Thermosensitive phenotype of transgenic mice overproducing human glutathione peroxidases

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ABSTRACT Exposure of humans and other mammals to hyperthermic conditions elicits many physiological responses to stress in various tissues leading to profound injuries, which eventually result in death. It has been suggested that hyperthermia may increase oxidative stress in tissues to form reactive oxygen species harmful to cellular functions. By using transgenic mice with human antioxidant genes, we demonstrate that the overproduction of glutathione peroxidase (GP, both extracellular and intracellular) leads to a thermosensitive phenotype, whereas the overproduction of Cu,Zn-superoxide dismutase has no effect on the thermosensitivity of transgenic mice. Induction of HSP70 in brain, lung, and muscle in GP transgenic mice at elevated temperature was significantly inhibited in comparison to normal animals. Measurement of peroxide production in regions normally displaying induction of HSP70 under hyperthermia revealed high levels of peroxides in normal mice and low levels in GP transgenic mice. There was also a significant difference between normal and intracellular GP transgenic mice in level of prostaglandin E2 in hypothalamus and cerebellum. These data suggest direct participation of peroxides in induction of cytoprotective proteins (HSP70) and cellular mechanisms regulating body temperature. GP transgenic mice provide a model for studying thermoregulation and processes involving actions of hydroxy and lipid peroxides in mammals.

The existence of significant levels of reactive oxygen species (ROS) in cells and tissues under physiological conditions requires the cells to have a defense system consisting of low molecular weight antioxidants [α-tocopherol, glutathione (GSH), etc.] and enzymes that can eliminate oxygen radicals (1). There are three major antioxidant enzymes, superoxide dismutase (SOD), catalase, and GSH peroxidase (GP) (2). In normal conditions, there is a steady-state balance in production of ROS and cellular antioxidant systems and in activities of different antioxidant enzymes. This balance is necessary to ensure optimal efficiency of antioxidant systems (3, 4). Interestingly, ROS play an important role in gene regulation by activating some transcription factors, which in turn mediate induction of proteins involved in cellular responses to changes in environmental conditions (5). Transgenic mice overexpressing antioxidant enzymes provide an excellent system to study the role of these enzymes in animal physiology under normal and stressed conditions. Indeed transgenic mice overexpressing SOD have been constructed in a number of laboratories and used as a model for various studies (6–9). In the present study, we have constructed transgenic mice overexpressing SOD and GPs by using the human genes for Cu,Zn-SOD and intracellular and extracellular GP (GPE and GPP, respectively). Here, we report that these transgenic mice exhibit a thermosensitive phenotype in comparison with normal mice and transgenic mice with human Cu,Zn-SOD, indicating that the level of GP activity may play an important role in regulation of body temperature under stress conditions.

MATERIALS AND METHODS

Transgenic Mice. Transgenic mice with human Cu,Zn-SOD, GPE, and GPP genes were produced as described by Hogan et al. (10). The coding regions of the human GPE (11), GPP (12), or Cu,Zn-SOD (13) genes were inserted into the unique BamHI site of the pHMG cassette vector (14). To generate transgenic mice, Nor I fragments of recombinant plasmids were microinjected into the pronuclei of the C57BL/6 × CBA/J hybrid. Mouse lines GPE23 and GPP17 (containing 200 copies of the human GPE gene and 2 copies of the human GPP gene, respectively) and SOD42 (containing 70 copies of the human SOD gene) were used for further studies. To obtain normal and transgenic animals for experiments, transgenic founders were bred with (C57BL/6 × CBA/J)F1 mice.

Northern Blot Analysis. Total RNA was prepared from tissues of transgenic and control mice by the guanidinium thiocyanate extraction protocol (15). Poly(A) mRNA was obtained by using the oligo(dT) column. Total RNA (20 μg) or poly(A) mRNA (3 μg) was denatured in 1× Mops electrophoresis buffer (16), 6.5% (vol/vol) formaldehyde, and 50% (vol/vol) formamide. RNA was loaded on 1.4% agarose gel with 1.1% formaldehyde. After electrophoresis, the gel was blotted and hybridized as described (16).

Induction of Hyperthermia. After measurement of body weights, hyperthermia was induced by placing 4- to 5-month-old female mice in an incubator (Forma Scientific, Marietta, OH) at 40°C, according to a protocol approved by the University Animal Care and Use Committee. Animals were placed in ventilated Plexiglas restraints to allow continuous monitoring of colonic temperature (Tc) by using a Digi-Sense scanning thermometer (Cole–Palmer) and a rectal type T thermocouple probe. After the treatment, animals were killed by decapitation. Tissues were removed and frozen for RNA isolation.

Analysis of HSP70 Expression. Groups of normal and transgenic animals were placed in partial restraints and Tc was continuously monitored during the exposure to 40°C. Total RNA was prepared from collected tissues and hybridized with labeled HSP70-specific oligonucleotide probe as described (17).

