Prolonged latency to CNS-O2 toxicity induced by heat acclimation in rats is associated with increased antioxidative defenses and metabolic energy preservation

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Eynan M, Ertracht O, Gancz H, Kashi Y, Arieli Y. Prolonged latency to CNS-O2 toxicity induced by heat acclimation in rats is associated with increased antioxidative defenses and metabolic energy preservation. J Appl Physiol 113: 595–601, 2012. First published June 21, 2012; doi:10.1152/japplphysiol.00228.2012.—We have previously shown that heat acclimation provides protection against central nervous system oxygen toxicity (CNS-OT). This was well correlated with increased levels of heat shock protein 72 (HSP72). We now examine other antioxidative defenses against CNS-OT that are correlated with heat acclimation. Two groups of male Sprague-Dawley rats were used. The heat-acclimated group (HA) was exposed for 4 wk to 32°C, and the control group (C) was maintained at 24°C. At the end of the acclimation period, rats were exposed to oxygen at 608 kPa. EEG was recorded continuously until appearance of the first electrical discharge. Brain samples were taken from each group after exposure to pressure. Levels of the antioxidant enzymes CuZnSOD, MnSOD, catalase, and glutathione peroxidase, as well as levels of HSP72, were quantified by Western blot. Comparative proteome analysis of the brains of HA and C rats was carried out using two-dimensional electrophoresis and mass spectrometry to define protein spot alterations. Levels of HSP72 and CuZnSOD were higher in HA rats. Levels of the other antioxidant enzymes were not affected significantly by heat acclimation. Differences in the levels of four protein spots identified as α-synuclein, valosin-containing protein, adenylate kinase 1 (AK1), and the mitochondrial H+-ATP synthase α subunit were found between HA and C rats. We conclude that elevation of HSP72, CuZnSOD, AK1, and the mitochondrial H+-ATP synthase α subunit and possible phosphorylation of α-synuclein—all proteins involved in oxidative stress or energy conservation—might contribute to the prolongation of latency to CNS-OT induced by heat acclimation.

Reactive oxygen species (ROS) and nitric oxide (NO) both play a major role in the generation of CNS-OT (9, 10, 14, 17, 34, 35, 40). Living cells have developed several mechanisms for coping with continuous exposure to ROS. These include antioxidant enzymes and low molecular weight antioxidants such as vitamin C, vitamin E, and glutathione, among others, which scavenge the ROS. The antioxidant enzymes include three kinds of SOD, which transform the superoxide ion into hydrogen peroxide (H2O2): 1) CuZnSOD, which is active in the cytosol; 2) MnSOD, which is active in the mitochondria; and 3) extracellular SOD, which is active in the interstitium. Catalase and glutathione peroxidase catalyze H2O2 to O2 and H2O. Under normal conditions, there is a state of equilibrium between ROS generation and ROS scavenging. In situations of stress, this equilibrium is disturbed, and an increase in the level of ROS might lead to oxidative stress and cell injury (2, 13, 16, 21). One way of preventing injury due to the intensified generation of ROS under conditions of oxidative stress is acclimation to heat.

A number of studies have shown that long-term heat acclimation provides cross-tolerance to various forms of stress, such as ischemia-reperfusion (28, 33), head injury (8, 37, 41), exposure to HBO (5), and exercise (22, 26). Heat acclimation may increase the level of ROS scavengers in the rat brain, protecting it from the destructive effects of ROS and NO (1, 8). Heat acclimation also increases the level of heat shock proteins (HSPs) in various organs (5, 31, 32). This group of proteins is known to serve as chaperones that protect cells from thermal, oxidative, and other types of injury. Several studies have thus associated HSP70 and HSP72 with cross-tolerance to various forms of stress (5, 23, 32, 43, 44, 46). Arieli et al. (5) showed that heat acclimation doubled the latency to CNS-OT. This was maintained for 2 wk during deacclimation and was associated with a significant increase in the level of HSP72 following heat acclimation, with a subsequent decrease during deacclimation.

