Increased Cytochrome c–Mediated DNA Fragmentation and Cell Death in Manganese–Superoxide Dismutase–Deficient Mice After Exposure to Subarachnoid Hemolysate

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Background and Purpose—We sought to investigate the mechanisms for oxidative injury caused by subarachnoid hemolysate, a pro-oxidant.

Methods—Injection of 50 μL of subarachnoid hemolysate or saline was performed in CD1 mice (n=75), mutant mice deficient in Mn–superoxide dismutase (Sod2+/−; n=23), and their wild-type littermates (n=23). Subcellular location of cytochrome c was studied by immunocytochemistry, immunofluorescence, and immunoblotting of cellular fractions. DNA fragmentation was assessed though DNA laddering and terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end-labeling (TUNEL). Cell death was examined through basic histology.

Results—Cytochrome c immunoreactivity was present in the cytosol of neurons at 2 hours after hemolysate injection and increased by 4 hours compared with saline-injected animals (P<0.02). Cytosolic cytochrome c was more abundant in Sod2+/− mutants. DNA fragmentation was evident at 24 hours, but not 4 hours, after hemolysate injection as determined by DNA laddering and TUNEL staining (P<0.02). DNA fragmentation colocalized to cells with cytosolic cytochrome c and iron. In Sod2+/− mutants, the extent of fragmentation was increased as determined by TUNEL staining (52% increase; P<0.02) and DNA laddering (optical density 0.819 versus 0.391; P<0.01). Cell death was evident on basic histology as early as 4 hours after hemolysate injection. No cell death was evident in controls. In Sod2+/− mutants, cell death was increased by 51% compared with wild-type littermates (P<0.05).

Conclusions—These results demonstrate that subarachnoid blood products are associated with the presence of cytochrome c in the cytosol and subsequent cell death in neurons. It appears that Mn–superoxide dismutase plays a role in preventing cell death after exposure to subarachnoid blood products. (Stroke. 2001;32:506-515.)

Key Words: hemoglobin ■ iron ■ stroke, experimental ■ subarachnoid hemorrhage ■ superoxide dismutase ■ mice

Oxidative stress has been implicated in the pathogenesis of cell injury after subarachnoid blood hemorrhage.1–3 Lysis of extravasated, subarachnoid red blood cells permits the release of blood constituents, including free hemoglobin.4 Hemoglobin is an iron-containing protein that is rapidly distributed throughout the subarachnoid space and has been linked to the development of lipid peroxidation, oxidative cell membrane injury, DNA damage, and cell death.5–10

Oxidative stress in brain has been associated with superoxide production, mutagenesis of nuclear genes, irreversible and reversible DNA damage, and mitochondrial injury.11–18 Cytochrome c, a mitochondrial membrane protein, functions in the respiratory chain to transport electrons from coenzyme QH2–cytochrome c reductase to the cytochrome oxidase.19 Recent attention has focused on its relationship to DNA fragmentation.20 Injury severe enough to release cytochrome c into the cytosol has been linked to subsequent fragmentation of DNA and cell death.15 Within the cytosol, cytochrome c activates caspase-3, which has been shown to trigger apoptosis.20,21 The presence of mitochondrial Mn–superoxide dismutase (Mn-SOD) appears to attenuate cytochrome c translocation after oxidative injury. This same protein is associated with protection from DNA fragmentation and cell death.15

Recently, cell death and DNA fragmentation have been described after intracranial hemorrhage.22,23 Prior studies have suggested that some cell damage may be a direct consequence of blood toxicity and independent of intracranial pressure changes and secondary ischemia.2,24 Pretreatment with antioxidants has been shown to attenuate cellular and...
vascular damage after exposure to subarachnoid blood.\textsuperscript{2,3} Although deposition of blood products in the subarachnoid space has been associated with oxidative injury, the specific subcellular location of their action has not been defined. It is also uncertain if their effect is deleterious enough to produce mitochondrial injury and to perturb the localization of cytochrome $c$. Therefore, we decided to investigate whether cytochrome $c$ is translocated to cytosol after exposure to subarachnoid hemolysate (“heme”) before cell death. We also investigated cell injury after exposure to heme in mutant mice with a heterozygous knockout gene encoding Mn-SOD ($Sod2^{+/−}$). We hypothesized that reduced expression of Mn-SOD would be associated with cytosolic cytochrome $c$, DNA fragmentation, and cell death after exposure to subarachnoid blood products.

