Oxidative Stress in Rats With Heatstroke-Induced Cerebral Ischemia

Chih-Ya Yang, MS; Mao-Tsun Lin, DDS, PhD

Background and Purpose—Heatstroke is associated with cerebral ischemia as well as increased levels of interleukin-1β, dopamine, and glutamate in the brain. These factors are known to increase free radical production. This study attempted to ascertain whether an excessive accumulation of cytotoxic free radicals in the brain and oxidative stress can occur during heatstroke.

Methods—Urethane-anesthetized rats underwent instrumentation for the measurement of mean arterial pressure, cerebral blood flow, neuronal damage score, and colonic temperature. Rats were exposed to heat stress (ambient temperature, 42°C) until mean arterial pressure and cerebral blood flow began to decrease from their peak levels, which was arbitrarily defined as the onset of heatstroke. Controlled rats were exposed to 24°C. Concentrations of dihydroxybenzoic acid, lipid peroxidation, rate of O₂⁻/H₂O₂ generation, superoxide dismutase, and catalase activity of the brain or other vital organs were assessed during heatstroke.

Results—The values of mean arterial pressure and cerebral blood flow after heatstroke onset were all significantly lower than those in control rats. However, the values of colonic temperature, dihydroxybenzoic acid levels in the striatum, and neuronal damage score were greater. The extent of lipid peroxidation in the brain and the rate of O₂⁻ generation in the brain, liver, and heart were all greater in rats after heatstroke onset. In contrast, the values of total superoxide dismutase in the brain, liver, and heart and the catalase activity in the brain were lower.

Conclusions—Taken together, these results indicate that hydroxyl radicals mediate cerebral ischemic injury associated with heatstroke. (Stroke. 2002;33:790-794.)

Key Words: cerebral ischemia • free radicals • heatstroke • neuronal damage • oxidative stress • rats

Oxygen free radicals, including superoxide anion, hydrogen peroxide, and hydroxyl radical, form during univalent reduction of oxygen to water. Normally, free radical production is low, and the organism can neutralize, metabolize, or substrate the toxic effects by free radical scavengers such as superoxide dismutase (SOD) and catalase. Nevertheless, under some pathophysiological conditions, the oxygen radicals accumulate and impair cells. Oxidative stress is caused by the imbalance between production of pro-oxidants and the antioxidant defenses.

We have performed several studies in a rat heatstroke model, showing that heatstroke is associated with cerebral ischemia, higher levels of interleukin-1β, and increased release of dopamine and glutamate in the brain. These factors are known to increase free radical production. It is not unexpected that an excessive accumulation of cytotoxic free radicals in the brain and oxidative stress can occur during the development of heatstroke.

To test this hypothesis, experiments were performed in the present study to detect in vivo hydroxyl radical production, extent of lipid peroxidation, total SOD, and catalase activity in the brain and rate of superoxide generation of submitochondrial particles in the brain of rats suffering from heatstroke compared with normothermic controls.

Materials and Methods

Experimental Animals
Adult Sprague-Dawley rats (weight, 300±15 g) were obtained from the Animal Resource Center of the National Science Council of the Republic of China (Taipei, Taiwan). The animals were housed 4 in a group at an ambient temperature of 22±1°C, with a 12-hour light/dark cycle. Pelleted rat chow and tap water were available ad libitum. All protocols were approved by the Animal Ethics Committee of the National Yang-Ming University School of Medicine. Adequate anesthesia was maintained to abolish the corneal reflex and pain reflexes induced by tail pinching throughout all experiments (approximately 8 hours) after a single intraperitoneal dose of urethane (1.4 g/kg body wt). At the end of the experiments, control rats and any rats that had survived heatstroke were killed with an overdose of urethane.

Surgery and Physiological Parameter Monitoring
The right femoral artery and vein of rats were cannulated with polyethylene tubing (PE 50), under urethane anesthesia, for blood
maintained at approximately 36.5°C with the use of an electric thermal mat before the start of the experiments.

