Hemin activation of an inducible isoform of nitric oxide synthase in vascular smooth-muscle cells

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Nitric oxide (NO) is a gaseous, free radical molecule that is integrally involved in intracellular signal transduction and plays an important role in the maintenance of cellular homeostasis. The enzyme responsible for producing NO, nitric oxide synthase (NOS), has been shown to be present in a variety of cell types, including vascular endothelial cells and neurons. These two types of cells constitutively express NOS, which then catalytically generates NO from the N-guanidino-terminal of L-arginine in the presence of oxygen, nicotinamide-adenine dinucleotide (NADPH), and reduced form flavin adenine dinucleotide (FAD). Although the endothelial type of NOS and brain type of NOS are encoded at distinct genomic sites, these NOS isoforms share similar characteristics. Their activities are strictly regulated by the levels of intracellular Ca++ and their rates of NO synthesis are of the order of seconds.

In contrast to these two constitutively expressed forms of NOS, an additional isoform of NOS has been identified that is regulated at the transcriptional level. This enzyme has therefore been termed the "inducible" isoform of NOS (iNOS). Although originally identified in macrophages, iNOS has subsequently been identified in a variety of cell types, including vascular smooth-muscle cells, vascular endothelial cells, glial cells, fibroblasts, and neurons. In contrast to the constitutively expressed isoforms of NOS, iNOS activity is independent of intracellular Ca++ concentrations and little is known about the posttranscriptional regulation of this enzyme. Because of its general lack of posttranscriptional regulation, when iNOS is stimulated it tends to produce extremely high amounts of NO. Excessive production of NO can exert injurious effects on cells by several distinct mechanisms. These mechanisms include: 1) inhibition of mitochondrial enzymes; 2) disruption of gene transcription; 3) increased oxidative injury and generation of highly reactive free radical species; and 4) increased lipid peroxidation. In addition, it has recently been reported that NOS is capable of catalyzing the production of not only NO but also other types of free radicals. Any or all of these events could have severe consequences for cellular viability. It is therefore important to characterize the conditions under which iNOS is activated and to identify the cellular elements responsible for excessive NO production.
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The types of molecules capable of triggering the production of iNOS are just beginning to be appreciated. Recent findings indicate that hemoglobin can augment the stimulatory effect on iNOS by interleukin-1β (IL-1β).41 This observation is of particular importance in the context of vascular injury after subarachnoid hemorrhage (SAH) because substantial evidence implicates red blood cell constituents in the pathophysiology of cerebral vasospasm.12,25 The present study examined the role of hemin, another product of the hemolysate, as a possible activator of NOS in vascular smooth-muscle cells. The findings demonstrate a profound stimulatory effect of hemin on NO production and indicate that this effect is mediated by the inducible isozyme of NOS.

Materials and Methods

Cell Culture

Smooth-muscle cells of rat thoracic aorta were cultured in Dulbecco’s modified Eagle’s medium plus 10% bovine calf serum in a 5% CO₂ atmosphere at 37°C as described previously.41 Aortic cells were used in this study because of the limited long-term viability of cerebrovascular smooth-muscle cells in culture. The cells were passaged weekly, and cells at six to nine passages were used. Cells were seeded into 24-well plates (50 x 10⁴ cells/well) and were grown for 3 days. The culture medium was then aspirated and replaced with 250 µl/well of minimum essential medium containing Earle’s salts, 2 mM glutamine, and 0.1% (w/vol) fatty acid–free bovine serum albumin for 24 hours. Hemin (1–100 µM) or human recombinant IL-1β (100 U/ml) was then added to the culture medium. In some experiments, an inhibitor of NOS, N⁶-nitro-l-arginine (300 µM) or an inhibitor of protein synthesis, cycloheximide (5 µg/ml) was added to the culture medium. In these experiments the inhibitors were added for 24 hours, and the concentration of l-arginine was reduced to 100 µM using a Select-Amine kit (GIBCO BRL, Grand Island, NY). In all other experiments, the concentration of l-arginine in the culture medium was 862 µM.

Hemin solutions were prepared by dissolving hemin chloride in 0.1 M NaOH, which was then diluted with culture medium to the desired concentrations. Hemin solutions were sterilized by filtration using a sterile acrodisc with a pore size of 0.2 µm (Gelman Sciences, Ann Arbor, MI).