**Materials and Methods**

**Animal Preparation**
For the first part of the study, CD1 male mice (aged 3 months; weight, 35 to 40 g) were studied. Because homozygous knockout mutants ($Sod2^{−/−}$) showed neonatal lethality due to dilated cardiomyopathy,\textsuperscript{24} heterozygous knockout mutants ($Sod2^{+/−}$) were used for the second part of this study. The $Sod2^{+/−}$ mice with a CD1/SV129 background were backcrossed with CD1 mice for 10 generations. These mice and their wild-type (Wt) littermates with an identical genetic background were studied. In prior studies that investigated oxidative stress from focal stroke, these same $Sod2^{+/−}$ mutants had Mn-SOD activity (0.1 U/mg protein) that was approximately 50% that of their Wt littermates (0.2 U/mg protein).\textsuperscript{25} In these same animals, CuZn-SOD activity was similar (5 U/mg protein) between $Sod2^{+/−}$ mutants and Wt littermates.\textsuperscript{26} For this study, Mn-SOD activity was measured in $Sod2^{+/−}$ mutants ($n=3$) and Wt littermates ($n=3$) by a method previously described.\textsuperscript{27} The $Sod2^{+/−}$ mutants had a level of Mn-SOD activity that was 33% less than that of Wt littermates ($P<0.05$).

**Experimental Model of Blood Exposure**
All procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by Stanford University’s Administrative Panel on Laboratory Animal Care. Subarachnoid injection of heme or saline was performed in male CD1 mice ($n=75$), $Sod2^{+/−}$ mutants ($n=23$), and Wt littermates ($n=23$) by a method previously described.\textsuperscript{21} Experimental animals were anesthetized with a mixture of 68% nitrous oxide, 30% oxygen, and 2% isoflurane by mask ventilation. The rectal temperature was controlled at 37±0.5°C with a homeothermic blanket. After cannulation, 50 µL of autologous blood was withdrawn from the femoral artery and placed into a sterile container. Blood was lysed by freezing and thawing in dry ice. An equivalent volume of normal saline was then replaced intraperitoneally.

Animals were then placed into a stereotaxic frame (Kopf Instruments). The posterior scalp was incised on the midline, and the skull was exposed at the junction of the parietal and occipital bones. A small burr hole was made 2 mm to the right of midline. The heme was then placed in a sterile 1-ml syringe with a 30-gauge needle. The needle was introduced through the burr hole and placed into the subarachnoid space over the dorsal aspect of the cortex beneath the coronal suture. Heme ($n=69$) or sterile saline for controls ($n=52$) was then slowly injected over 1 minute. The needle was removed, and the burr hole was filled with sterile bone wax. The incision was then closed.

**Cytochrome c Immunocytochemistry**
At 2, 4, or 24 hours after heme or saline injection, subjects ($n=6$ for each group) were anesthetized and killed by transcardiac perfusion with heparinized 0.1 mol/L PBS (pH 7.4) followed by 3.7% formaldehyde. After endogenous peroxidase and nonspecific binding were blocked, sections (25 µm) were reacted with a rabbit polyclonal anti–cytochrome $c$ antibody (1/100, Santa Cruz Biotechnologies) for 48 hours at 4°C. The rabbit polyclonal anti–cytochrome $c$ antibody has been shown to detect cytosolic but not mitochondrial cytochrome $c$. This antibody is not able to penetrate consistently through the mitochondrial membrane; consequently, minimal immunostaining is observed in normal animals, and appearance of mitochondrial staining is distinctly different from cytosolic staining.\textsuperscript{28–30} Sections were reacted with biotin-conjugated goat anti-rabbit IgG antibody (1/300, Vector Laboratories) and visualized with the use of avidin-biotin horseradish peroxidase (ABC kit, Vector Laboratories) followed by 0.02% diaminobenzidine and 0.06% H$_2$O$_2$. The sections were counterstained with methyl green. As a negative control, some sections were incubated without primary antibody.