**CBF Monitoring**

Local CBF in the corpus striatum was monitored with a Laserflo BPM2 laser-Doppler flowmeter (Vasemetics). A 24-gauge stainless steel needle probe (diameter, 0.58 mm; length, 40 mm) was inserted into the right corpus striatum with the following coordinates: anterior, 0.2 mm; lateral, 3.4 mm from the midline; and horizontal, 5 mm from the top of the skull.

**Neuronal Damage Score**

At the end of the experiments, the brain was removed, fixed in 10% neutral buffered formalin, and embedded in paraffin blocks. Serial (10-μm) sections through the frontal cortex, striatum, hippocampus, or hypothalamus were stained with hematoxylin and eosin for microscopic evaluation. The extent of cerebral neuronal damage was scored on a scale of 0 to 3, modified from the grading system of Pulinselli et al.,16 in which 0 is normal, 1 indicates that approximately 30% of the neurons are damaged, 2 that approximately 60% of the neurons are damaged, and 3 that 100% of the neurons are damaged. Each hemisphere was evaluated independently without the examiner knowing the experimental conditions.

**Extracellular Hydroxyl Radical Monitoring**

A microdialysis probe (CMA20) with a 4-mm-long dialysis membrane was vertically implanted into the right striatum with the following coordinates: anterior, 0.2 mm; lateral, 3.4 mm; horizontal, 7.5 mm. The concentrations of hydroxyl radicals were measured by a modified procedure based on the hydroxylation of sodium salicylate by hydroxyl radicals, leading to the production of 2,3-DHBA and 2,5-DHBA.11–13 These 2 compounds in dialysates were then measured by high-performance liquid chromatography with electrochemical detection. A Ring- er’s solution (0.860 g NaCl, 0.030 g KCl, 0.033 g CaCl₂ per 100 mL) containing 2 mmol/L sodium salicylate was perfused through the microdialysis probe at a constant flow rate (1.2 μL/min). After 2 hours of stabilization, the dialysates from the striatum were collected at 20-minute intervals. A reverse-phase C18 column (BAR, phase II, ODS 3UM; 100×3.2 mm) was used, and the mobile phase consisted of a mixture of 75 mmol/L monochloroacetic acid, 0.7 mmol/L disodium EDTA, 1.5 mmol/L sodium 1-octanesulfonate, and 45 mL/L acetonitrile (pH 3.0). The retention times of 2,3-DHBA and 2,5-DHBA were 9.07 and 5.44 minutes, respectively.

**Measurement of Lipid Peroxidation**

At 20 minutes after the onset of heatstroke, 8 rats were decapitated. The 8 normothermic rats were used as a control group. The brains were removed and dissected into several parts, including the cortex, striatum, hippocampus, and hypothalamus. A fluorescence assay procedure was used to measure lipid peroxidation in these areas.14,15 Each brain area was homogenized with water for 2 seconds in an ultrasonic tissue disruptor. The homogenates were mixed with 400 μL chilled chloroform and 200 μL methanol and then subjected to vertical motion for 1 minute and left on ice for 15 minutes. After centrifugation at 8000g for 5 minutes, 400 μL of the chloroform layer was transferred to another tube containing 100 μL of methanol and was scanned with a spectrophotometer. The lipid peroxidation was determined by measuring the levels of malondialdehyde and its dihydropyridine polymers at 356 nm excitation and 426 nm emission.

**Isolation of Mitochondria and Preparation of Submitochondrial Particles**

All steps in the isolation of mitochondria and the preparation of submitochondrial particles were performed at ice-cold temperature. Mitochondria from the brain were isolated according to Ozawa et al.,16 Briefly, the brain was dissected into different areas, then rinsed knowing the experimental conditions.

**Experimental Groups**

Animals were randomly assigned to 1 of the following 2 groups. One group of rats was exposed to an ambient temperature of 42°C (with relative humidity of 60% in a temperature-controlled chamber). The time when both MAP and local CBF in the striatum began to decrease from their peak levels was taken as the onset of heatstroke, as shown in Figure 1. The second group was exposed to ambient temperature of 24°C for at least 90 minutes to reach thermal equilibrium and was used as control. Their colonic temperature was
resulting pellet was resuspended in 40 mL of isolation buffer and centrifuged further at 5000g for 10 minutes. The aforementioned brown pellet was resuspended in 30 mmol/L potassium phosphate buffer (pH 7.0).