HSP72 is probably just one of many possible defenses against CNS-OT whose level in the rat brain is elevated following heat acclimation. Because ROS play a major role in the generation of CNS-OT, we studied the effect of heat acclimation on the antioxidant enzymes CuZnSOD, MnSOD, catalase, and glutathione peroxidase by measuring their expression in the heat-acclimated (HA) rat brain and also by conducting a comparative proteome analysis with a control group (C).

MATERIALS AND METHODS

Animals and maintenance. The Animal Care Committee of the Israel Ministry of Defense approved the experimental procedure, and...
Twenty male Sprague-Dawley rats with an initial weight of 100 ± 10 g (mean ± SD) were used. The animals’ weight at the end of the experiment appears in RESULTS. Rats were divided into two groups: eight rats were in the HA group, and 12 animals served as a C group. Rats were housed in the animal unit in a specially thermoregulated Plexiglas acclimation cage (50 × 30 × 20 cm) continuously aerated with dry air, four rats to a cage. Heat acclimation for the HA group was achieved by exposing the rats to 32 ± 1°C for 4 wk (5). Rats in the C group were housed in a similar cage with the temperature set at 24 ± 1°C. The animals were maintained on an 8:16-h light-dark cycle. Food and water were provided ad libitum. After 3 wk of acclimation (7 days before the experiment), all of the rats had an EEG electrode implanted in the skull under equithesin anesthesia (0.3 ml/100 g body wt ip) for recording of the EEG during HBO exposure (3–5).

Experimental system and procedure. At the end of the 4-wk acclimation period, all of the animals were subjected to two exposures in the hyperbaric chamber separated by an interval of at least 2 days. The rat was placed in the experimental cage, which was placed in a 150-l hyperbaric chamber (Roberto Galeazzi, La Spezia, Italy) (3–5). The flow of gas through the cage was controlled by two needle valves. A small portion of the outgoing gas was directed out of the pressure chamber (controlled by another needle valve), passed through a flowmeter, and sampled by an oxygen analyzer (Model 571; Servomex, Crowborough, East Sussex, UK), which monitored the concentration of O2 in the experimental cage. The temperature in the cage was maintained within a thermoneutral range (27 ± 1°C) to avoid any effect of temperature on CNS-OT (3–5). The EEG was recorded on a data logger (CODAS; DATAQ Instruments, Akron, OH) and was displayed on the computer screen. When the desired pressure was reached (608 kPa), a period of 20 min was allowed for acclimation to the experimental conditions, during which air flowed through the cage at −5 l/min. At the end of this period, the flow of air was replaced immediately by pure oxygen at a fast flow rate of 15 l/min for 1 min to replace the cage’s atmosphere. When the oxygen level reached 95%, oxygen flow into the experimental cage was reduced to 5 l/min. The exposure was terminated 10 s after the appearance of the first electrical discharge (FED) in the EEG, and latency to the FED was measured starting from the time at which O2 reached a level of 95%. Ten seconds after the onset of the FED, we changed the inflowing gas back to air and reduced pressure at a rate of 100 kPa/min. At the end of decompression, we immediately killed the animals; the entire brain was removed quickly, placed in liquid nitrogen, and kept at −80°C until analysis.