**Cytochrome c Immunofluorescent Double Labeling**
After reaction with the primary anti–cytochrome $c$ antibody, sections were reacted with goat anti-rabbit Fab fragments (1/50, Jackson Immunoresearch) followed by Texas red–conjugated donkey anti-goat antibody (1/100, Jackson Immunoresearch) in PBS. Sections were then incubated with a rabbit anti-neurofilament antibody (1/500, Serotec) for 48 hours at 4°C followed by a biotin-conjugated goat anti-rabbit antibody (1/200, Vector Laboratories) and fluorescein-conjugated avidin D (1/200, Vector Laboratories).

**Western Blot Analysis of Cytochrome c**
Protein extraction of both the mitochondrial and cytosolic fractions was performed as described.\textsuperscript{20,28} Animals were killed at 2 or 4 hours after heme or saline injection ($n=6$ for each group). Approximately 50 mg of neocortex was removed from the hemorrhagic or saline-injected hemisphere. Tissue was homogenized in 7 volumes of cold suspension buffer with protease inhibitors. Homogenates were centrifuged at 750g at 4°C. Supernatants were then centrifuged at 8000g at 4°C for 20 minutes. The 8000g pellets were resuspended and used as the mitochondrial fraction. Supernatants were further centrifuged at 100,000g at 4°C for 1 hour. The final cytosolic supernatants were then removed. Protein concentrations were determined by the Bradford method (Bio-Rad).

Proteins (6.2 µg of cytosolic fraction or 2.6 µg of mitochondrial fraction) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 10% to 20% Tris-glycine gel (Novex) and transferred to a polyvinylidene difluoride membrane (Novex). Western blots were reacted with the use of a rabbit polyclonal antibody against mouse cytochrome $c$ (Santa Cruz Biotechnologies) at a dilution of 1:1000 or 1 µg/mL of 20E8C12 mouse monoclonal antibody to cytochrome oxidase subunit IV (COX, Molecular Probes). Immunoblots were visualized with the use of enhanced chemiluminescence Western blotting detection reagents (Amer sham). A densitometric analysis was made on the mitochondrial fractions and cytosolic fractions from both hemorrhage and control hemispheres. The films were scanned by GS-700 imaging densitometer (Bio-Rad), and the results were quantified with the use of the Multi-analyst software program (Bio-Rad). Western blot analysis of $β$-actin was performed with the use of a mouse monoclonal antibody with horseradish peroxidase–conjugated anti-mouse IgG reagents (Amer sham).

**In Situ Labeling of DNA Fragmentation**
At 4 or 24 hours after heme or saline injection, subjects ($n=6$ CD1/saline, CD1/heme, $Sod2^{+/−}$/heme, Wt/heme, $Sod2^{+/−}$/saline, Wt/saline) were anesthetized and killed by transcardiac perfusion. Sections (25 µm) were reacted with Proteinase K (20 mg/L, Boehringer Mannheim) in 0.01 mol/L Tris-HCl (pH 8.0) and equilibrated with 1X terminal deoxynucleotidyl transferase (TdT) buffer (Life Technologies) for 15 minutes. Sections were then reacted with TdT enzyme (375 U/mL buffer, Life Technologies) and
bionylated 16–dUTP (60 nmol/mL buffer, Boehringer Mannheim) in 1× TdT buffer at 37°C for 60 minutes. Slides were washed with 2× SSC (150 mmol/L sodium chloride, 15 mmol/L sodium citrate, pH 7.4) followed by 2% bovine serum albumin. Staining was visualized with the use of avidin–biotin horseradish peroxidase solution (ABC kit, Vector Laboratories) followed by 0.025% diaminobenzidine, 0.04 mol/L nickel sulfate, and 0.075% H2O2 in 0.175 mol/L sodium acetate. Sections were counterstained with methyl green.