The heart mitochondria were isolated according to the procedure of Arcos et al.17 Pieces of the heart were homogenized in 10 volumes (wt/vol) of isolation buffer consisting of 0.3 mol/L sucrose, 30 mmol/L nicotinamide, and 20 mmol/L EDTA (pH 7.4). The homogenate was centrifuged at 700g for 10 minutes, and the supernatant was recentrifuged at 700g for 5 minutes. The supernatant was centrifuged at 10,000g for 10 minutes, and the aforementioned pellet was resuspended in 30 mmol/L phosphate buffer.

The liver mitochondria were isolated according to the procedure of Sohal et al.18 Pieces of the liver were homogenized in 10 volumes (wt/vol) of isolation buffer containing 0.22 mol/L mannitol, 0.07 mol/L sucrose, 3 mmol/L EDTA, and 1 mmol/L Tris HCl (pH 7.4). The homogenate was centrifuged at 270g for 20 minutes, and the supernatant was centrifuged at 1080g for 20 minutes again. The supernatant was recentrifuged at 3015g for 10 minutes, and the aforementioned pellet was resuspended in 30 mmol/L phosphate buffer.

Sub mitochondrial particles were made by resuspending the mitochondrial preparation in 4 volumes of 30 mmol/L potassium phosphate buffer (pH 7.0). This mixture was sonicated 3 times for 30 seconds each at 1-minute intervals. The sonicated mitochondria were centrifuged at 8250g for 10 minutes to sediment unfragmented mitochondria, and the supernatant was recentrifuged at 80,000g for 40 minutes to pellet the submitochondrial particles. The aforementioned pellet was resuspended in 30 mmol/L phosphate buffer and used for further assay or storage at −80°C.18,19

**Superoxide Generation Monitoring**

The rate of superoxide (O2−) generation by submitochondrial particles was measured according to Boveris.20 Both the test and reference cuvettes contained 20 to 40 µL submitochondrial homogenates, 0.1 mol/L potassium phosphate buffer (pH 7.4), 7.2 µmol/L cytochrome c, 0.6 µmol/L antimycin A, and 7 mol/L sucinate. The reference cuvette was added into 200 µL SOD (Sigma). The reduction of cytochrome c was spectrophotometrically monitored at 550 nm. Since both the test and reference cuvettes contained identical ingredients except that the latter included SOD, the measured rate of cytochrome c reduction was specific because of its interaction with O2−.

**SOD and Catalase Activity Monitoring**

Tissues from the brain, heart, and liver were homogenized in 10 volumes (wt/vol) of 0.1% Triton X. The homogenate was centrifuged at 3000g for 5 minutes, and the supernatant was centrifuged at 50,000g for 30 minutes. The resulting supernatant was used for measurement of the activity of SOD and catalase. The SOD activity was measured according to Misra and Fridovich.21 To measure SOD activity, 20 to 40 µL of the homogenate was added to 2 mL of reaction mixture consisting of 50 mmol/L potassium phosphate buffer, 0.033 mmol/L EDTA, 0.25 mmol/L diansisidine, and 12.5 mmol/L riboflavin (pH 7.0). The optical density of sample at 460 nm was determined before and after illumination with 20-W fluorescent tubes in a box; the change in absorbance observed in a blank (with approximately 200 µg bovine serum albumin but no homogenate) was subtracted from each sample. A standard curve was established by using known units of SOD. Fridovich SOD units were obtained by dividing the change in optical density of the sample by the change in each unit of SOD (from the standard curve).

Catalase activity was measured by the method of Luck.22 Both the blank and the system cuvettes contained 20 to 40 µL homogenate and 3 mL 1/15 mol/L phosphate buffer. The system cuvette contained additional 1 mmol/L H2O2. The time required for the optical density at 240 nM of a mixture of homogenate, H2O2, and buffer to change from 0.45 to 0.4 seconds was used as a measure of catalase activity; 1 unit of catalase activity is equal to 17 seconds. Thus, the activity could be calculated from the rate of the reaction.

