Oxyhemoglobin-Induced Cytotoxicity and Arachidonic Acid Release in Cultured Bovine Endothelial Cells

Katsunobu Takenaka, MD; Neal F. Kassell, MD; Patricia L. Foley, DVM; and Kevin S. Lee, PhD

Background and Purpose: An impairment of endothelial function is associated with vasospasm after subarachnoid hemorrhage. Oxyhemoglobin is considered to be a critical trigger in the pathogenesis of vasospasm. The present studies examined the direct effects of oxyhemoglobin on cultured endothelial cells from bovine carotid artery.

Methods: Confluent endothelial cells were treated with oxyhemoglobin, and the following were studied: 1) cell morphology, 2) cell density, and 3) the release of radiolabel from [3H]arachidonic acid–treated cells.

Results: Endothelial cells exposed to oxyhemoglobin exhibited detachment vacuoles, and cell density was significantly decreased in time- and dose-dependent manners. Superoxide dismutase, a free radical scavenger, provided partial protection against the cytotoxic effects of oxyhemoglobin. The release of radiolabel from [3H]arachidonic acid–treated cells was increased by oxyhemoglobin in time- and dose-dependent manners. Treatment with an inhibitor of phospholipase A2 or a calcium chelator inhibited the effects of oxyhemoglobin on arachidonic acid release and cellular viability.

Conclusions: Oxyhemoglobin exerts a direct cytotoxic effect on cultured endothelial cells, and this effect is associated with increased release from [3H]arachidonic acid–labeled cells. Phospholipase A2 and free radicals appear to participate in the pathogenesis of endothelial cell damage. Oxyhemoglobin-induced compromise of endothelial cells may contribute to cerebrovascular pathology. (Stroke 1993;24:839–846)

Key Words • cytotoxicity • oxyhemoglobins • vasospasm

Cerebral vasospasm is a major complication in patients with subarachnoid hemorrhage resulting from ruptured aneurysms. Despite many investigations, the pathogenesis of vasospasm is still a matter of discussion.1–4 It has been generally accepted that constituents of erythrocytes and substances produced during hemolysis of subarachnoid blood clots are closely associated with the induction of cerebral vasospasm. Among the substances found in the hemolysate, oxyhemoglobin is considered to play a key role in this process4–5; however, its precise mechanism of action is still controversial.7,10,11

It has recently been suggested that impairment of the vasodilatory activity of cerebral arteries after subarachnoid hemorrhage contributes to the pathogenesis of vasospasm.12,13 One explanation for impaired vasodilation is that normal endothelial function is disrupted during the development of vasospasm. It is well documented that endothelial cell damage occurs after subarachnoid hemorrhage.14–16 The present experiments investigated whether oxyhemoglobin exerts a cytotoxic effect on cultured endothelial cells and examined the possible participation of arachidonic acid generation in this process.

Materials and Methods

Bovine hemoglobin type I, fatty acids, indomethacin, superoxide dismutase (SOD) from bovine erythrocytes, ethylene glycol-bis (β-aminooxyether) N,N′,N″,N‴-tetraacetic acid (EGTA), and 4-bromophenacyl bromide (BPB) were purchased from Sigma Chemical Co., St. Louis, Mo. Dulbecco’s modified Eagle’s medium (DMEM) and bovine calf serum (BCS) were purchased from Gibco, Grand Island, N.Y. [3H]Arachidonic acid (79.9 Ci/mmol) was obtained from NEN Research Products, Boston, Mass. Goat anti-human factor VIII–related antiserum, peroxidase conjugated donkey anti-goat immunoglobulin G, normal goat serum, and normal rabbit serum were purchased from ICN Biomedicals, Inc., Costa Mesa, Calif. All other reagents were of the highest grade available commercially.