Cytochrome c Immunostaining With In Situ Double Labeling of DNA Fragmentation
Adjacent sections were removed from subjects killed at 24 hours after heme or saline injection (n=6 each group). Sections were stained first for cytochrome c and subsequently for DNA fragmentation with the techniques described above.

Iron Histology With In Situ Double Labeling of DNA Fragmentation
To clarify the role of iron deposition, adjacent sections were removed from subjects killed at 24 hours after heme or saline injection (n=6 in each group). Sections were washed in deionized water and reacted with 0.024 mol/L potassium ferrocyanide (Sigma) in 0.37% HCl for 15 minutes. Sections were then reacted with 0.025% diaminobenzidine, 0.075% H2O2, and 0.1% Tween-20 for 15 minutes. Sections were then stained for DNA fragmentation.

Gel Electrophoresis
At 4 or 24 hours after heme or saline injection, subjects (n=6 CD1/saline, CD1/heme, Sod2+/−/heme, Wt/heme, Sod2+/−/saline, Wt/saline) were anesthetized and killed by decapitation. Approximately 40 to 50 mg of neocortex was dissected from the region underlying the area of injection. Samples were incubated in 0.6 mL of lysis buffer (0.5% SDS, 0.01 mol/L Tris-HCl, and 0.1 mol/L EDTA) with 0.6 mg Proteinase K at 55°C for 20 hours. The DNA was extracted with equal volumes of phenol and phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated in 0.2 mol/L sodium chloride in 100% ethanol at −80°C for 24 hours. DNA was resuspended in DNase-free H2O (Sigma Chemical), and concentration was measured with the use of To-Pro-1 dye (Molecular Probes).

Gel electrophoresis for detecting DNA laddering was performed as described.24 Before electrophoresis, 1 μg of DNA was incubated with 50 μg/L DNase-free RNase (Boehringer Mannheim) and then reacted with Klenow enzyme and dNTP in 1× Klenow buffer (Trevigen). Samples were mixed with loading buffer and subjected to electrophoresis on a 1.5% agarose gel. The gel was washed sequentially with 0.25 mol/L HCl, 0.4 mol/L NaOH/0.8 mol/L NaCl, and 0.5 mol/L Tris buffer, pH 7.5. DNA was transferred to a nylon membrane overnight in 10× SSC and reacted with streptavidin–horseradish peroxidase conjugate (Trevigen) for 30 minutes. Bands were visualized by the chemiluminescence method with the use of PeroxyGlow (Trevigen) followed by exposure to chemiluminescence film. Bands were compared by densitometry, as described above.

Histological Assessment of Cell Death
At 4 or 24 hours after heme or saline injection (n=6 CD1/saline, CD1/heme, Sod2+/−/heme, Wt/heme, Sod2+/−/saline, Wt/saline), subjects were anesthetized and killed. After transcardiac perfusion fixation, 25-μm sections were mounted on slides and dried overnight. Sections were then dehydrated, delipidized, rehydrated, and stained with cresyl violet for 5 minutes. Sections were dehydrated and coverslipped.

Statistical Analysis
For TdT–mediated dUTP–biotin nick end-labeling (TUNEL) staining and basic histology, 12 serial sections were taken at 300-μm intervals beginning at the coronal suture and continuing posteriorly.