Protein concentration in the aforementioned experiments was determined by the method of Lowry et al.23

**Statistical Analysis**

Data from experiments were expressed as mean ± SEM for each time point. Statistical analysis was conducted by ANOVA with Scheffe’s multiple comparisons. A P value < 0.05 was considered statistically significant.

**Results**

Figure 1 shows the effects of a high ambient temperature (42°C) on colonic temperature, MAP, striatal CBF, and DHBA (2,3-DHBA and 2,5-DHBA) concentrations in the striatum in 8 rats. Another 8 rats maintained at an ambient temperature of 24°C served as controls. As shown in Figure 1, the values of colonic temperature, MAP, CBF, and DHBA in the striatum were increased with increase in the heat exposure time. At a certain point in the rats in the heatstroke group, both MAP and CBF in the striatum began to decrease from their peak levels; this moment was considered the onset of heatstroke. The latency of the onset of heatstroke and the time to death (interval between the onset of heatstroke and cardiac arrest) were 87.8 ± 1.7 and 21.5 ± 3.4 minutes, respectively. In contrast, 10 minutes after the onset of heatstroke, the values of MAP and CBF were significantly lower than those of the controls. The last microdialysate was collected from 100 to 120 minutes because the dialysate was collected every 20 minutes. Verification of the last microdialysate revealed that the DHBA surged to the highest level before the animal’s death. The very small value of SEM indicates the good reproducibility of the measurements.

In separate studies, normothermic controls and rats in the heatstroke group were killed 10 minutes after the onset of heatstroke for pathological verification. As shown in the Table, compared with the normothermic, control rats, rats in the heatstroke group had higher neuronal damage score values in their frontal cortex, striatum, hippocampus, and hypothalamus 10 minutes after the onset of heatstroke.

Figure 2 shows the extent of lipid peroxidation in several different brain areas. In rats killed at 20 minutes after or 10 minutes before the onset of heatstroke, the degree of lipid peroxidation in the cortex, striatum, hippocampus, and hypothalamus was measured.
amus was in all cases greater than that of rats killed at 0 minutes before the start of heat exposure.

Figure 3 shows the effects of a high ambient temperature (42°C) on the rate of \( \text{O}_2^- \) generation in the submitochondrial particle of different organs. It was found that the values of rate of \( \text{O}_2^- \) generation were greater in rats killed at 10 minutes after the onset of heatstroke than those of rats killed at 0 minutes, before the start of heat exposure. On the other hand, rats killed at 10 minutes after the onset of heatstroke had a lower value of total SOD activity in the cortex, striatum, hippocampus, hypothalamus, liver, and heart homogenates than those of rats killed at 0 minutes, before the start of heat exposure. Moreover, the values of catalase activity in the striatum, hippocampus, and hypothalamus homogenates were also lower in rats killed at 10 minutes after heatstroke onset than those of rats killed at 0 minutes, before the start of heat exposure.

Discussion

In this study a well-characterized rat model of heatstroke was used to demonstrate that the heatstroke-induced cerebral ischemic insults were associated with an increased production of free radicals, specifically hydroxyl radical and superoxide, higher lipid peroxidation, and lower enzymatic antioxidant defenses. The present study is original because it shows for the first time that oxidative stress occurs after the onset of heatstroke. Pre-treatment with hydroxyl radical scavengers such as mannitol and \( \alpha \)-tocopherol prevented increased production of hydroxyl radicals, increased levels of lipid peroxidation, and ischemic neuronal damage in different brain structures attendant to heatstroke and increased subsequent survival time (C-Y. Yang, MS, et al, unpublished data, 2001). The present results are consistent with many studies showing that reactive oxygen species are involved in brain injuries in many neurological disorders such as cerebral ischemia and reperfusion, hypoxia, inflammation, and fever. Nevertheless, the specificity of heatstroke on the brain is in question since other vital organs, such as the heart and the liver, are also under oxidative stress. Thus, the causal effect of the heat on brain and on animal mortality cannot be readily established from the model. In fact, thus far we are not able to detect any lesion or nitric oxide synthase expression in the heart or liver in rats suffering from heatstroke (C.P. Chang, MS, unpublished data, 2001).