Determination of Peroxides Level by the 2',7'-Dichlorofluorescein Fluorescence. Intracellular peroxide levels in brains of transgenic and control mice were determined by a modified method of Tsuchiya et al. (18). Briefly, mid- and parasagittal

Abbreviations: ROS, reactive oxygen species; SOD, superoxide dismutase; GP, glutathione peroxidase; GPE, intracellular glutathione peroxidase; GPP, extracellular glutathione peroxidase; GSH/GSSG, reduced/oxidized glutathione; HSF, heat shock factor; HSP, heat shock protein; Tc, colonic temperature; PG, prostaglandin.
slices (0.6–0.8 mm lateral) of brains in OCT embedding compound (Miles) were snap-frozen in liquid nitrogen. Sections (25–30 μm), mounted on poly(l-lysine)-coated slides, were incubated in 100 mM 2',7'-dichlorofluorescein diacetate (Molecular Probes) in 100 mM sodium/potassium phosphate-buffered saline at 37°C for 1 h. Slides were given four 5-min rinses in sodium/potassium phosphate buffer and analyzed for fluorescence intensity by using a Meridian Instruments (Lansing, MI) model 570 anchored cell analysis system. Cerebellum and choroid plexus were read by using a 10% neutral density filter, which eliminated 90% of the irradiating light.

**Extraction and Measurement of Prostaglandin (PG) E₂.** Mice were exposed to 40°C as described above. Immediately after decapitation, brain tissue samples (~20 mg) were removed and then sonicated in 500 μl of buffer (50 mM Tris-HCl, pH 7.0/100 mM NaCl/0.2 mM EDTA). Extraction of the eicosanoids from homogenate was performed on ethyl C₂ Amrep mini-columns by the manufacturer’s procedure (Amersham). PGE₂ was measured by Biotrak PGE₂ immunoassay system (Amersham).

**RESULTS**

Transgenic Mice with SOD and GP Genes. A series of transgenic mice lines overproducing human Cu,Zn-SOD, GPE, and GPP were constructed. The human genes are under the control of a mouse hydroxymethylglutaryl-coenzyme A reductase promoter (14), as shown in Fig. 1. Northern blot analyses of the expression of transgenes in different tissues of lines GPE23, GPP17, and SOD42, which had the highest levels of expression, are shown in Fig. 2. In the GPE transgenic mouse line (line 23), GP activity was 4-fold higher in brain, 1.6 times higher in muscle, 1.27 times higher in liver, and 1.5 times higher in kidney than in the control mice. However, in the GPP transgenic mice (line 17), the activity increased by 55% in blood compared to the control. This increase was as expected since GPP is an extracellular enzyme. In the SOD transgenic mice (line 42), Cu,Zn-SOD activity was 2.5 times higher in brain, 3.9 times higher in lung, 2.7 times higher in muscle, 3.9 times higher in kidney, and 3.2 times higher in liver than that in control.

**Heat-Induced Hyperthermia in Transgenic Mice.** Normal and transgenic animals were placed in an incubator at 38°C, 40°C, or 42°C. The Te was continuously measured. It was found that transgenic mice with either GPE or GPP were more sensitive, surviving for a significantly shorter time period at elevated temperatures (Fig. 3). Average survival times during exposure to 40°C were 96 min (SE, ±2.9 min) for GPE transgenic mice and 129 min (SE, ±3.9 min) for GPP mice in contrast to 165 (SE, ±10 min) and 175 (SE, ±7 min) min for SOD and normal mice, respectively. Exposure of normal and transgenic mice to 38°C and 42°C demonstrated similar patterns of temperature sensitivity as seen in Fig. 3, except that for all groups survival times increased correspondingly at 38°C and decreased at 42°C (data not shown). After the initial phase, mice exhibited an adaptive stage, the duration of which was different depending upon the transgenic mice lines (Fig. 3). After this, the Te increased steadily until death. Histopathological examinations of sections of the heart, lung, liver, spleen, kidney, and brain of the transgenic and normal mice revealed acute pulmonary congestion without overt pathology such as necrosis. These results strongly suggest that the cause of death was heat-induced hyperthermia and that the overproduction of GPE or GPP may have increased sensitivity of mice to heat-induced acute physiologic death without detectable anatomic changes.

