Age-related Effects of Heat Stress on Protective Enzymes for Peroxides and Microsomal Monooxygenase in Rat Liver

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To evaluate the age-related response of essential cell functions against peroxidative damage in hyperthermia, we studied the biochemical response to heat stress in both young and aged rats. Passive hyperthermia was immediately observed in rats after exposure to hot environments. In aged rats, the rectal temperature maintained thermal homeostasis and increased to the same degree as in young rats. In these aged animals, the damage from heat stress was more serious than in young animals. In aged rats under normal environmental conditions, hepatic cytosolic glutathione peroxidase (GSH peroxidase) activities were markedly higher than those activities in younger rats. Hepatic cytosolic GSH peroxidase activities were induced by heat stress in young rats but were decreased by hot environments in aged rats. Hepatic catalase activities in young rats were not affected by hot environments, whereas in aged rats, hepatic catalase activities were seriously decreased. Catalase activities in the kidney of aged rats were also reduced by hot environments. Lipid peroxidation in the liver was markedly induced in both young and aged rats. Because the protective enzymes for oxygen radicals in aged rats were decreased by hot environments, lipid peroxidation in the liver was highly induced. In aged rats, lipid peroxidation in intracellular structures such as mitochondria and microsomes was also markedly induced by hot environments. In both young and aged rats, hyperthermia greatly increased the development of hypertrophy and vacuolated degeneration in hepatic cells. In aged rats, both mitochondria and endoplasmic reticulum of the hepatic cells showed serious distortion in shape as a result of exposures to hot environments. Microsomal electron transport systems, such as cytochrome P450 monooxygenase activities, were seriously decreased by heat stress in aged rats but not in young rats. Although the mitochondrial electron transport systems were not affected by acute heat stress in young rats, their activities were simultaneously inhibited after long-lasting heat exposure. In isolated hepatic cells and polymorphonuclear leukocytes in animals, the 70-kDa heat shock-induced proteins were markedly increased by heat stress. In conclusion, the heat stress-inducible oxygen radical damage becomes more severe according to the age of rats. Because aging and hyperthermia have a synergistic effect on lipid peroxidation, protective enzyme activities for oxygen radicals may be essential for surviving and recovering from thermal injury in aged animals and also in humans. *Key words*: aging, catalase, cytochrome P450, electron transport, glutathione peroxidase, heat stress, hyperthermia, lipid peroxidation, monooxygenase, oxygen radicals. *Environ Health Perspect* 105:726–733 (1997)

A 1996 report by a task group convened by the World Health Organization (WHO), the World Meteorological Organization (WMO), and the United Nations Environmental Programme (UNEP) (*1*) indicated that one of the most important direct impacts of global warming would be a greater frequency and greater duration of exposure to hotter temperatures, especially during the summer months.

Typical hyperthermia sometimes occurs during severe heat waves in summer and as a result of hard exercise throughout the world. In some temperate large cities, extreme heat stress is associated with an enhanced heat island effect. The incidence of heat-related morbidity, such as heat stroke in aged persons, has been shown to increase as a result of exposure to extremely hot temperatures in summer (*2–4*). Heat stroke is caused by severe hyperthermia, and rectal temperatures of typical patients are higher than 40°C. Many organs including the central nervous system are damaged by severe hyperthermia and thrombus infarct, and death from heat stroke may be caused by injury of these organs.

In animals and humans, some physiological and biochemical adaptations could occur to protect essential cell functions against heat stress and to permit a rapid recovery from moderate hyperthermic damage (*5–7*); however, each tissue and organ has a different sensitivity for sustaining thermal injury (*8–11*). Therefore, it is necessary to study the biochemical mechanism of hyperthermic damage and age-related response under hot environmental conditions.

Many biological processes, such as ischemia-reperfusion, inflammation, and an uncoupling reaction of electron transport systems in mitochondria and microsomes, produce oxygen radicals from molecular oxygen (*12,13*). Heat stress sometimes increases oxygen radicals, possibly by the disruption of the electron transport assemblies of the membrane (*14*).

