Human Serum Albumin and its N-Terminal Tetrapeptide (DAHK) Block Oxidant-Induced Neuronal Death

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Background and Purpose—Studies using animal models of stroke have shown that human serum albumin (HSA) significantly ameliorates cerebral ischemic injury after both transient and permanent ischemia, even when administered after the onset of ischemia or reperfusion. The mechanism of this effect remains uncertain, and prior studies suggest both indirect hemodynamic and direct cytoprotective effects. HSA is a potent antioxidant, in part because of its strong copper-binding capacity. Here we examined the effect of HSA on oxidant-induced neuronal death in a cortical cell culture system.

Methods—Murine cortical cultures were exposed to oxidative stress generated by hydrogen peroxide and by a mixture of copper plus ascorbic acid. We examined the ability of HSA and a tetrapeptide occupying its N-terminus (DAHK) to prevent neuronal death after these challenges.

Results—H₂O₂ and CuCl₂/ascorbic acid were used at concentrations that, in the absence of HSA, killed >90% of the neurons. HSA provided complete protection at a concentration of 37.5 μmol/L and 50% protection at 3.75 μmol/L. The copper-binding tetrapeptide DAHK had nearly identical potency and efficacy. HSA and DAHK were also equally effective in preventing neuronal death induced by CuCl₂/ascorbic acid.

Conclusions—HSA has potent antioxidant properties, probably due to binding of copper and other transition metals. HSA extravasation into ischemic brain may provide neuroprotection by limiting metal-catalyzed oxidant stress. The tetrapeptide DAHK may be an effective, small-molecular-weight alternative to HSA as a therapeutic agent for stroke. (Stroke. 2004;35:590-595.)

Key Words: albumins ■ antioxidants ■ copper ■ ischemia ■ neurons
tetrapeptide or HSA itself. It was recently demonstrated that a novel synthetic analogue of DAHK could improve recovery of rat hearts from an ischemia/reperfusion insult, suggesting that this approach may similarly be useful in the treatment of stroke.

We hypothesized that the antioxidant properties of this 4-amino acid terminus of HSA might explain, in part, its neuroprotective effects after ischemia/reperfusion injury in brain. To test this hypothesis, we utilized HSA and DAHK in a neuronal cell culture model of oxidative injury. Both HSA and DAHK were neuroprotective in cell culture models of hydrogen peroxide– and copper ascorbate–induced neurotoxicity.

Materials and Methods
HSA was obtained from Centeon L.L.C. as a 25% solution, and DAHK was obtained from Genemed Synthesis Incorporated. All other reagents were obtained from Sigma-Aldrich, except where otherwise noted. Adventitious metals were removed from HSA by applying it to a column of iminodiacetic acid attached to cross-linked polystyrene, after which it was reconcentrated to a 25% solution.

Cell Cultures
The studies were conducted in accordance with National Institutes of Health guidelines and with the use of protocols approved by the local institutional committee on animal studies. Mice were anesthetized with isoflurane before being killed for harvesting of brains. Cortical cell cultures were prepared in a 2-step process, which has previously been described in detail. Astrocytes were plated at 1.5 × 10^5 cells per well. Neurons were plated on the astrocyte layers at a density of 6 × 10^5 cells per well.

Experimental Procedures
Experiments were begun by replacing the culture medium with a balanced salt solution (BSS), as previously described. The pH of the BSS was adjusted to pH 7.2 and during equilibration in a 5% CO_2 atmosphere. Osmolarity was measured with a Wescor vapor pressure osmometer and adjusted with H_2 O or NaCl when necessary to achieve 280 to 320 mOsm. Test compounds were prepared as ×100 stock solutions in distilled deionized water and were diluted to working concentrations in BSS before use. The test compounds were added to the cultures in BSS, and the cultures were then replaced in working concentrations in BSS before use. The resulting concentration-response curve showed a threshold effect on neuronal survival (Figure 1), similar to previous reports. Exposure to 100 μmol/L caused near-complete neuronal death, and this concentration was used for all subsequent studies.

We evaluated the efficacy of HSA as a neuroprotective agent by adding various concentrations of HSA to the culture simultaneously with H_2 O_2. HSA had a dose-dependent neuroprotective effect, with neuronal death reduced to a level comparable to control conditions at concentrations of ≥15 μmol/L (Figure 2A). To determine whether the chelating tetrapeptide DAHK would also prevent neuronal death resulting from H_2 O_2, cultures were incubated with 100 μmol/L H_2 O_2 in the presence of a range of DAHK concentrations. These studies showed a potent, dose-dependent effect of DAHK on H_2 O_2-induced neuronal death (Figure 2B). Photomicrographs showing the effect of HSA on H_2 O_2-induced neuronal death are shown in Figure 3.

We tested the ability of HSA to protect neurons against the mixture of 25 μmol/L CuCl_2 and 50 μmol/L ascorbic acid (Figure 4), which in oxygenated solutions generates oxygen-derived free radical species. In the absence of HSA, this exposure killed >95% of the neurons, but in the presence of 150 μmol/L HSA neuronal death was reduced to control values. HSA has a high-affinity binding site for copper and other transition metals at its N-terminus in the form of a DAHK tetrapeptide. Synthetic DAHK tetrapeptide alone also completely blocked Cu/ascorbic acid–dependent neurotoxicity when added at concentrations of ≥50 μmol/L, although the potency of DAHK was >100-fold greater than HSA when the concentrations of both were expressed as...
percent weight/volume. H₂O₂ requires interaction with a transition metal to produce reactive oxygen species.²⁴

The antioxidant effect of HSA could, in principle, occur in either the extracellular or the intracellular compartments, since HSA can be internalized by neurons under some conditions.²⁵ As a possible way to determine the compartment in which the neuroprotective effect of HSA was exerted, we assessed the ability of HSA to prevent N-methyl-D-aspartate (NMDA)–induced neuronal death, a process that is mediated in part by intracellular production of oxygen free radicals and is blocked by cell-permeant oxygen free radical scavengers.²⁶,²⁷ HSA had no effect on NMDA neurotoxicity. This suggests that the action of HSA occurs in the extracellular space (Figure 5).