This encompassed a region of 3.6 mm adjacent to the area of heme injection. Counts of TUNEL–positive cells and dead cells were then performed by a blinded reviewer using a light microscope equipped with a 250×250-μm grid. Counts were completed for all 12 sections in each animal. Thereafter, the codes were broken, and average cell counts for Wt and Sod2+/− mutants were compared with an unpaired Student’s t test (2-tailed). For assessment of mortality and evaluation of the numbers of mice with cytochrome c–immunopositive cells, comparisons were made with a 2-tailed Fisher exact test. Finally, for densitometric analysis, average optical densities were obtained from Sod2+/− mutants (n=6) and Wt littermates (n=6). Average optical densities were also obtained from heme- (n=6) and saline-injected (n=6) subjects for cytochrome c and COX analysis. Results were compared with an unpaired Student’s t test (2-tailed). Significance was determined by a P value <0.05.

Results
After injection of heme, an 8% mortality rate was observed. Differences in survival were not statistically significant compared with saline-injected animals or between Sod2+/− mutants and Wt littermates. With heme injection, subarachnoid blood was evident in the region overlying the dorsal, right neocortex beneath the parietal bone. The appearance of the heme was similar in Sod2+/− mutants and Wt mice (Figure 1) and was consistent with prior reports using a similar method.23

Cytochrome c Immunocytochemistry, Immunofluorescence, and Immunoblotting
Minimal cytochrome c immunoreactivity was detected in saline-injected animals at 2, 4, and 24 hours (Figure 2A), a result consistent with the minimal immunoreactivity of mitochondrial cytochrome c.26,28 Cytochrome c immunoreactivity was only evident in sparse, small fragments, consistent with the occasional staining of mitochondria. This result compares with prior studies and is consistent with the fact that primary antibodies do not usually penetrate through mitochondrial membranes.28,29 Sparse immunoreactivity was evident at 2 hours when the primary antibody was eliminated, suggesting some immunoreactivity of the secondary antibody (Figure 2B). However, after heme injection, significant cytosolic cytochrome c immunoreactivity was observed in the ipsilateral neocortex (5/6 animals at 2, 4, and 24 hours; Figure 2C through 2E). These results were significant when compared...
with controls for each interval ($P<0.02$). Double-immunofluorescent labeling demonstrated these cells to be neurons (Figure 2F and 2G). Immunostaining decreased with distance from the injection site.

After saline injection, COX and cytochrome c immunoblots of mitochondrial fractions demonstrated positive bands characteristic of these proteins, while no positive bands were present in the cytosol (Figure 3A). After either heme or saline injection, COX immunoblotting was observed in the mitochondrial fractions but not in the cytosolic fractions (Figure 3A). The intensity (Figure 3B) of the mitochondrial COX bands did not differ between heme and saline. In contrast, the intensity of mitochondrial cytochrome c immunoblotting (optical density $=0.146 \pm 0.013$; Figure 3B) was significantly diminished after heme injection compared with the saline-injected animals (optical density $=0.182 \pm 0.028$; $P<0.05$).

After heme injection, cytochrome c immunoblotting was evident in the cytosolic fractions (Figure 3C and 3E). When compared with controls at 2 and 4 hours (optical density $=0.105 \pm 0.010$ and $0.102 \pm 0.017$, respectively), the degree of cytochrome c immunoblotting (Figure 3D) was significant at 2 (optical density $=0.241 \pm 0.052$; $P<0.02$) and 4 hours (optical density $=0.396 \pm 0.165$; $P<0.02$).

Cytosolic cytochrome c was also observed in Sod2+/− mutants after heme injection (Figure 3E). The optical density of these immunoblots increased (Figure 3D and 3E) in Sod2+/− mutants (optical density $=0.641 \pm 0.221$) compared with Wt littermates at 4 hours (optical density $=0.351 \pm 0.145$; $P<0.05$). β-Actin immunoblotting of cytosolic fractions showed bands of similar intensity between saline and heme subjects (Figure 3C and 3E).