Cellular and intracellular membrane damage and denaturation of enzymes might be important in the pathogenesis of heat injury. The oxygen free radical damage of biological membranes and high molecules is a destructive phenomenon that is associated with many types of cellular damage (*15,16*). In the guinea pig, significant lipid peroxidation in liver occurred in passive hyperthermia caused by a hot environment. Because the peroxidation of lipids in biological membranes is a destructive phenomenon that is associated with a variety of cellular damage, hyperthermia has been shown to develop pathological degeneration in hepatic cells (*11*).

For the protection of cell function from lipid peroxidation, two types of glutathione peroxidases (GSH peroxidase; EC 1.11.1.9), such as selenium GSH peroxidase and non-selenium GSH peroxidase are very important (*17,18*). The activities of these enzymes are markedly different among animal species. Selenium GSH peroxidase activities in human liver are very low in comparison with that in rat liver, and selenium GSH peroxidase is not active in guinea pig liver (*19*).

It has been proven that the heat stress-inducible GSH peroxidase is a selenium GSH peroxidase (*11*). Therefore, it is necessary to prove that a relationship exists between age-related induction of GSH peroxidase and lipid peroxidative damage under hot environmental conditions. Since GSH peroxidase activities were not induced in guinea pig liver, marked peroxidative damage has been shown to occur in mitochondrial electron transport systems under hot environmental conditions (*11*).

Peroxidative damage is related to the production of the protective enzymes for

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peroxides such as GSH peroxidase, GSH transferase, and catalase (EC 1.11.1.6). Therefore, animal experiments were carried out to study the relationship between age-related changes of protective enzyme activities and the peroxidative damage of heat stress on intracellular structure, such as mitochondria and endoplasmic reticulum.

Materials and Methods

Materials. Cytochrome c and glutathione reductase (EC 1.6.4.2) were purchased from the Sigma Chemical Co. (St. Louis, MO). NADH, NADPH, and GSH were purchased from the Wako Pure Chemical Co. (Tokyo, Japan). Sephadex G-25 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), and an ^{125}I-corticosterone radioimmunoassay kit was purchased from ICN Biochemicals Inc. (Costa Mesa, CA). All other chemicals were of the highest purity that was commercially available.

Animal treatment. One hundred and twenty SPF-grade 5-week-old male Fisher rats (Clea Japan Inc., Tokyo, Japan) were housed in 23 ± 0.5°C under a 12-hr light:12-hr dark cycle. All animals were allowed free access to laboratory chow (Clea Japan Inc.) and distilled water. When rats were 7 weeks old, 12 months old, 17 months old, or 25 months old, rats were randomly divided into the following experimental groups. Eighty 7-week-old rats were divided into four treatment groups. Each rat was housed at 25 ± 0.5°C, 30 ± 0.5°C, 32 ± 0.5°C, and 35 ± 0.5°C at 40 ± 10% relative humidity for 7–42 days. Ten rats from each of the 12-, 17-, or 25-month-old groups were divided into two treatment groups and housed at 25 ± 0.5°C and 35 ± 0.5°C at 40 ± 10% relative humidity for 3 or 7 days, respectively. After exposure to various environmental temperatures, animals were sacrificed between 10:00 A.M. and noon by severing the main abdominal artery while under ether anesthesia. Tissues and organs were prepared for photomicrographic and electronmicrographic examination. Serum and tissue cell samples were prepared for biochemical analysis.

Electronmicroscopy. The tissue samples were fixed in a solution consisting of 2% paraformaldehyde/2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.5) for 2 hr at 4°C. The tissue samples were rinsed in several changes of fixative solution over a period of 2 hr and were then fixed in 1% osmium tetraoxide/sodium phosphate buffer (pH 7.5) using an automatic sample fixative apparatus (Rotex RX-90, Australian Biochemical Co., Mordialloc, Australia). The samples were dehydrated, sectioned, and stained in 8% saturated uranyl acetate followed by lead citrate. Observation was carried out using a JEX-1200EXII electron microscope (Jeol Co., Tokyo, Japan).