Oxyhemoglobin was prepared as described previously.11 Briefly, bovine hemoglobin type I containing a mixture of oxyhemoglobin and methemoglobin was reduced with sodium dithionite (Na2S2O4). After removal of the reducing agent by extensive dialysis against
distilled water, the purity of oxyhemoglobin was determined spectrophotometrically. Endothelial cells from bovine internal carotid artery were isolated by the method of Gospodarowicz et al. Briefly, the carotid artery was cut open lengthwise with a scalpel, and the intimal surface was washed with Ca²⁺-free phosphate-buffered saline (PBS) to remove blood. The endothelial cell layer was removed by gently scraping the intimal surface with a grooved director. The grooved director was dipped in 10 mL of DMEM with 10% BCS. This technique yielded cell populations composed of 99% endothelial cells. The medium containing the cells was transferred to a 15-cm tissue culture dish, and 20 mL of DMEM with 10% BCS was added to the plates. After a 24-hour incubation period, aggregates or single cells started to attach to the bottom of the dishes; the medium was renewed at 48 hours. The cells were cultured in DMEM plus 10% BCS in a 5% CO₂ atmosphere at 37°C and passaged weekly. Cells at nine to 12 passages were used for the current study. The cells were grown in the serum-containing growth medium for 4 days to confluence.

Cells were grown on two-well chamber slides (Lab-Tek, Miles Laboratories, Naperville, Ill.) and fixed in 95% ethanol for 10 minutes. Immunohistochemical staining for factor VIII–related antigen was performed using the indirect immunoperoxide method. To minimize nonspecific staining, the fixed cells were treated with methanol containing 0.3% hydrogen peroxide for 30 minutes. After washing in PBS, the cells were treated at room temperature with normal rabbit serum for 20 minutes. Cells were then incubated for 30 minutes with goat anti-immunoglobulin G (Fab') fragments diluted 1:200 in PBS containing 5% normal rabbit serum. Normal goat serum, which was diluted 1:20 as above, was used for control incubations. The cells were washed three times with PBS and incubated for 30 minutes with peroxidase conjugated donkey anti-goat immunoglobulin G (Fab') fragments diluted 1:500 in PBS. After washing three times, the peroxidase binding sites were detected by staining with a solution of 4-chloro-1-naphthol.

Confluent endothelial cells were incubated under various conditions in medium containing 5% BCS. To investigate the effect of SOD or BPB on oxyhemoglobin-induced changes, cells were exposed to these compounds for 30 minutes before and during oxyhemoglobin treatment. The effect of EGTA was examined by adding it to the incubation medium only in the presence of oxyhemoglobin. After incubation for various periods of time, cell density was determined by using a dye uptake method described in detail elsewhere. Briefly, the cells were stained with 0.5% crystal violet (water:methanol, 4:1) for 15 minutes and then washed with water three times. Then the dye was eluted by 33% (vol/vol) acetic acid, and the absorbance at 600 nm was measured (model U-2000 spectrophotometer, Hitachi, San Jose, Calif.). SOD was denatured by titrating the protein to a pH of 10 and incubating at room temperature for 12 hours in the presence of a 10-fold excess of hydrogen peroxide followed by two-step dialysis.

Confluent endothelial cells were incubated with 1 mL of 0.5 µCi/mL [³H]arachidonic acid for 24 hours. The cells were rinsed five times with DMEM to remove unincorporated [³H]arachidonic acid. After washing, the cells were exposed to “test” medium (i.e., DMEM containing 5% BCS with or without hemoglobin). To investigate the effect of indomethacin or BPB, cells were exposed to these compounds for 30 minutes before and during the presentation of test medium. The effect of EGTA was also tested by addition to the medium. At various times, 100 µL of the medium was collected and counted with a RackBeta liquid scintillation counter (model 1209, LKB, Gaithersburg, Md.) as described previously.

Values are reported as mean±SD. Analysis of variance and Student’s t test were used for statistical analysis. A value of p<0.05 was considered significant.

Results

At confluence, the endothelial cells formed a monolayer, most of which demonstrated the uniform cobblestone appearance typical of endothelial cells (Figure 1, top panel). Positive staining for factor VIII–related antigen was observed in almost all cultured cells. Staining was localized to the perinuclear space (Figure 2), and this staining was specific; no positive staining was observed when incubations were performed in the absence of antibody. Almost all of the cultured cells were identified as endothelial cells because they incorporated Dil-Ac-LDL (1,1′-dioctadecyl-3,3′,3′-tetramethyl-indocarbocyanine perchlorate).