Western immunoblotting. The brains of eight HA and eight C rats were homogenized with SDS buffer (20% glycerol and 6% SDS in 0.12 M Tris buffer with a pH of 6.8), centrifuged at 14,000 rpm for 20 min at 4°C, and boiled for 10 min. The protein concentration of the brain specimens was quantified by the Bradford method (Bio-Rad Laboratories, Hercules, CA). Prepared samples were diluted further in sample buffer to allow loading of 50 mg total protein in each gel well. Protein was separated on a 10% or 15% polyacrylamide gel, according to the expected kDa of each protein studied, under denaturing conditions. The samples were diluted in dissociation buffer (10% SDS, 200 mM EDTA, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue), blended, and heated again to a temperature of 95°C for 2 min. Electrophoresis was conducted at 50 mA (120 V) for 2 h. After separation, proteins were transferred onto nitrocellulose (190 mA, 4°C, 1 h) by Western blotting. Each nitrocellulose membrane contained samples from the two groups studied, a group of molecular weight markers, and a control sample prepared from a pooled brain homogenate of a known amount of protein, which served as a reference and was immunoblotted together with all of the experimental series. The nitrocellulose sheets were washed in PBS containing 5% milk powder for 2 h. After washing, the sheets were incubated at 4°C overnight with monoclonal IgG cross-reactive to the inducible HSP72 antibody diluted 1:1,000; CuZnSOD antibody diluted 1:10,000; MnSOD antibody diluted 1:10,000 (all three from Stressgen Biotechnologies, Sidney, BC, Canada); catalase antibody diluted 1:60,000 (Rockland Immunochemicals, Gilbertsville, PA); and glutathione peroxidase antibody diluted 1:10,000 (Chemicon International, Temecula, CA). After repeated washing in PBS with 0.2% Tween 20, the membranes were incubated at room temperature for 1 h with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Sigma, St. Louis, MO) at 1:10,000 dilution for HSP72 or with peroxidase-conjugated AffiniPure goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA) at 1:10,000 dilution for the other antibodies. The membrane was then developed by chemiluminescence (Amer sham Laboratories, Bucks, UK) to enhance detection and exposed to X-ray film (Kodak, Rochester, NY). Levels of HSP72 and the antioxidant enzymes were measured by scanning the immunoblots with a laser densitometer (Vilber Lourmat, Marne-la-Vallée, Cedex, France). Band density was calculated by integrating the area in pixels and was normalized to the level of the reference sample. Each band density was measured five separate times and averaged.

Protein isolation protocol for proteome analysis. The brain tissues from four HA and four C rats were put into TriReagent solution (Sigma) and homogenized in a glass Teflon homogenizer. The TriReagent suspensions were mixed thoroughly with chloroform and centrifuged (12,000 g, 10 min, 4°C). The organic phase containing the proteins was treated with 100% ethanol and centrifuged (10,000 g, 10 min, 4°C). The protein was precipitated with isopropanol and centrifuged (12,000 g, 10 min, 4°C). The protein pellet was washed three times with 0.3 M guanidine hydrochloride in 95% ethanol, according to the manufacturer’s protocol (Sigma). Protein content was determined using the Bradford method.

Two-dimensional gel electrophoresis and image analysis. Two-dimensional (2D) PAGE was performed under conditions essentially as described in the manufacturer’s protocol (Bio-Rad Laboratories), using 110 mm, pH 3–10, immobilized pH gradient (IPG) strips and Criterion (4–20% gels). Protein samples were dissolved in buffer containing 7 M urea, 2 M thiourea, 65 mM DTT, 0.125% (vol/vol) Biolytes 3–10, 2% 3-[3-(cholamidopropyl)dimethylammonio]-1-propane sulfonate, and bromophenol blue. For the first dimension, 200 μg of protein was applied to a dehydrated IPG strip, and the isoelectric focusing was carried out at room temperature as follows: passive rehydration for 1 h, at 50 V for 12 h, at 200 V for 1 h, at 500 V for 1 h, at 1,000 V for 1 h, followed by a linear gradient to 8,000 V over a period of 30 min, and finally, at 8,000 V to 62,000 V. Before the second-dimension separation, the gel strips were equilibrated for 15 min for disulfide reduction in 37.5 mM Tris-HCL (pH 8.8) containing 6 M urea, 2% (wt/vol) SDS, 30% (wt/vol) glycerol, 0.5% DTT, and 0.1% bromophenol blue and then re-equilibrated for 15 min for carbamoylmethylation in the same buffer containing 2% iodoacetamide in place of DTT. Strips were placed on Criterion gels, and second-dimension separation was carried out at 200 V for 75 min. Dual Color markers were applied as a molecular standard. Following electrophoresis, gels were visualized by SeeBend Forte protein staining solution per the manufacturer’s protocol (Bio-Rad Laboratories). The gel images were acquired with a Bio-Rad Fluor-S Multilager, and spots were indexed using the PDQuest 2D software for comparison and quantitation of 2D gel spots.