Preparation of samples. Mitochondria, microsomes, and cytosols for enzyme assay were prepared from liver according to the method described previously (*11*). Liver was thoroughly perfused with cold 0.9% NaCl and homogenized with 9 volumes of cold 0.15 M KCl, 10 mM HEPES buffer (pH 7.4) in a Potter-Elvehjem glass-Teflon homogenizer (Wheaton, Millville, NJ) in an ice bath. The homogenate was then centrifuged at 900*g* for 10 min. The supernatant was then centrifuged 7,000*g* for 10 min and at 105,000*g* for 60 min. The washed mitochondria and microsomes were suspended in 0.15 M KCl, 10 mM HEPES (pH 7.4).

To get the purified mitochondria, the density gradient isopycnic centrifugation of mitochondria was performed according to the modified method of Neuberger et al. (*20*). Homogenates, mitochondria, microsomes, and cytosols were immediately placed in liquid nitrogen and stored at -80°C.

Analytical methods. Rectal temperature and eyeball temperature were measured using a digital thermometer (TD-300; Shibaura Electron, Co., Urawa, Japan) and an infrared radiation thermometer (505-S; Minolta Co., Osaka, Japan), respectively. Serum corticosterone was measured by radioimmunoassay (RIA) using a kit from ICN Biochemicals according to the manufacturer's instructions. Cytochrome P450 and cytochrome b_5 in liver microsomes were determined according to the modified method of Omura and Sato (*21*) and Estabrook and Werringloer (*22*), with extinction coefficients of 91/mM/cm (between 490 and 450 nm) and 185/mM/cm (between 426 and 409 nm), respectively. The concentration of microsomes for assays of cytochrome P450 and cytochrome b_5 was 3 mg protein/ml. Protein was determined using a commercial kit (Bio-Rad Laboratories, Inc., Hercules, CA) for the Bradford protein assay (*23*).

Thiobarbituric acid reacting substances (TBARS) were determined according to the modified method of Ohkawa et al. (*24*) and Miller et al. (*25*). Hepatic cytosolic glutathione content was determined according to the method of Tietze (*26*).

Preparation of heat-stressed cells. Isolated hepatic cells from rats were obtained according to the modified method of Seglen (*27*). Hepatic cells and polymorphonuclear leukocytes (PMNs) were grown at 37°C or under heat stress at 43°C for 60 min and then labeled with [^{35}S]methionine at 37°C for 120 min. After labeling, the cells were harvested and the labeled proteins were analyzed by SDS-gel electrophoresis. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (*28*) using 12.5% (w/v) acrylamide gels, and the electrophoresed protein was assayed using autoradiograms.

Enzyme assay. To determine the activities of mitochondrial cytochrome c oxidase, the oxidation of reduced cytochrome c was assayed according to Ando et al. (*11*) using a UV-240 spectrophotometer equipped with a computer (Shimadzu Co., Kyoto, Japan). To determine the activities of the mitochondrial cytochrome c reductase system, the reduction of oxidized cytochrome c was assayed (*11*).

Aminopyrine *N*-demethylase activities (AP demethylase) were assayed as the formation of formaldehyde by the method of Orrenius (*29*) using 5′-AMP to prevent the breakdown of NADPH by microsomal pyrophosphatase (*30*). Assay of tetramethylparaphenylenediamine (TMPD) peroxidase activities were performed as described by O'Brien and Rahimtula (*31*). Reaction rates were corrected for TMPD oxidation without cumene hydroperoxide and were measured by following the rate of Wurster's blue free radical formation at 610 nm, with an extinction coefficient of 11.6/mM/cm.

NADPH cytochrome P450 reductase and NADH cytochrome b_5 reductase activities were assayed using the methods of Omura and Takesue (*32*) and Takesue and Omura (*33*), respectively.