DNA Fragmentation
DNA fragmentation was analyzed with the in situ labeling of DNA breaks (TUNEL) at 4 and 24 hours after heme or saline injection. DNA fragmentation was not evident after injection of saline at 4 or 24 hours (0/6 in each group; Figure 4A). At 4 hours after heme exposure, no TUNEL-positive cells were observed adjacent to the area of injection (Figure 4B). At 24 hours after heme exposure, DNA fragmentation was observed in the ipsilateral neocortex in 5 of 6 subjects (Figure 4C). TUNEL-positive cells were observed adjacent to the site of heme injection and had morphologies consistent with both apoptosis and necrosis.32,33 The difference in the number of TUNEL-positive subjects at 4 and 24 hours after SAH (0/6 versus 5/6; $P<0.02$) was statistically significant and suggested that the onset of DNA fragmentation was a delayed phenomenon.

DNA fragmentation was not evident after injection of saline in either Sod2+/− mutants or Wt mice (Figure 4D). DNA
Fragmentation was observed in the ipsilateral neocortex of Sod2+/− mutants and Wt mice after injection of heme (Figure 4E and 4F). The abundance of TUNEL-positive cells differed significantly between Sod2+/− mutants and Wt mice (Figure 4G). Sod2+/− mutants had a significantly greater number of TUNEL-positive cells per square millimeter (±SD) (1052 ± 247) than Wt mutants (692 ± 154; P, 0.02).

Colocalization of fragmented DNA and either cytochrome c or cytosolic ferric iron was investigated 24 hours after heme injection through double staining. At 24 hours, cytochrome c–immunopositive cells were also TUNEL-positive (Figure 4H). Additionally, if cells were stained for intracellular ferric iron with Prussian blue,31 the accumulation of intracellular iron occurred primarily in TUNEL-positive cells (Figure 4I). However, some cells were TUNEL-positive without iron, while others stained positive for iron but not TUNEL (Figure 4I).

To investigate further the time course of DNA fragmentation as characterized by intranucleosomal DNA fragments, we analyzed by gel electrophoresis DNA extracted from neocortex 4 or 24 hours after heme or saline injection. No DNA laddering was evident in the saline-injected animals at 24 hours, nor was it present 4 hours after heme injection (Figure 5A). DNA laddering was present in the neocortex 24 hours after heme injection (Figure 5A). In Wt mice and Sod2+/− mutants, DNA laddering was not evident 24 hours after injection of saline (not shown). DNA laddering was present in neocortex 24 hours after heme injection in Wt mice and Sod2+/− mutants (Figure 5B). The intensity of the DNA laddering ±SD was significantly greater (Figure 5C) in Sod2+/− mutants (optical density = 0.819 ± 0.318) than in Wt littermates (optical density = 0.391 ± 0.055; P < 0.01).

**Cell Death**

The presence of cell death was evaluated by histological assessment after cresyl violet staining. Irreversibly damaged neocortical cells demonstrated shrunken or condensed nuclei,
often with a prominent nucleolus. After injection of saline, no cell death was evident in neocortex in either Wt mice or Sod2+/− mutants (Figure 6A and 6B). Cell death was evident at 4 hours after injection of heme in Wt mice (Figure 6C). Cell death was abundant at 24 hours in both Wt mice and Sod2+/− mutants. Necrotic cells were evident closest to the site of heme injection (Figure 6C through 6E). The abundance of dead cells per square millimeter (±SD) was significantly greater in Sod2+/− mutants (1603±466) than in Wt littermates (1057±364; P<0.05; Figure 6F).

Discussion

Cytochrome c Immunostaining and Immunoblotting

After subarachnoid injection, blood products have been observed to disseminate throughout the brain within a few hours and to be sequestered by neurons and microglia. Intracranial blood products have been associated with the development of DNA fragmentation in neocortical regions of highest blood concentration. In our study cytochrome c immunoreactivity was observed at 2 hours in neocortical neurons closest to the site of heme injection. Because cytochrome c is a water-soluble protein, the diffuse intracellular immunoreactivity was presumably a consequence of protein dispersion throughout the cytosol after mitochondrial perturbation. Cytochrome c immunoreactivity was not observed in the contralateral hemisphere after heme injection, nor was it apparent in the brains of saline-injected animals. This result was consistent with recent observations that, with the use of current immunocytochemical methods, mitochondrial cytochrome c itself does not produce immunopositivity.