Figure 1 shows the morphology of oxyhemoglobin-treated (10 µM) endothelial cells after 3 and 10 days of incubation. After treatment with oxyhemoglobin, cells became vacuolated and detached, and cell density decreased significantly. The time courses for the effects of several concentrations of oxyhemoglobin (1–100 µM) on confluent bovine endothelial cells are shown in Figure 3. The cytotoxic effect of oxyhemoglobin on the cells was both time dependent and dose dependent. The cytotoxic effect elicited by 1 or 10 µM oxyhemoglobin increased gradually with prolonged incubation lasting up to 15 days. A high concentration of oxyhemoglobin (100 µM) elicited a complete loss of cells within 4 days’ incubation. In contrast, methemoglobin (100 µM) did not elicit a cytotoxic response (Table 1).

The effects of two free fatty acids, palmitic acid and arachidonic acid, were tested on endothelial cells. Figure 4 shows the effect of fatty acid concentration on the cytotoxic response of the cells. Arachidonic acid inhibited cell growth in a dose-dependent manner, whereas palmitic acid had no effect at concentrations up to 100 µM. To investigate the mechanisms whereby oxyhemoglobin elicits cytotoxicity, the effects of indomethacin (cyclooxygenase inhibitor), SOD (free radical scavenger), EGTA (calcium ion chelator), and BPB (phospholipase A₂ inhibitor) on oxyhemoglobin-induced cytotoxicity were examined. BPB and SOD produced dose-dependent reductions in oxyhemoglobin-induced cytotoxicity (Table 1). EGTA and low-calcium medium also provided a substantial reduction in cytotoxicity. In contrast, indomethacin (10⁻⁴ M) had no protective effect. SOD inactivated with hydrogen peroxide also had no inhibitory effect (Table 1).

The effect of oxyhemoglobin on release of radiolabel was studied in endothelial cell cultures treated with [³H]arachidonic acid. After incubation of cells with [³H]arachidonic acid for 24 hours, the amount of radioactivity incorporated into cells was approximately 100,000 disintegrations per minute per 10⁶ cells. The addition of oxyhemoglobin (100 µM) to the cells in-
Figure 1. Morphology of control endothelial cells and oxyhemoglobin-treated cells. Confluent endothelial cells were incubated with Dulbecco's modified Eagle's medium supplemented with the following reagents: 5% bovine calf serum without oxyhemoglobin for 10 days (top left panel); 10 μM oxyhemoglobin for 3 days (top right panel); and 10 μM oxyhemoglobin for 10 days (left panel). Bar, 30 μm.
creased the release of radioactivity in a time-dependent manner (Figure 5). The levels measured between 30 minutes and 5 hours after oxyhemoglobin addition were significantly higher than those of controls (p<0.01). Figure 6 illustrates the dose dependence of the oxyhemoglobin-stimulated release of radiolabel from the cells.

One explanation for oxyhemoglobin-induced release of radiolabel is the activation of phospholipase A₂ and the release of labeled arachidonic acid from phospholipids. To examine this idea, cultures were exposed to BPB, an inhibitor of phospholipase A₂, before applying oxyhemoglobin. BPB at a concentration of 100 μM completely abolished the effects of oxyhemoglobin (Table 2). In contrast, indomethacin had no effect on the production of arachidonic acid in hemoglobin-treated endothelial cells (Figure 6).

Discussion

Hemorrhage and subsequent blood clot lysis in the subarachnoid space elicit a complex series of events that severely affect cellular constituents of the cerebrovascular wall. Whereas the prominent actions on vascular smooth muscle cell function are most apparent during subarachnoid hemorrhage-induced vasospasm, several modifications in endothelial cell function also occur. For instance, endothelium-dependent vasodilation is impaired in conjunction with cerebral vasospasm.₁₂,₁₆,₂₁ In addition, the barrier formed by the arterial wall in major cerebral arteries is compromised as a result of injury to the endothelial cells.₁₄,₁₅,₂₂,₂₃ Direct damage to endothelial cells is therefore thought to be responsible for components of the pathogenic response to subarachnoid hemorrhage.