Gel proteolysis and mass spectrometry analysis. Protein spots, which increased or decreased in size by >50% or underwent modification, were analyzed further. The proteins in the gel were reduced with 10 mM DTT (60°C for 30 min) and modified with 100 mM iodoacetamide in 10 mM ammonium bicarbonate (room temperature for 30 min). The gel pieces were dehydrated with acetonitrile and rehydrated with 10% acetonitrile in 10 mM ammonium bicarbonate containing trypsin (Promega Biosciences, San Luis Obispo, CA). The tryptic peptides were resolved by reverse-phase chromatography on 0.1 × 200 mm fused silica capillaries (100 μm ID; J&W Scientific, Rancho Cordova, CA) and packed with reversed-phase material (Vy-
Body weight and latency to the FED. After 4 wk of acclimation to heat, the weight of the HA rats was significantly lower compared with the C rats (236 ± 21 and 282 ± 40 g, respectively; \( P < 0.01 \)). Latency to the FED was significantly longer in the HA rats compared with the C group (\( P < 0.01 \); Fig. 1).

Levels of HSP72 and antioxidant enzymes. Levels of HSP72 and CuZnSOD in the rats’ brains are presented in Figs. 2 and 3, respectively. There was a significant increase in HSP72 and CuZnSOD levels in the HA group compared with the C group (\( P < 0.01 \) and \( P < 0.05 \), respectively). No differences were found between the groups for the levels of the other antioxidant enzymes examined, MnSOD, catalase, and glutathione peroxidase.

Proteome analysis. To find other parameters with a similar response time to heat acclimation, we screened the overall expression of proteins in the rats’ brains. Proteins were extracted from the brain tissue of HA and C rats and analyzed by 2D gels. More than 200 protein spots were detected. Figure 4 displays the overall 2D patterns of the protein extracts for the two experimental groups. The expression of a number of proteins differed between the HA and C rats, and these were identified by mass spectrometry and database searches. Table 1 lists the putative protein identifications and associated analysis information for each gel spot marked on the representative gels in Fig. 5. The spot with sample spot protein (ssp) 1203 observed in the gels of the C samples was not detected in the HA gels. In the gels of the HA samples, we detected a spot with ssp 1204. The position of ssp 1204 in the HA gels was slightly shifted into the low pH area compared with the ssp 1203 spot obtained in the C gels (Fig. 5). Both ssp 1203 and 1204 were identified as α-synuclein protein, but there were differences in the intensity of the spots between the groups. Protein shifting into a low pH area in the course of isoelectrofocusing of experimental samples may be explained by protein modification, and phosphorylation in the brain due to the heat acclimation procedure is one of the possible causes of such a shift. There were two spots (ssp 3805 and ssp 7203) that were detected only in the HA group (Fig. 5). Protein ssp 3805 was identified as a valosin-containing protein, and ssp 7203 was identified as adenylate kinase 1 (AK1). Protein ssp 8702 was identified as a subunit of ATP synthase.

DISCUSSION

In the present study, we investigated possible mechanisms by which long-term heat acclimation may prolong the latency to CNS-OT. We found that latency to CNS-OT was significantly longer in HA rats compared with C. Prolongation of latency to CNS-OT in the HA group corroborates the findings of our previous study (5), which were also associated with smaller weight gain in the HA group. This has been reported...
previously by a number of studies examining the physiological alterations associated with heat acclimation (28, 33). HSP72 and CuZnSOD were elevated in the HA group compared with the C animals. HSP70 and HSP72 act as chaperones protecting the cell from comprehensive damage and probably also as antioxidant molecules that act to reduce the level of NO (32). Reduction of NO levels by administration of a NO inhibitor can delay seizures following HBO exposure to five atmospheres absolute in the rat (9, 14).

CuZnSOD is one of the major cellular defense enzymes that plays a vital role in protecting cells against the toxic effect of superoxide radicals by dismutasing the superoxide ion to H$_2$O$_2$. Of all of the antioxidant enzymes investigated in the present study, only CuZnSOD was altered after heat acclimation, increasing significantly compared with the C group. A number of studies have examined the relationship between HSP70 and CuZnSOD. Choi et al. (11) found that the expression and activity of CuZnSOD were reduced in HSP70 knockout mice, whereas no difference was found in the activity of catalase and glutathione peroxidase between the knockout mice and the wild-type. Other investigations suggested a positive correlation between the regulation of HSP70 and CuZnSOD (24, 27, 46, 47). It was reported that heat shock may induce expression of the HSP70 gene (46), as well as the CuZnSOD gene through the heat shock element (47). In transgenic mice overexpressing CuZnSOD, there was increased expression of HSP70 mRNA following focal cerebral ischemia (24) and global cerebral ischemia (27). Our findings provide further circumstantial evidence for this positive correlation.