GSH peroxidase activities were measured by a modification of the coupled assay procedures of Paglia and Valentine (*34*). The reaction mixture for total GSH peroxidase activities contained 60 µg hepatic cytosolic protein/ml, 0.25 mM GSH, 0.12 mM NADPH, 1 unit/ml GSH reductase, 1.5 mM sodium cyanide, 0.1 mM EDTA, and 0.2 mM cumene hydroperoxide in 50 mM HEPES buffer (pH 7.6). The reaction mixture for selenium GSH peroxidase activities contained 60 µg hepatic cytosolic protein/ml, 0.25 mM GSH, 0.12 mM NADPH, 1 unit/ml GSH reductase, 1.5 mM sodium cyanide, 0.1 mM EDTA, and 0.2 mM *tert*-butyl hydroperoxide in 50 mM HEPES buffer (pH 7.6).

Cytosolic superoxide dismutase (SOD; EC 1.15.1.1), cytosolic glutathione *S*-transferase (GSH transferase; EC 2.5.1.18), and catalase (EC 1.11.1.6) activities were assayed as described by Ponti et al. (*35*), Habig et al. (*36*), and Aebi (*37*), respectively, using 50 µg of hepatic protein/ml. The enzyme activities were assayed by recording the absorbance change using the UV-240 spectrophotometer.

Statistical evaluation. Statistical analysis was carried out using the F and *t*-test of Snedecor and Cochran (*38*); $p<0.05$ was considered significant.

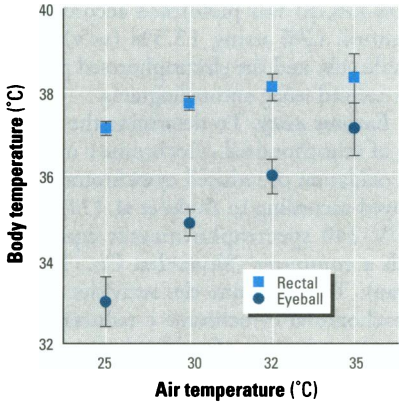

Figure 1. Body rectal temperature and eyeball temperature records in 7-week-old rats under various environmental temperatures at 40 ± 10% relative humidity for 6 days. Each value represents the mean temperature ± standard deviation for five rats.

Results

As shown in Figure 1, typical passive hyperthermia was observed in 7-week-old rats; rectal temperature immediately increased after exposure to hot environmental temperatures. In rats, rectal temperature obviously exhibited the thermal homeostasis to hot environments during heat stress. In aged rats, the rectal temperature also maintained the thermal homeostasis and increased the same degree as in 7-week-old rats, but in aged rats, the damage from heat stress was more serious than for young rats. In 17-month-old rats, it was difficult for animals to survive for 7 days at 35°C; therefore, heat stress experiments were carried out for 3 days.

Exposure to hot environments induced serious histochemical changes in various organs such as liver, kidney, lung, testis, and heart. As shown in Figure 2A, the hepatic cells around the hepatic vein developed hypertrophy to a great extent, and vacuolated degeneration was observed during 1–6 weeks in a hot environment. The electron micrograph shows that heat stress also affects the intracellular structure of the hepatic cell, including mitochondria, smooth endoplasmic reticulum, and peroxisomes (Fig. 2B–D). In aged rats (Fig. 2C), these histochemical and intracellular changes were more serious than those in young rats (Fig. 2B).

In aged rats, the distorted shapes of mitochondria of hepatic cells were markedly increased under hot environmental conditions (Fig. 2D). There was a significant increase in peroxisome proliferation. Lipid droplets and the distorted shape of smooth endoplasmic reticulum were also observed under hot environmental conditions (Fig. 2B,C).