Nitrate/Nitrite Assay

An assay that measures both nitrate and nitrite was used to estimate NO production in the smooth-muscle cells (Fig. 1). The nitrate assay is a straightforward and widely accepted method of estimating the amount of NO produced.2 Nitric oxide is a highly reactive molecule and is easily oxidized into nitrite, which is stable in an aqueous solution.13 However, in the presence of an oxidant such as hemin, nitrite tends to be oxidized into nitrate, which is not detectable using the Griess reagent-based method.15,19 Therefore, in the present study, nitrite was assayed after enzymatic reduction of nitrate into nitrite to avoid underestimation of the amounts of NO produced.15

Culture medium was decanted and centrifuged at 10,000 rpm for 5 minutes. The supernatant was removed and used for the determination of nitrate and nitrite. Nitrate was reduced to nitrite according to the method of Gilliam, et al.,13 with slight modification (Fig. 1). Briefly, a final reaction volume of 250 µl contained 100 µl of 0.14 M KH₂PO₄ buffer (pH 7.5), 75 µl of double-distilled water, 6.25 µl of 100 µM FAD, 2 µl of 12.5 mM NADPH, and 62.5 µl of specimen or standard. This solution was mixed thoroughly and allowed to stand at room temperature for 3 minutes. Then 4.25 µl of 3.5 U/ml nitrate reductase was added, and the solution was again mixed thoroughly. After 10 minutes, 250 µl of Griess reagent (1% sulfanilamide, 0.2% N-(1-naphthyl)ethylenediamine dihydrochloride, and 2% phosphoric acid) was added, and the solution was allowed to stand for 10 minutes after a further thorough mixing. The absorbance at 540 nm was read with an automated microplate reader (EL311SX; Bio Tek Instruments, Winooski, VT). The concentration of nitrate was estimated from a standard curve using sodium nitrite. In the following descriptions, the accumulated amounts of these compounds will be referred to as “nitrite/nitrate accumulation.”

Cell Density

Cell density was determined using a standard dye method.18 Briefly, the cells were washed twice with normal saline and then incubated in 0.5% crystal violet solution (water/methanol, 4:1) for 15 minutes at 37°C. The cells were washed with water three times to remove excessive dye and air-dried. The dye was eluted by acetic acid (33% vol/vol), and the absorbance at 600 nm was measured with the automated microplate reader.

Statistical Analysis

All values are expressed as mean ± standard deviation. Differences between means within each experiment were evaluated by analysis of variance (Bonferroni/Dunn). If a significant difference was demonstrated, then the unpaired Student’s t-test was used to determine which pairs of means were significantly different. Probabilities less than 5% (p < 0.05) were considered significant.

Results

Hemin-Induced NO Production

The first series of experiments examined the effect of hemin on the nitrite/nitrate accumulation in the culture medium of vascular smooth-muscle cells. Hemin (1, 10, and 100 µM) was applied to the cell culture medium and incubated for 24 hours as described above. The nitrite/nitrate accumulation was increased by hemin in a concentration-dependent manner in samples taken at the 24-hour time point (Fig. 2 left). It is possible that the increase in nitrite/nitrate accumulation could reflect an increase in the number of viable cells in the cultures treated with hemin. The density of cells was measured in control and hemin-treated cultures to evaluate this issue. As shown in Fig. 3, the density of cells was not affected in the hemin-treated cultures.

A second series of experiments examined the effect of removing hemin from the culture medium after 24 hours and testing the cells at a later time point. These experiments used the same protocol as above except that the cells were washed three times at the 24-hour time point, and fresh culture medium was added to thoroughly remove the hemin. The accumulated amount of nitrite/nitrate was then assayed another 24 hours after the washing and medium change. Even after the removal of hemin from the culture medium, the accumulated amount of nitrite/nitrate continued to increase in the treated cells (Fig. 2 right); this effect was concentration dependent.
The time course of the hemin (100 μM)-induced increase in nitrite/nitrate levels was compared with that of a well-established activator of iNOS, IL-1β (100 U/ml). After a lag time of several hours, both hemin and IL-1β increased the nitrite/nitrate accumulation in a time-dependent fashion (Fig. 4).

Effect of Inhibitors on Hemin-Induced NO Production

The delay of several hours for hemin to elevate the levels of nitrite/nitrate suggests that the transcriptionally regulated form of NOS is responsible for this effect. To further examine this issue, two different types of inhibitors were applied to the culture medium together with 100 μM hemin (Fig. 5). Both an inhibitor of NOS, Nω-nitro-L-arginine (300 μM), and an inhibitor of protein synthesis, cycloheximide (5 μg/ml), significantly inhibited hemin-induced nitrite/nitrate accumulation (Fig. 5).

