest that the concentrations of \( \text{H}_2\text{O}_2 \) used in these experiments might be achieved at inflammatory sites of PMN accumulation and that the \( \text{H}_2\text{O}_2 \) thus released from adherent PMNs might be protected from rapid inactivation by extracellular antioxidant enzymes.

Although quantitatively less than that induced by direct chemotactic factor activation of PMNs or cytokine or LPS stimulation of cultured endothelium (see Table 1), adhesion of PMNs to \( \text{H}_2\text{O}_2 \)-treated endothelium has been previously reported to be twofold to fourfold above control values (Lewis et al\(^{23} \)), a value replicated by the studies reported here using either

![Graph](http://circ.ahajournals.org/)

**Figure 10.** Graph shows effect of the platelet-activating factor antagonist WEB2086 (5 \( \mu \text{mol/l} \) final concentration) on \( \text{H}_2\text{O}_2 \)-induced (1 mmol/l [mM]) polymorphonuclear leukocyte (PMN) retention examined in four perfused canine carotid arteries. Perfusion protocol was identical to that described in Figure 1 except that WEB2086 was added to the perfusate of all arteries except those serving as controls. WEB2086 (5 \( \mu \text{mol/l} \) final concentration) also was incubated with radiolabeled PMNs for 10 minutes before instillation in the vessel lumen. Four additional groups of vessels were perfused with 1% zymosan-activated dog serum (ZADS) with and without WEB2086 (5 \( \mu \text{mol/l} \)) or R15.7 (10 \( \mu \text{g/ml} \)) for 10 minutes before instillation in the vessel lumen as described in Figure 1. **\( p \leq 0.05 \) compared with control, control+WEB2086, and 1 mmol/l \( \text{H}_2\text{O}_2 \)+WEB2086.

### Table 2. Effect of WEB2086 on Canine Neutrophil Adhesion to Canine Endothelial Cells In Vitro

<table>
<thead>
<tr>
<th>PMN*</th>
<th>DJVEC†</th>
<th>( n )</th>
<th>Percent adherence (±SEM)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control</td>
<td>11</td>
<td>9.3±1.8</td>
</tr>
<tr>
<td>WEB2086</td>
<td>Control</td>
<td>5</td>
<td>9.6±2.9</td>
</tr>
<tr>
<td>Control</td>
<td>( \text{H}_2\text{O}_2 )</td>
<td>6</td>
<td>26.3±1.1§</td>
</tr>
<tr>
<td>WEB2086</td>
<td>( \text{H}_2\text{O}_2 )</td>
<td>6</td>
<td>8.3±1.8§</td>
</tr>
<tr>
<td>PAF</td>
<td>Control</td>
<td>6</td>
<td>60.3±1.2§</td>
</tr>
<tr>
<td>PAF, WEB2086</td>
<td>Control</td>
<td>6</td>
<td>19.8±1.3§</td>
</tr>
</tbody>
</table>

PMN, polymorphonuclear leukocyte; DJVEC, canine jugular vein endothelial cells.

*PMNs were either suspended throughout the adhesion assay in phosphate-buffered saline (PBS), 10 \( \mu \text{g/ml} \) WEB2086, or 100 ng/ml platelet-activating factor (PAF) as indicated.

†Endothelial monolayers were pretreated with 20 mmol/l \( \text{H}_2\text{O}_2 \) in PBS or PBS alone for 10 minutes and washed in PBS before the assay.

‡Percent cells remaining attached to the endothelial monolayer ± SEM.

§\( p < 0.01 \) compared with control without stimulus.

||| Values given are means of four experiments for adherence studies and three experiments for flow cytometry studies.

### Table 3. Effect of PAF and PAF Receptor Antagonist WEB2170 on CD18 Surface Expression and Neutrophil Adhesion

<table>
<thead>
<tr>
<th>Stimulant*</th>
<th>Inhibitor†</th>
<th>Adherence‡</th>
<th>CD18§</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>5±2%</td>
<td>135</td>
</tr>
<tr>
<td>PAF, 1 ng/ml</td>
<td>None</td>
<td>98±2%</td>
<td>215</td>
</tr>
<tr>
<td>PAF, 1 ng/ml</td>
<td>WEB2170, 1 ( \mu \text{g/ml} )</td>
<td>6±1%</td>
<td></td>
</tr>
<tr>
<td>PAF, 1 ng/ml</td>
<td>Anti-C5a, 1:500</td>
<td>94±8%</td>
<td>226</td>
</tr>
<tr>
<td>ZAS, 0.3%</td>
<td>None</td>
<td>75±8%</td>
<td>275</td>
</tr>
<tr>
<td>ZAS, 0.3%</td>
<td>WEB2170, 1 ( \mu \text{g/ml} )</td>
<td>72±9%</td>
<td>257</td>
</tr>
<tr>
<td>ZAS, 0.3%</td>
<td>Anti-C5a, 1:500</td>
<td>10±5%</td>
<td>142‡</td>
</tr>
</tbody>
</table>

*Stimulants: PAF, platelet-activating factor (1-O-hexadecyl-2-acetyl-glycero-3-phosphocholine); ZAS, zymosan-activated dog serum used at a concentration of 0.3%. Stimulation time 30 minutes, 37°C for CD-18 surface expression and 5 minutes for adherence.