**Expression of HSP70.** Levels of HSP70 mRNA in brain, lung, and muscle of both normal and GPE transgenic mice exposed to 40°C were examined. Northern blot analysis (Fig. 4A) indicates that when the Te of transgenic animals reached
42°C and of normal mice reached 40°C, the levels of induced HSP70 mRNA in brain were very similar despite differences in $T_c$, but in lung and muscle of the control mice, the levels were higher than those in transgenic mice (compare lanes 1 and C2, Fig. 4A). When $T_c$ of normal animals reached 42°C, a higher level of HSP70 mRNA was observed in all tissues examined (compare lanes C1 and lanes T1, Fig. 4A). These data suggest that there is impairment of HSP70 induction in the three tissues of GPE transgenic mice. Similar data were obtained for the line of GPP mice, although the difference in HSP70 expression between normal and transgenic mice was less than that between GPE and normal mice. Heat shock factor (HSF) DNA-binding activity was higher in normal mice than GPE mice in brain only (data not shown).

Level of Peroxidation in Brain of Transgenic and Control Mice Under Hyperthermia. Transgenic mice were maintained at external temperature of 40°C until core temperature reached 42°C at which time brains were removed for analysis. The body temperature of normal animals reached only ~40°C for the same period of exposure. An additional group of normal animals was permitted to achieve a core temperature of 42°C. Although the levels of peroxide were minimal in both normal and GPE transgenic mice in the hypothalamus, cerebellum, or choroid plexus at room temperature, the former exhibited approximately twice as much peroxide-induced fluorescence as transgenic animals (Fig. 5 Upper). An increase in the body temperature to near 40°C did not cause significant elevation in the intensity of fluorescence observed in the hypothalamus and choroid plexus of normal mice (see Fig. 5 Lower A and G). However, strong fluorescence was observed in apical cerebellar granule cells (Fig. 5 Lower D). A marked increase in peroxide-induced fluorescence was also seen in the molecular layers, while cerebellar white matter remained unaffected. Elevation of the body temperature of normal mice to 42°C resulted in large increases in peroxide-induced fluorescence particularly in the white matter of hypothalamus and in the choroid plexus (Fig. 5 Lower B and H). Increased fluorescence was also observed in all cerebellar layers (Fig. 5 Lower E). In contrast, in GPE transgenic mice with a body temperature of 42°C, there was no significant production of peroxides in either the cerebellum or choroid plexus (Fig. 5 Lower F and I). Only mild fluorescence was observed in the white matter tracts and neuropil of the lateral hypothalamus. These data strongly indicate that under hyperthermic conditions, the accumulation of peroxides occurs in regions of brain participating in neurohormonal responses to stress conditions. Furthermore, in the presence of elevated levels of GP, the quantity of peroxides was significantly decreased in the brain of transgenic mice compared to normal animals. The present procedure is most likely to be detecting intracellular lipid peroxides, which are more stable than H2O2 and may be formed at least partially as secondary products of increased ROS production.

PGE2 Levels in Hypothalamus and Cerebellum Under Hyperthermia. To measure levels of PGE2, groups of normal and transgenic mice were exposed to hyperthermic conditions, as described above. At normal temperature, the quantity of PGE2 in hypothalamus was the same in both strains of mice (276 ± 26.6 pg/mg in normal mice and 248 ± 24.3 pg/mg in GPE mice). In cerebellum, PGE2 level was 57% higher in normal (89 ± 8.3 pg/mg) than in the transgenic (56.6 ± 3.5 pg/mg) mice. An increase in body temperature in normal animals leads to a drop in detectable PGE2 in hypothalamus and cerebellum. At 40°C, quantity of PGE2 in hypothalamus was 242 ± 16.7 pg/mg and in cerebellum was 68.5 ± 3.5 pg/mg, but at 42°C it was 19.8 ± 4.5 pg/mg and 42 ± 9.0 pg/mg, in the two regions, respectively. In transgenic animals with a body temperature of 42°C, PGE2 level decreased in hypothalamus alone (up to 41 ± 3.5 pg/mg). The level was still 2.1 times higher when compared to control animals at the same body temperature. Under hyperthermia, the quantity of PGE2 in the cerebellum of GPE mice was slightly increased in comparison to the nonexposed mice (72 ± 14.4 pg/mg vs. 56.6 ± 3.5 pg/mg).

DISCUSSION

The temperature sensitivity of independently obtained strains of transgenic mice with two genes (GPE or GPP) strongly suggests that the heat-sensitive phenotype is a direct consequence of the overproduction of GP. This enzyme scavenges hydroperoxides by using GSH as an electron donor, thus playing a critical role in protecting cells against oxidative stress. Lowering the reductive equivalents (GSH) in cells may lead to increased thermal sensitivity (19, 20). When GSH and oxidized GSH (GSSG) levels in normal and transgenic animals were measured after a 30-min exposure to 40°C, no significant differences were detected in various organs. However, a local GSH/GSSG imbalance (for example, in regulatory regions of brain) cannot be excluded.