The cellular and molecular mechanisms responsible for endothelial cell damage after vasospasm remain a
TABLE 1. Effect of Superoxide Dismutase, Ca\(^{2+}\), and Bromophenacyl Bromide on Oxyhemoglobin-Induced Cytotoxicity in Cultured Endothelial Cells

<table>
<thead>
<tr>
<th>Treatment for 2 days</th>
<th>Cell density (OD(_{600}))</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.06±0.09</td>
<td>12</td>
</tr>
<tr>
<td>Hb (10(^{-4}) M)</td>
<td>0.32±0.07</td>
<td>9</td>
</tr>
<tr>
<td>MetHb (10(^{-4}) M)</td>
<td>2.09±0.02</td>
<td>6</td>
</tr>
<tr>
<td>SOD (1,000 units/mL)</td>
<td>2.07±0.03</td>
<td>6</td>
</tr>
<tr>
<td>Hb (10(^{-4}) M)+SOD (1 unit/mL)</td>
<td>0.34±0.03</td>
<td>7</td>
</tr>
<tr>
<td>Hb (10(^{-4}) M)+SOD (10 units/mL)</td>
<td>0.34±0.03</td>
<td>8</td>
</tr>
<tr>
<td>Hb (10(^{-4}) M)+SOD (100 units/mL)</td>
<td>0.43±0.10*</td>
<td>12</td>
</tr>
<tr>
<td>Hb (10(^{-4}) M)+SOD (300 units/mL)</td>
<td>0.63±0.05*</td>
<td>9</td>
</tr>
<tr>
<td>Hb (10(^{-4}) M)+SOD (1,000 units/mL)+H(_2)O(_2)</td>
<td>0.76±0.05*</td>
<td>7</td>
</tr>
<tr>
<td>Hb (10(^{-4}) M)+SOD (1,000 units/mL)+H(_2)O(_2)</td>
<td>0.37±0.03</td>
<td>7</td>
</tr>
<tr>
<td>EGTA (1 mM)</td>
<td>2.09±0.03</td>
<td>6</td>
</tr>
<tr>
<td>Hb (10(^{-4}) M) in low-Ca(^{2+}) medium</td>
<td>1.48±0.09*</td>
<td>6</td>
</tr>
<tr>
<td>Hb (10(^{-4}) M)+EGTA (1 mM)</td>
<td>1.53±0.06*</td>
<td>11</td>
</tr>
<tr>
<td>BPB (10(^{-4}) M)</td>
<td>2.09±0.02</td>
<td>6</td>
</tr>
<tr>
<td>Hb (10(^{-4}) M)+BPB (10(^{-5}) M)</td>
<td>0.33±0.02</td>
<td>6</td>
</tr>
<tr>
<td>Hb (10(^{-4}) M)+BPB (3×10(^{-5}) M)</td>
<td>0.56±0.02*</td>
<td>6</td>
</tr>
<tr>
<td>Hb (10(^{-4}) M)+BPB (10(^{-4}) M)</td>
<td>1.02±0.01*</td>
<td>6</td>
</tr>
<tr>
<td>Hb (10(^{-4}) M)+indomethacin (10(^{-4}) M)</td>
<td>0.36±0.04</td>
<td>6</td>
</tr>
</tbody>
</table>

Values are mean±SD of at least three separate experiments performed in duplicate. Control value is cell density of endothelial cells incubated with Dulbecco's modified Eagle's medium supplemented with 5% bovine calf serum without hemoglobin for 2 days. OD, optical density; Hb, oxyhemoglobin; MetHb, methemoglobin; SOD, superoxide dismutase; EGTA, ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid; BPB, bromophenacyl bromide.

*p<0.05 compared with exposure to oxyhemoglobin alone.

matter of discussion. However, an attractive candidate mechanism for inducing endothelial cell damage is the release of oxyhemoglobin during lysis of the subarachnoid clot. Among the actions of oxyhemoglobin that could contribute to endothelial cell damage are the initiation and propagation of lipid peroxidation\(^{24}\) and the release of free radicals.\(^{24,25}\) Oxyhemoglobin auto-oxidizes spontaneously to methemoglobin, releasing superoxide anion radical (O\(_2^-\)), which is thought to initiate and propagate lipid peroxidation by the Haber-Weiss mechanism. However, a candidate mechanism for inducing endothelial cell damage is the release of oxyhemoglobin during lysis of the subarachnoid clot. Among the actions of oxyhemoglobin that could contribute to endothelial cell damage are the initiation and propagation of lipid peroxidation\(^{24}\) and the release of free radicals.\(^{24,25}\) Oxyhemoglobin auto-oxidizes spontaneously to methemoglobin, releasing superoxide anion radical (O\(_2^-\)), which is thought to initiate and propagate lipid peroxidation by the Haber-Weiss mechanism.