†Inhibitors: Neutrophils were preincubated with WEB2170 for 5 minutes before addition of stimulus. Stimulus was preincubated with the anti-C5a (rabbit polyclonal serum obtained from Dr. Roger Rossen, Baylor College of Medicine, Houston, Tex.) for 5 minutes before addition of neutrophils.

‡Adherence: Neutrophil adherence to serum-treated glass expressed as percent cells remaining attached after a contact period of 500 seconds ± SD.

§CD18: Mean fluorescence channel, flow cytometry, FACScan. Neutrophils were prepared by using R15.7 IgG (anti-CD18) and FITC-labeled goat anti-mouse IgG.

| \( p < 0.01 \) | Values given are means of four experiments for adherence studies and three experiments for flow cytometry studies. |
cultured endothelium or perfused vessels. The reasons for this quantitative difference between H$_2$O$_2$- and cytokine- or hormone agonist-mediated PMN adherence to endothelium has not been clearly identified but may relate to differences in intracellular signals that mediate the actions of H$_2$O$_2$ versus hormonal agonists such as histamine, bradykinin, thrombin, and angiotensin II because the former occurs without apparent receptor activation via a transient increase in cytosolic calcium, whereas the latter requires receptor-coupled signaling, probably through a pertussis toxin-insensitive G protein.

That the adhesion of PMNs to H$_2$O$_2$-treated vessels or cultured endothelium was dependent on CD18 and ICAM-1 was supported by the observations that specific Mabs completely inhibited adhesion both in vivo and in vitro. We have shown in other studies that these antibodies inhibit CD18- and ICAM-1-dependent adhesion of canine neutrophils to canine endothelial cells in vitro. This ligand-specific adhesion was lost in vessels mechanically denuded of endothelium, further indicating that the H$_2$O$_2$ effect was on PMN–endothelial cell adhesion.

Although the molecular mechanisms by which H$_2$O$_2$ increased endothelial adhesiveness are not addressed directly in the current experiments, previous work has shown that CD18- and ICAM-1–dependent adhesion is enhanced by several different stimuli. Protein synthesis is required because there appear to be no storage pools for ICAM-1 within the endothelial cell and the increase in surface ICAM-1 is not detected until about 1 hour after stimulation. We were unable to show that H$_2$O$_2$ exposure caused increases in surface ICAM-1 on cultured endothelial cells, indicating that this mechanism is unlikely to account for the increased adhesion. However, ICAM-1 is constitutively expressed on endothelial cells and appears to be a ligand for stimulated PMNs.

Another potential mechanism whereby H$_2$O$_2$-treated endothelial cells induce CD18-dependent adhesion of neutrophils relies on the fact that chemotactic stimulation of neutrophils markedly augments CD18-dependent adhesion. Human endothelial cells appear to produce PAF after H$_2$O$_2$ exposure. Most if not all of this newly synthesized PAF appears to remain associated with the endothelial cell. PAF is capable of stimulating human neutrophils to increase their surface levels of Mac-1 and of increasing CD18-dependent adhesion to endothelial cells and protein-coated foreign surfaces. We have found that the PAF receptor antagonist WEB2086 markedly inhibits the ability of PAF to increase canine neutrophil adhesion to endothelial cells and protein-coated foreign surfaces and to upregulate surface levels of Mac-1. It also completely inhibits the increased adhesion of canine neutrophils to H$_2$O$_2$-treated vessels and cultured endothelial monolayers. These observations support the hypothesis that the adhesiveness induced by H$_2$O$_2$ results from the stimulation of neutrophils by endothelially derived PAF. Our findings of an inhibitory effect of WEB2086 on PMN adhesion in the H$_2$O$_2$-treated vessels are consistent with the continued association of PAF with the endothelial cell because any PAF synthesized by the endothelial cells in response to H$_2$O$_2$ and then released would have been washed out of the vessel by the perfusion buffer and, therefore, would not have been available to sustain PAF-dependent H$_2$O$_2$-induced PMN adhesion. The specific α-subunit of the CD18 integrins involved in this adhesion cannot be inferred from current results because there is evidence from studies of human cells that both LFA-1 and Mac-1 can interact with ICAM-1.

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**Key Words**: platelet-activating factor • vascular endothelium • reactive oxygen species • catalase • dimethylthiourea
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