The exact role of oxidative stress in hyperthermia is unknown at present. It was suggested that hyperthermia may induce oxidative stress due to increased production of ROS and/or the promotion of cellular oxidation events (21–23). These data also support the idea that ROS may be involved in hyperthermic induction of heat shock protein (HSP) (24), which is known to play an essential role in protecting cells from irreversible cell damage by heat (25). Inducible HSP expression under stress conditions involves transcriptional activation mediated by the transcription factor HSF (26). The level of HSP70 induction in different organs of rats and mice exposed to elevated temperatures has been shown to directly correlate with heat-induced increase in $T_c$ (17, 27). Measurements of HSP70 mRNA induction in transgenic and normal mice under hyperthermic conditions demonstrate that the amounts of the mRNA are less in all tested tissues of transgenic mice overproducing GP than in the corresponding tissues of the normal mice at the same body temperature. HSF DNA-binding activity was found to be lower only in brain of the transgenic mice under hyperthermic conditions. The present results suggest that transgenic mice overproducing GP are unable to induce HSP70 sufficient to protect from cytotoxic effects of hyperthermia. This effect is unlikely to be attributed to the reduction of the HSF DNA-binding activity. Recently, failure in several types of cells to elicit transcriptional activity of the HSP70 gene under heat shock conditions even in the presence of significant HSF DNA-binding activity was reported (28, 29). This may be due to the presence of inhibitory proteins or the lack of specific
Fig. 5. Peroxide production in brains of control and GPE transgenic mice. (Upper) Pseudocolor representations of peroxide-induced fluorescence in the hypothalamus (A and B), cerebellum (C and D), and choroid plexus of the lateral ventricle (E and F) of control (A, C, and E) and transgenic (B, D, and F) mice at normal temperature. (Lower) Pseudocolor representations of peroxide-induced fluorescence in the hypothalamus (A–C), cerebellum (D–F), and choroid plexus of the lateral ventricle (G–I) in brains of control and GPE transgenic mice under hyperthermic condition (40°C). The measurement was carried out at the body temperature of control mice at 40°C (A, D, and G) or 42°C (B, E, and H) and of transgenic mice at 42°C (C, F, and I).

Factors interacting at sequences around the heat shock element. Our data suggest that these factors may be regulated by peroxides or may be sensitive to the ox/redox status of the cells. Both H₂O₂ and lipid peroxides, substrates of GP, are effective inducers of HSP70 in several types of cells (30, 31). Therefore, peroxides may play a very important role in signaling or modulate signaling pathway for mobilization of cell defense mechanisms. Removal of this signal by GP may lead to acute hyperthermia in GP transgenic mice under the heat-stress conditions. Similarly, heat-shocked primary cell cultures from transgenic mice revealed decreased amounts of HSP70 mRNA in comparison to cells from normal mice (unpublished results).

There is increasing interest in the expression of HSP in the nervous system and the role that these proteins may play in neural trauma, fever, degenerative disorders, and hyperthermia. Exposure to high temperature leads to the induction of HSP70 in the cerebellum, choroid plexus, and brain regions probably coordinating neuroendocrine stress responses (32). The fact that only selected brain regions show high levels of HSP70 RNA induction with heat stress suggests that factors other than differential heating of cells in these regions leads to
induction of HSP. It is possible that in very active brain regions under hyperthermic condition, levels of H$_2$O$_2$/peroxides are significantly increased leading to the activation of HSP. Indeed, according to one theory of thermoregulation, response to elevated temperature involves interaction of noradrenergic, cholinergic, and serotonergic pathways (33). Importantly, oxidative metabolism of monoamine neurotransmitters are one of the major sources of H$_2$O$_2$ and other ROS in neurons (34).

We measured levels of peroxides in normal and transgenic mice in regions of brain known to express high levels of HSP70 during hyperthermic conditions. Our data support the hypothesis that hyperthermia leads to oxidative stress in regulatory regions of brain in normal animals. Formation of ROS in the presence of elevated levels of GP is inhibited in GP transgenic mice leading to the failure of induction of cellular protective mechanisms and, possibly, malfunction of temperature regulation systems.

Body temperature is regulated almost entirely by nervous system feedback mechanisms through temperature-regulatory centers located in the hypothalamus (33). Hyperthermia occurs when thermoregulatory mechanisms are overcome by excessive metabolism, impaired dissipation, or excessive environmental heat. It was proposed that the hypothalamic set point may be modulated by PGs involved in the actions of cytokines. The role of these active lipids in normal temperature regulation and fever is still debatable (35). Several experiments have suggested that “peroxide tone” can regulate the production of eicosanoids, which are produced in response to stimuli (36). Indeed, our data indicate that the metabolism of PGs is altered in transgenic mice. However, the importance of changes in the PG levels in the observed phenotype remains to be further proven.

Our data suggest that ROS are involved in thermoregulation. Transgenic mice overproducing GP significantly modulate levels of lipid peroxides under conditions of induced stress and may be a good model system to study diverse pathological conditions such as cancer, ischemia–reperfusion damage, inflammation, etc.

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