**FIGURE 4.** Line graph showing effect of fatty acids on cellular viability. Confluent cells were incubated with various concentrations of arachidonic acid or palmitic acid for 5 days; cell density then was determined as described in "Materials and Methods." Values are mean±SD of triplicate determinations from three separate experiments. *p<0.05.

**FIGURE 5.** Line graph showing effect of oxyhemoglobin (oxyHb) on release of radiolabel from \(^{3}H\)arachidonate-treated endothelial cells. Confluent endothelial cells were incubated with \(^{3}H\)arachidonate (0.5 \(\mu\)Ci/mL) for 24 hours. The cells were then washed three times with Dulbecco's modified Eagle's medium and incubated with Dulbecco's modified Eagle's medium containing 5% bovine calf serum with (○) or without (□) 100 \(\mu\)M oxyhemoglobin. At the indicated times, an aliquot was drawn from the cultured medium, and the amount of radioactivity was counted. Each value shows average of duplicate determinations from three separate experiments. SD levels were <10% at each point and are not shown.
reaction and Fenton chemistry. This sequence of events could contribute directly to vasospasm because lipid peroxides are known to cause both vasoconstriction and structural damage to cerebral arteries in vitro and in vivo.

Several lines of evidence support the idea that oxyhemoglobin contributes to the injury of cellular elements in the vascular wall. Oxyhemoglobin causes myonecrosis, invasion of myointimal cells in the tunica intima, transformation of nerve endings, changes in endothelial cell basement membrane, and the detachment of endothelial cells in vivo. In addition, oxyhemoglobin applied to exposed rat basilar artery results in cell craters, blebs, and vacuoles in endothelial cells, as well as severe vasoconstriction. The observed cytopathological effects on endothelial cells might be the result of a direct action of oxyhemoglobin on the endothelial cells but could also be due to secondary effects resulting from severe vasoconstriction. This issue is difficult to resolve using in vivo models of cerebrovascular spasm, and evidence as to whether oxyhemoglobin can directly affect the function of endothelial cells has not been available. The present study therefore sought to determine whether oxyhemoglobin is capable of exerting a direct cytotoxic effect on cerebrovascular endothelial cells maintained in vitro. Our results demonstrate that oxyhemoglobin elicits a time- and dose-dependent cytotoxic effect on cultured, cerebral endothelial cells. These observations indicate that direct effects of oxyhemoglobin on endothelial cell viability could contribute to vascular pathology, such as occurs after subarachnoid hemorrhage.

A critical issue in understanding this type of cellular damage is to identify the type of molecular mechanism(s) involved in the pathological response. The present studies indicate that activation of phospholipase A2 is integrally involved in this process. An inhibitor of phospholipase A2 (BPB) blocked the cytotoxic effects of oxyhemoglobin and inhibited the release of radiolabel from cells pretreated with [3H]arachidonic acid. The involvement of arachidonic acid in the cytotoxic effect is further supported by the observation that exogenously applied arachidonic acid is cytotoxic to the cells. The cytotoxic effect of arachidonic acid is dose dependent and not mimicked by palmitic acid, a structurally related free fatty acid. These observations suggest that the production of arachidonic acid and/or one of its metabolites by phospholipase A2 plays a critical role in damaging endothelial cells. This issue was examined by testing the effects of an inhibitor of cyclooxygenase (indomethacin). Cyclooxygenase is an enzyme responsible for producing prostaglandins from arachidonic acid, and increased production of prostaglandins has been observed in a variety of pathophysiological conditions. Pretreatment of endothelial cells with indomethacin, however, did not affect the extent of oxyhemoglobin-induced cytotoxicity. Taken together, these observations indicate that arachidonic acid production by phospholipase A2 is a critical event in the endothelial cell toxicity, but that metabolism of arachidonic acid to prostaglandins does not appear to be a necessary step for cytotoxicity.

The participation of superoxide free radical and calcium in oxyhemoglobin-induced cytotoxicity is also indicated by the current studies. Scavenging of superoxide radicals by SOD or reducing calcium concentrations attenuated the cytotoxic effect of oxyhemoglobin on cultured endothelial cells. Further studies will be necessary to provide a more complete picture of the precise sequence of pathological events elicited by oxyhemoglobin in endothelial cells.