Discussion

Substantial evidence indicates the involvement of red blood cell–derived substances in the pathogenesis of cerebral vasospasm after aneurysmal SAH. Although hemoglobin is considered the leading candidate in this process, its potential to induce vasoconstriction may not be strong enough to be the sole factor responsible for cerebral vasospasm. Consequently, it is important to examine other blood clot–derived factors that may directly or indi-

Fig. 2. Effects of hemin (1, 10, and 100 μM) on nitrite/nitrate accumulation in the culture medium. Values are expressed as mean ± standard deviation. Data were obtained from three separate experiments performed in duplicate. *p < 0.01; **p < 0.001; and ***p < 0.0001 compared to control. Left: Rat aortic smooth-muscle cells were incubated with hemin for 24 hours, after which time the culture medium was removed. Hemin elicited nitrite/nitrate accumulation in a concentration-dependent fashion. Right: Delayed production of nitrite/nitrate after incubation with hemin for 24 hours. Cells were treated as for the early experiment (left) but were then washed three times to remove hemin and interleukin-1β thoroughly, and incubated for an additional 24 hours in normal medium without hemin. The accumulated amount of nitrite/nitrate was then assayed.

Fig. 3. Cell density of rat aortic smooth-muscle cells was estimated using a dye method after incubation for 24 hours with hemin (1–100 μM). There were no significant changes in cell density after treatment with hemin. Values are expressed as mean ± standard deviation. Data were obtained from three separate experiments performed in duplicate.
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directly contribute to the development of cerebral vasospasm.

Hemin is an important candidate for this type of analysis. It is readily derived from methemoglobin, which is distributed widely in the subarachnoid space after SAH. In a canine model of SAH, hemin reaches concentrations of 300 μM or more in the subarachnoid clot. The concentrations of hemin used in the present in vitro study (1–100 μM) are therefore within a reasonable range relative to those achieved in vivo after SAH. The present study provides the first evidence that hemin stimulates a substantial increase in the production of NO in vascular smooth muscle and that this effect is mediated by an inducible isofom of NOS. The conclusion that NOS is responsible for the elevation of nitrite/nitrate is based on the observation that a general inhibitor of NOS (N\textsuperscript{G}-nitro-L-arginine) is capable of inhibiting this effect. The participation of the inducible isofom of NOS in this response is indicated by two findings. First, the delay to elevation of nitrite/nitrate was several hours in duration; a delay of this length is consistent with the time required for transcriptional induction. Second, inhibition of protein synthesis by cycloheximide attenuated the hemin-induced increase in nitrite/nitrate. Together, the prolonged delay to elevated NO production and the reliance on de novo protein synthesis for this effect are consistent with an induction of iNOS by hemin.

Increased production of NO by hemin represents a somewhat paradoxical outcome when considering possible pathogenetic roles for hemin in the development of cerebral vasospasm. Nitric oxide is a well-established vasodilatory substance, the production of which would presumably oppose any tendency toward vasoconstriction. However, extensive recent evidence supports a pathophysiological role for NO when high levels are produced following the induction of iNOS. As enumerated in the introduction to our article, these cytotoxic effects include the inhibition of basic homeostatic mechanisms and the production of injurious free radical species. Although the roles of free radicals and lipid peroxidation in the development of vasospasm are not well elucidated, several recent studies indicate that free radical scavengers and iron chelators are effective in limiting cerebral vasospasm. The induction of iNOS can elicit free radical generation and lipid peroxidation by generating large amounts of NO under a variety of pathophysiological conditions. Nitric oxide itself is a free radical, but its most severe cytotoxic impact might occur via the production of more potent free radical species, such as peroxynitrite and the hydroxyl radical. Other possible mechanisms through which excessive NO may aggravate vascular function include a direct inhibition of NO production by complementary NOS. The activity of the more physiological, constitutively expressed forms of NOS may thus be suppressed by NO derived from iNOS. Another possible impact may be through intercellular injury. Cytotoxic actions of NO on vascular endothelial cells have also been reported, and it is conceivable that smooth muscle–derived NO could damage endothelium-dependent vasodilatory mechanisms. A goal of future studies will be to characterize the precise mechanisms through which high concentrations of NO can aggravate vascular function.

The role of hemin in relation to other clot-derived substances also remains to be clarified. Hemoglobin is the best characterized of these substances and clearly plays a central role in the pathophysiology of vasospasm. However, with respect to the activation of iNOS, hemin and hemoglobin appear to play quite different roles. A recent study reported that hemoglobin is capable of augmenting IL-1β–induced NO production in vascular smooth-muscle cells. However, the direct stimulatory action of hemoglobin on iNOS was minimal when tested in the absence of added cytokines. In contrast, hemin is capable of potently inducing iNOS by itself. These observations indicate different roles for hemoglobin and hemin in the activation of iNOS. Because of its ability to induce iNOS independently, hemin can be considered a more direct trigger of NO-mediated free radical generation and lipid peroxidation.

In conclusion, the present study demonstrates the induction of iNOS activity by hemin in vascular smooth-muscle cells. Hemin may represent a critical trigger of SAH-induced injury to the cerebrovasculature. In addition, excessive production of NO by iNOS may contribute to vascular injury during the evolution of cerebral vasospasm after aneurysmal SAH.

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