The cytochrome c protein is synthesized in the endoplasmic reticulum but becomes associated with the mitochondria. Immunoblotting of subcellular fractions demonstrated the cytosolic presence of cytochrome c after heme exposure. Presumably, the cytosolic appearance of the cytochrome c protein could have arisen from impaired transport or increased synthesis after heme exposure. Although the endoplasmic reticulum and ribosomes were removed by ultracentrifugation and the increase in cytosolic cytochrome c was matched by a decrease in mitochondrial cytochrome c, a distinct possibility existed that protein transport to the mito-

Figure 4. TUNEL staining of mouse neocortex. No TUNEL-positive cells were observed after injection of saline (A) or 4 hours after injection of heme (B). TUNEL-positive cells were observed at 24 hours after injection of heme (C, arrows), with some TUNEL-positive cells (C, arrowheads) being larger and paler in staining—an appearance consistent with necrosis. No positive cells were observed in the Sod2+/− mutants after injection of saline (D). At 24 hours after injection of heme, TUNEL-positive cells were observed in Wt littermates (E, arrows), with the quantity of TUNEL-positive cells (F, arrows) significantly increased in Sod2+/− mutants. Counts of TUNEL-positive cells (G) demonstrated a significant (P<0.02) increase in Sod2+/− mutants (1052±297) vs Wt littermates (692±154). Double staining for DNA fragmentation and either cytochrome c (H) or iron (I) revealed colocalization (arrows in H, I). Some cells that stained for iron (I, arrowheads) were not TUNEL-positive. Some TUNEL-positive cells (I, small arrowheads) did not stain for iron. Bar=30 μm (A through C), 25 μm (D through F, I), and 10 μm (H).
chondria was impaired and that such impairment accounted for the presence of the cytosolic cytochrome c. Certainly, disruption of the mitochondrial membrane could have permitted cytochrome c to enter the cytosol. However, a severe disruption of the mitochondrial membrane presumably would have allowed COX to enter the cytosol, and our results with COX immunoblotting did not support this hypothesis. The last possibility is that cytochrome c was translocated to the cytosol of neurons after exposure to heme. Exploring the specific mechanism by which oxidative stress increases cytosolic cytochrome c will form the basis for further studies.

TUNEL Staining and DNA Electrophoresis
In this study we primarily observed a pattern of TUNEL staining consistent with the intranucleosomal DNA fragmentation of apoptosis. TUNEL-positive cells were observed 24 hours after heme but not at 4 hours. Prior studies have demonstrated that the presence of TUNEL-positive cells and DNA laddering in the brain does not necessarily equate with archetypal apoptosis, although their presence is consistent with some form of DNA fragmentation. TUNEL-positive cells may also represent cell death by necrosis, albeit with a different appearance. In this study some TUNEL-positive cells were indeed observed that were consistent with necrosis.

The presence of DNA laddering on gel electrophoresis, however, suggested that most TUNEL-positive cells in neocortex had undergone DNA fragmentation in a pattern consistent with apoptosis. After we used both of these methods to examine DNA fragmentation, it was evident that this phenomenon did not occur early but was rather delayed in onset after both heme exposure and the presence of cytochrome c in the cytosol.

DNA Fragmentation and Mn-SOD
Recent evidence has shown DNA fragmentation to be linked to elevated superoxide production in mitochondria. One of the principal scavengers of this anion is Mn-SOD. Reduction of its expression has been associated with an increase in DNA fragmentation and cell injury after focal ischemia. In our study reduction of Mn-SOD expression was associated with the presence of cytochrome c in the cytosol, DNA fragmentation, and cell death.