Participation of the events described above in cerebrovascular pathology in vivo remains to be established. It is important to note, however, that recent immunocytochemical studies from this laboratory show that oxyhemoglobin can penetrate into the endothelial layer of the rabbit basilar artery after subarachnoid hemorrhage. Thus, oxyhemoglobin can gain access to the appropriate cellular target after subarachnoid hemorrhage. Taken together with the current findings, it is plausible that oxyhemoglobin derived from the lysis of a perivascular clot exerts direct and detrimental effects on endothelial cells in cerebral arteries.

**Figure 6.** Line graph showing oxyhemoglobin-stimulated release from [3H]arachidonate-treated cells and effect of indomethacin. Confluent endothelial cells were incubated with [3H]arachidonic acid (0.5 μCi/mL) for 24 hours. Then the cells were washed three times with Dulbecco’s modified Eagle’s medium and incubated with various concentrations of oxyhemoglobin in the absence (●) or presence (○) of 10 mM indomethacin. At 5 hours, an aliquot was drawn from the culture medium, and the amount of radioactivity was counted. Each value shows average of duplicate determinations from three separate experiments. SD levels were <10% at each point and are not shown.

**Table 2.** Effect of Bromophenacyl Bromide and Ca²⁺ on Release of [3H] in Oxyhemoglobin-Stimulated Cultured Endothelial Cells

<table>
<thead>
<tr>
<th>Treatment for 30 minutes</th>
<th>Release of [3H]arachidonate (dpm)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>478±42</td>
<td>6</td>
</tr>
<tr>
<td>Hb (10⁻⁴ M)</td>
<td>1721±101</td>
<td>6</td>
</tr>
<tr>
<td>Hb (10⁻⁴ M)+BPB (3×10⁻⁴ M)</td>
<td>1146±52*</td>
<td>3</td>
</tr>
<tr>
<td>Hb (10⁻⁴ M)+BPB (10⁻⁴ M)</td>
<td>509±93*</td>
<td>3</td>
</tr>
<tr>
<td>Hb (10⁻⁴ M)+EGTA (1 mM)</td>
<td>1428±28*</td>
<td>3</td>
</tr>
</tbody>
</table>

Values are mean±SD of at least three separate experiments performed in duplicate. Control value is release of [3H] from endothelial cells incubated with Dulbecco’s modified Eagle’s medium supplemented with 5% bovine calf serum without oxyhemoglobin for 30 minutes. Hb, oxyhemoglobin; BPB, bromophenacyl bromide; EGTA, ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid.

*p<0.05 compared with exposure to oxyhemoglobin alone.
Acknowledgment
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References

Editorial Comment

Unlike most forms of stroke, hemorrhagic stroke has not decreased significantly in incidence over the last 40 years. In patients with hemorrhagic stroke or subarachnoid hemorrhage (SAH), one of the most enigmatic and life-threatening complications is vasospasm of the large cerebral arteries. In part, the morbidity and mortality associated with cerebral vasospasm can be attributed to our incomplete understanding of the pathophysiology of this clinical entity. The article by Takenaka et al addresses some interesting and unifying pathophysiologic mechanisms applicable to cerebral vasospasm.

In general, cerebral vasospasm could develop from either impairment of endogenous vasodilatory substances or production of vasoconstrictor substances. Well-documented examples that demonstrate both mechanisms exist, and some debate has arisen from these differing observations. The vasodilatation from the endothelial-derived relaxing factors (EDRFs) prostacyclin1 and nitric oxide2 is impaired by SAH and oxyhemoglobin (Hgb). Others have shown that hemoglobin causes increased production of vasoconstrictor prostaglandins without impairment of endothelium-dependent dilation.3

The present study presents important data that unite these contrasting observations. Hemoglobin is well known to generate oxygen radicals by autoxidation4 in biologic systems; in the cell culture studies presented above, Hgb causes direct cytotoxic effects on endothelial cells that are inhibited by superoxide dismutase and a phospholipase A2 inhibitor. This raises the possibility that Hgb can simultaneously cause radical-mediated...
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