In young rats, hepatic cytosolic GSH peroxidase activities, especially selenium GSH peroxidase activities, were greatly induced in response to increased environmental temperatures. Increased GSH peroxidase activities in liver continued during

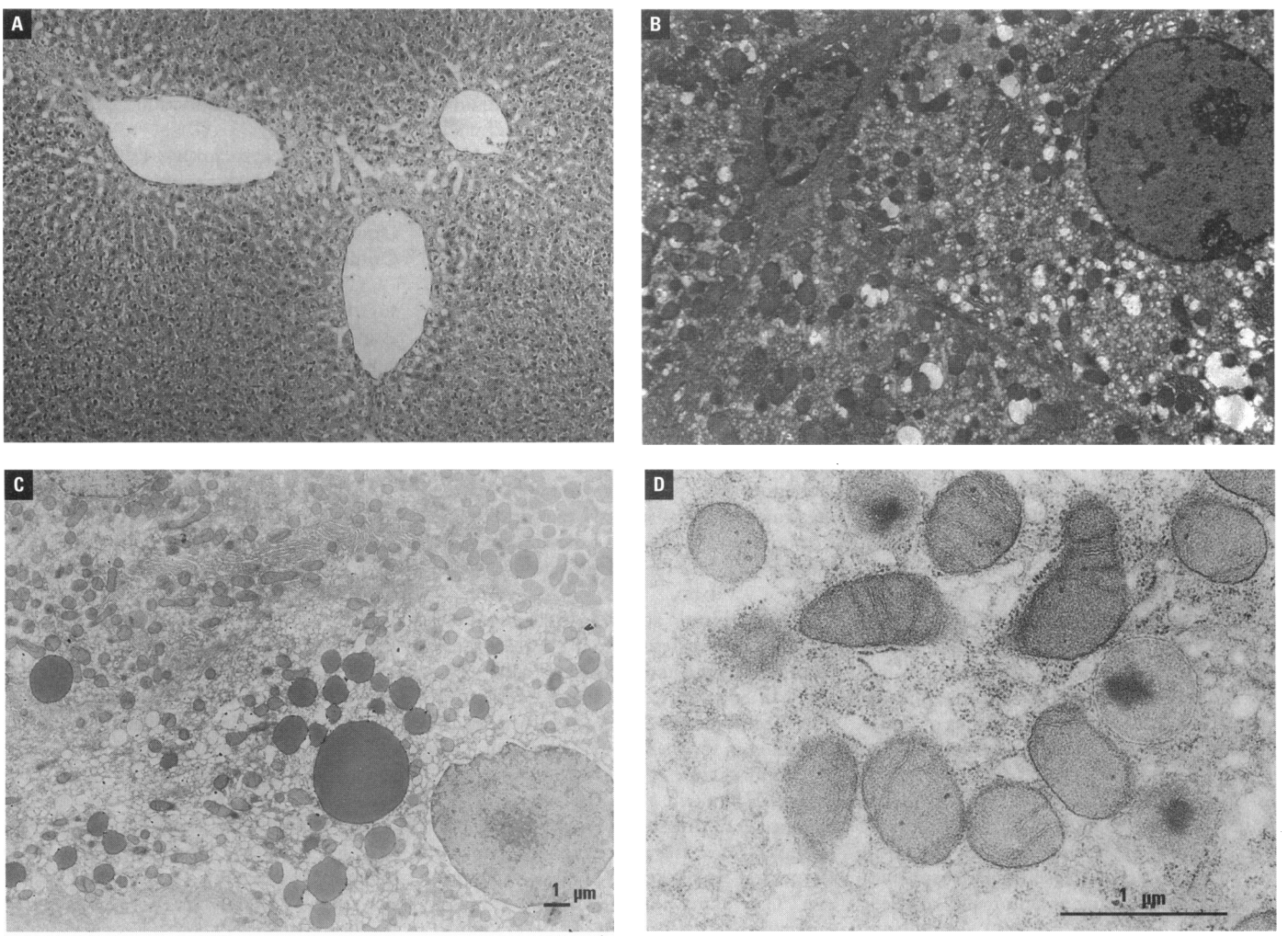

Figure 2. Photomicrograph [(A) ×25] and electron micrographs of hepatic cells in (B) a 7-week-old rat (×3,000) and in a 12-month-old rat [(C) ×3,000 and (D) ×20,000] during 7 days in a hot environment (35°C). Hypertrophy and vacuolated degeneration of hepatic cells are present around the hepatic vein (A). In aged rats, the histochemical and intracellular changes were more serious than in young rats, and distorted shapes of mitochondria and markedly increased numbers of peroxisomes and lipid droplets were observed (C,D).

the period of long-term heat exposure as shown in Figure 3.