### Table 1. Summary of differences in the expression of proteins in the brains of heat-acclimated and control rats identified by mass spectrometry

<table>
<thead>
<tr>
<th>SSP</th>
<th>Gi Accession #</th>
<th>Identified Protein</th>
<th>Spot Intensity</th>
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<tr>
<td>1203</td>
<td>9507125</td>
<td>$\alpha$-Synuclein (14.5 kDa)</td>
<td>850.0 ± 6.3</td>
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<tr>
<td>1204</td>
<td>9507125</td>
<td>$\alpha$-Synuclein (14.5 kDa)</td>
<td>no</td>
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<tr>
<td>3805</td>
<td>17865351</td>
<td>Valosin-containing protein (89.3 kDa)</td>
<td>1,617.7 ± 60.4</td>
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<tr>
<td>7203</td>
<td>13242235</td>
<td>Adenylate kinase 1 (21.6 kDa)</td>
<td>949.8 ± 79.1</td>
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<tr>
<td>8702</td>
<td>40538742</td>
<td>Mitochondrial H$^+$-ATP synthase a subunit (59.7 kDa)</td>
<td>2,222.1 ± 33.9</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD. SSP, sample spot protein; Gi, GenInfo identifier; C, control; HA, heat-acclimated.
that brains shifted into the low pH zone. This alteration may be however, a later study using a rat model demonstrated the nuclein fibrillization and enhances neurotoxicity in vitro (19).

However, a later study using a rat model demonstrated the thase /H11001 valosin-containing protein, adenylate kinase 1, mitochondrial H-/ATP synthase /H9251 Parkinson's disease. However, the mechanism of toxicity of which are considered to contribute to the pathogenesis of neurodegenerative disorders such as Parkinson's disease and dementia (15). This protein is natively normal function remains unclear. It has been implicated in the terminals. It is a principal component of Lewy bodies, and its specific protein that is normally localized in presynaptic ter-

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Previous studies have investigated energy sparing in HA rats (especially in the myocardium) under conditions of stress, such as ischemia/reperfusion. Heat acclimation resulted in adaptation of metabolic performance, represented by lower cardiac oxygen consumption in the rat (28, 29), and larger preischemic endogenous glycogen stores in conjunction with a quantitative increase in glycolysis but at a slower rate (18). This resulted in much better coping with stress situations such as ischemia/reperfusion. Schwimmer et al. (36) investigated the global genomic response in the hypothalamus, a key regulator of body homeostasis and an integrator of a variety of functions, one of which is energy balance. They found that after long-term heat acclimation (induced as in the present study), there was enhancement of gene transcription related to functions of food intake, metabolic rate, and osmotic regulation, as demonstrated by using $^{32}$P-labeled RNA hybridized onto cDNA Atlas array membranes.

We conclude that heat acclimation improves resistance to CNS-OT, as demonstrated by prolongation of the latency to CNS-OT in the HA rats compared with the C group. This was associated with elevation of HSP72, CuZnSOD, AK1, and the mitochondrial H+-ATP synthase $\alpha$ subunit and the possible phosphorylation of $\alpha$-synuclein in the brain of the HA rats, all of which may have played a part in improving the ability of these animals to cope better with CNS-OT.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.E., O.E., Y.K., and Y.A. conception and design of experiments; M.E. and O.E. prepared figures; M.E. drafted manuscript; O.E., Y.K., and Y.A. interpreted results of experiments; M.E., O.E., and H.G. performed experiments; M.E., H.G., and Y.A. contributed reagents/materials/analysis tools; M.E. and Y.A. edited and revised manuscript; M.E. and O.E. approved final version of manuscript; M.E. and O.E. acquired funding; M.E., O.E., H.G., Y.K., and Y.A. organized or managed the project. There are no funding agencies involved. All authors reviewed the manuscript.

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