![Figure 2. A. HSA decreased H₂O₂-induced neuronal death. The cultures were simultaneously treated with 100 μmol/L H₂O₂ plus various concentrations of HSA for 60 minutes. Twenty-four hours after treatment, neuronal death was assessed by propidium iodide staining. n=24 to 30, pooled from 6 independent experiments. **P<0.01. B. DAHK decreased H₂O₂-induced neuronal death. Cultures were exposed to H₂O₂ plus various concentrations of DAHK for 60 minutes. Neuronal death was assessed 24 hours after treatment. n=16 to 20, pooled from 4 independent experiments. **P<0.01.](image)

To test whether it is the DAHK moiety of HSA that is primarily responsible for its antioxidant effects, we compared HSA with several other proteins that do not contain this tetrapeptide at the N-terminus: lactalbumin, γ-globulin, bovine serum albumin, and casein. Somewhat surprisingly, each of these proteins also protected neurons against H₂O₂ toxicity with potencies roughly similar to that of HSA (Figure 6).

**Discussion**

These results show that the neurotoxic oxidant stress induced by either hydrogen peroxide or copper/ascorbic acid can be blocked by HSA and by a tetrapeptide that is the same sequence as the N-terminus of HSA. These results may be relevant to the recent series of articles that describe the protective effect of HSA in animal models of stroke and traumatic brain injury.²⁻⁵,⁹

In vivo, the concentration of HSA in cerebrospinal fluid is approximately 3.7 μmol/L.²⁸ This concentration of HSA produced an approximately 40% reduction in H₂O₂-induced neuronal death under the conditions used in the cell culture studies described here. Additional HSA may enter the brain from the plasma compartment after stroke through an open blood-brain barrier.²⁵ Since the concentration of HSA in serum is approximately 588 μmol/L,²⁹ even a small movement of serum proteins across the blood-brain barrier could substantially raise HSA concentrations in the extracellular space surrounding postischemic neurons and increase resistance to oxygen free radicals in the extracellular space.
For the purpose of testing the neuroprotective effects of HSA and DAHK in cultured neurons, 2 different models of oxidant-mediated neuronal injury were developed. The first uses hydrogen peroxide as the stressor, and the second uses a copper/ascorbic acid–driven free radical–generating system. Both oxidants were used under conditions that, in the absence of HSA or DAHK, killed nearly 100% of the neurons in the cultures. Copper-ascorbic acid–driven stress involves a transition metal, and it is almost certain that the hydrogen peroxide neurotoxicity also requires transition metals. In the absence of metals, hydrogen peroxide is extremely stable, with a calculated half-life for its uncatalyzed unimolecular homolysis at 30°C of $10^4$ years. H₂O₂ interaction with organic molecules requires transition metals, and chelation of transition metals prevents this interaction.

Transition metals (in particular, iron and copper) are capable of cycling between their reduced and oxidized states and, in the process, generating an electron that can create a free radical. In many cases, transition metals themselves may be bound by a lipid, protein, or nucleic acid molecule, and a
free radical causes site-specific damage at or near its binding site. It is this type of activity that is most likely responsible for copper-dependent cell death in culture. If copper and/or iron is decompartmentalized by ischemia/reperfusion, the neurotoxic effects may be accelerated, and the presence of HSA or its N-terminal tetrapeptide may prevent its binding to sites where it could contribute to cellular injury. Alternatively, normal levels of transition metals in the extracellular space may have no deleterious effects under normal conditions but may become highly deleterious in the presence of H$_2$O$_2$, formed by the superoxide generated during ischemia/reperfusion.

The N-terminus DAHK of HSA is not the only amino acid sequence that can bind transition metals or copper specifically. In the present studies DAHK was found to be equipotent with HSA in preventing H$_2$O$_2$ or copper/ascorbic acid neurotoxicity, despite the fact that HSA is 126-fold larger than DAHK. This suggests that the DAHK N-terminal tetrapeptide is the primary locus of HSA antioxidant activity. However, the other proteins examined had effects similar to those of HSA, despite absence of the DAHK domain. It is possible that relatively weak interactions between sulfhydryl groups and/or amino acids (particularly tryptophan or histidine) on these proteins and transition metals produce a chelating effect that, in aggregate, is comparable to that achieved with HSA or DAHK. The relatively high-affinity, nonspecific binding of copper to proteins has been previously reported to inhibit its capacity to generate hydroxyl radicals, which may explain in part the neuroprotective effects of proteins other than HSA. Regardless of the mechanism of this effect, these results suggest that other proteins may also contribute to brain antioxidant effects during blood-brain barrier breakdown. This possibility has not been tested in vivo.

In summary, we have demonstrated that HSA and its N-terminal tetrapeptide DAHK can block oxidant-driven neuronal injury produced with the use of 2 different oxidant-generating systems: hydrogen peroxide and copper/ascorbic acid. The fact that the peptide and HSA can block the neurotoxicity of the latter generating system in a stoichiometric fashion implies that they are both binding copper and can stop its redox cycling. The efficacy of DAHK in the hydrogen peroxide-driven system suggests that copper is also involved in oxidant-driven neurotoxicity in vitro and may be involved in tissue injury after ischemia and reperfusion in vivo. DAHK may be a useful alternative to HSA for the treatment of stroke.

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