The physiological mechanisms underlying cerebral ischemic injury resulting from increased production of pro-oxidants as well as decreased production of antioxidant defenses have been extensively studied. Although oxygen radicals, by virtue of their reactivity, can injure neurons and other brain cells directly, increased evidence has pointed to the role of redox signaling of oxygen radicals. Such redox signaling targets mitochondrial cytochrome \( c \) release, DNA repair enzymes, and transcriptional factor nuclear factor-\( \kappa B \), which might lead to neuronal damage. Transgenic and knockout animals that either overexpress or are deficient in pro-oxidant or antioxidant enzymes might provide unique tools to dissect out the molecular mechanisms of cell damage (necrosis or apoptosis or both) after the onset of heatstroke.

In the present results, increased production of extracellular DHBA in the brain, increased production of lipid peroxidation in brain homogenates, increased generation of superoxide anion radicals (\( \text{O}_2^- \)) in brain mitochondria, and hyperthermia (approximately 42°C) occurred at 10 minutes before the onset of heatstroke. Ten to 20 minutes after the onset of heatstroke, arterial hypotension (approximately 40 mm Hg) and hyperthermia (approximately 42°C) were associated with a further rise of DHBA, lipid peroxidation, and \( \text{O}_2^- \) production in the brain. In contrast, decreased activity of total SOD and catalase in brain homogenates and hyperthermia occurred at 10 minutes before the onset of heatstroke.
arterial hypotension and hyperthermia were associated with a further reduction of total SOD and catalase activities in brain homogenates. During heatstroke, these endogenous antioxidative defenses are likely to be perturbed as a result of overproduction of oxygen radicals by cytosolic pro-oxidant enzymes and mitochondria, inactivation of detoxification systems, consumption of antioxidants, and failure to adequately replenish antioxidants in heat-injured (because of hyperthermia) and ischemic (because of ensuing hypotension) brain tissue. It has been demonstrated in numerous studies that these reactive oxygen species are directly involved in oxidative damage with cellular macromolecules in ischemic tissues, which leads to cell death.28 Our previous results have also demonstrated that rats with heatstroke display an increased level of dopamine, serotonin, glutamine, or interleukin-1β in brain.4 A recent report showed that nitric oxide mediated the cerebral ischemic injury associated with heatstroke in rats (C.P. Chang, MS, unpublished data, 2001). All these substances may cause the increase of reactive oxygen species3,29 and result in ischemic neuronal damage in the brain.

Detecting free radical production is difficult because the half-life values of free radicals are so short. Of the reactive oxygen species that are known to exist in vivo, the hydroxyl radical is considered the most hazardous.30 The salicylate-trapping method combined with microdialysis was used in the present study to detect hydroxyl radicals in vivo.13 The hydroxylation of sodium salicylate by hydroxyl radicals leads to the production of 2,3-DHBA and 2,5-DHBA, which can be detected by high-performance liquid chromatography/electrochemical detection. In the present results an increased production of both 2,3-DHBA and 2,5-DHBA in the striatum of rats suffering from heatstroke reflected an excessive accumulation of extracellular hydroxyl radicals in the striatum. Since this heatstroke model produced edema only in the cortex,31 an increased concentration of DHBA in the striatum may not be due to concentration effect resulting from edema.

Finally, in the present study the rate of superoxide generation and the activities of total SOD and catalase were measured in submitochondrial particles obtained from isolated mitochondria and in brain homogenates, respectively.

Increased production of $O_2^-$ as well as decreased activity of total SOD or catalase in the brain occurred during heatstroke, implicating numerous enzymatic oxidation reactions that had taken place in the cytosolic compartments or subcellular organelles and mitochondria.

Acknowledgments

This work was supported by grants from the National Science Council (NSC90-2811-B-010-005) and the Ministry of Education (89-B-FA22-1-02) of the Republic of China. The authors appreciate Dr Hung-Hai Ku (Department of Anatomy and Cell Biology, National Yang-Ming University) and Dr Wynn Hwai-Tzong Pan (Institute of Pharmacology, National Yang-Ming University) for their valuable discussion of the manuscript.

References