After subarachnoid injection, blood products are rapidly distributed throughout the brain, and hemoglobin is sequestered by neurons and microglia. Hemoglobin has been linked to cell injury and oxidative stress. DNA fragmentation has been observed in areas of highest blood concentration. In our study DNA fragmentation was associated with the pres-
ence of both ferric iron and cytochrome c in the cytosol. It is conceivable that the sequestration of blood products by the brain could directly produce significant mitochondrial oxidative stress. Superoxide anions may form the toxic hydroxyl radical through the Haber-Weiss reaction in the presence of hydrogen peroxide and iron.36 The hydroxyl radical and oxidative stress have been shown to mediate DNA damage.8,17,18 Decreased levels of Mn-SOD may have permitted greater superoxide production and, in the presence of iron, greater hydroxyl radical formation.

Cell Death and Mn-SOD
In addition to the degree of DNA fragmentation, overall cell death was significantly increased in Sod2+/− mutants compared with Wt littermates after exposure to subarachnoid heme. Cell death may arise as a consequence of necrosis or DNA fragmentation after an oxidative insult.32 Reactive oxygen species have been implicated in both pathways.12,17,18,25,37 As described above, it was possible that superoxide in the presence of the iron carried by heme may have formed the reactive hydroxyl radical as a mediator of cell death.36

Another possible route to cell death and DNA damage may have been through formation of peroxynitrite from nitric oxide and superoxide.12,17,38 Peroxynitrite may damage cells through the formation of the hydroxyl radical and nitrogen dioxide, which may attack endothelial membranes.38 Diminished levels of Mn-SOD may have permitted increased formation of peroxynitrite, which would ultimately have led to DNA damage, DNA fragmentation, and cell death.12

In the endothelium, it was also possible that increased formation of peroxynitrite may have reduced the amount of

Figure 6. Histological assessment of neocortical cell death. Normal neurons were evident (arrowheads) after saline injection in Wt (A) and Sod2+/− mutants (B). At 24 hours (C through E) after injection of heme, necrotic cells (C, Necrosis) were numerous in the regions of neocortex adjacent to the subarachnoid heme (C, arrows). Normal neurons were evident (C, arrowheads) at a greater distance from the heme. Necrotic cells were present in Wt littermates (D, arrows) and Sod2+/− mutants (E, arrows). Overall cell death was less in Wt mice, with some cells surviving (D, arrowheads). Quantification of dead cells (F) revealed a significant (P<0.05) difference between Wt littermates (1057±364) and Sod2+/− mutants (1603±466). Bar=20 μm (A, B, D, E); bar=100 μm (C).
nitric oxide available for endothelial relaxation. This mechanism could have exacerbated vasospasm and produced secondary ischemia, especially since heme has been a known spasmogen.\textsuperscript{39} It remains uncertain how blood products begin to increase mitochondrial reactive oxygen species. Investigation of the specific mechanisms will form the basis for future studies.

Reduced expression of Mn-SOD may also have exacerbated brain injury after heme exposure through glutamate neurotoxicity. Intracranial blood has been associated with elevated levels of glutamate in regions around the hematoma.\textsuperscript{40} Excitotoxicity has been associated with increased mitochondrial reactive oxygen species, mitochondrial injury, necrosis, and apoptosis.\textsuperscript{12,13,37} Consequently, after exposure to heme, the glutamate neurotoxicity cascade could have developed with subsequent superoxide formation, cell death, and DNA fragmentation. It is conceivable that blocking the excitotoxicity cascade may protect against cell injury after exposure to high concentrations of blood products. Exploring this mechanism of neuroprotection will form the basis for future studies.

In summary, our results have demonstrated that cytochrome \(c\) is present in the cytosol of neurons as early as 2 hours after exposure to heme and that such presence precedes cell death by DNA fragmentation. Reduction of Mn-SOD increases the abundance of cytochrome \(c\) in the cytosol, DNA fragmentation, and overall cell death after exposure to subarachnoid heme.

**Acknowledgments**

This study was supported by a Bayer Research Fellowship (Dr Matz) and in part by grants NS14543, NS25372, NS36147, and NS38653 (Dr Chan). Dr Chan is a recipient of a Javits Neuroscience Investigator Award.

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*Stroke*. 2001;32:506-515
doi: 10.1161/01.STR.32.2.506

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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