Under normal environmental conditions, hepatic cytosolic GSH peroxidase activities markedly increased according to the age of animals, whereas the activities of hepatic GSH peroxidase in aged rats were not induced, but were slightly decreased by a hot environment, as summarized in Table 1. In young rats, hepatic cytosolic GSH transferase activities were also slightly induced during the period of long-term heat exposure as shown in Figure 4.

As summarized in Table 1, hepatic catalase activities in young rats were not affected by hot environments. Under normal environmental conditions, the catalase activities markedly decreased according to the age of animals. In aged rats, catalase activities in liver and kidney were seriously decreased by hot environments. Both in young rats and aged rats, GSH peroxidase in organs such as heart, kidney, and brain were not affected by hot temperatures.

The antioxidant components, such as SOD and GSH, did not change with a hot environment in this experiment. Activities of hepatic cytosolic SOD in young rats changed from 13.2 ± 0.5 to 12.9 ± 1.5 unit/mg protein after exposure to hot temperature (35°C). Cofactors of antioxidant enzymes such as GSH and oxidized glutathione (GSSG) changed from 5.50 ± 0.31 to 5.41 ± 0.44 μmol/g liver and from 0.26 ± 0.021 to 0.24 ± 0.029 μmol/g liver, respectively, after exposure to a hot environment.

In young rats, lipid peroxidation in liver was induced in response to the increased environmental temperatures. Increases in lipid peroxidation in liver continued during long-term heat exposure (Fig. 5). Since the protective enzymes for oxygen radicals in aged rats such as GSH peroxidase, GSH transferase, and catalase were decreased by hot environments, lipid peroxidation in liver was greatly induced, (see Table 2). Induction of TBARS in liver was observed when the rats were exposed to 35°C, whereas the production of lipid peroxidation was not affected by exposure to 32°C or less (Fig. 5).

In young rats, the production of TBARS in intracellular structures of hepatic cells such as mitochondria and microsomes were not affected by hot temperatures. In aged rats, the productions of TBARS in mitochondria and microsomes were markedly induced by hot environments (Table 2).

In young rats, lipid peroxidation in the kidney was not affected by hot environments, whereas in aged rats, lipid peroxidation in the kidney was significantly induced by hot temperatures. Both in young rats and aged rats, lipid peroxidation in other organs such as heart and brain was not affected by hot environments (data not shown). Serum corticosterone levels were slightly affected by heat stress (data not shown).

Because endoplasmic reticulum of hepatic cells in aged rats showed distorted shapes under a hot environment, microsomal electron transport systems were assayed. In aged rats, hepatic microsomal electron transport systems, such as AP demethylase activities, were seriously affected by hyperthermia as summarized in Table 3. Cytochrome P450 reductase activities were also slightly reduced in hyperthermia. In young rats, microsomal electron transport systems were not affected by hyperthermia.

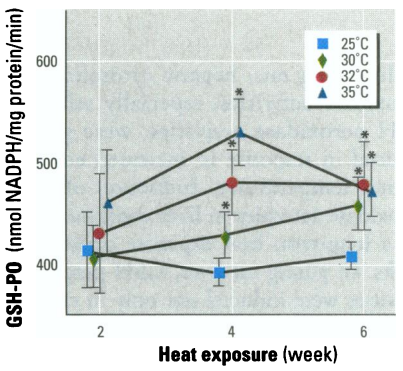

Figure 3. Effect of long-term heat exposure on hepatic glutathione peroxidase (GSH-PO) activities in young rats. Each value represents mean activity ± standard deviation of five rats. *Significantly different from control values measured at 25°C ($p<0.05$).

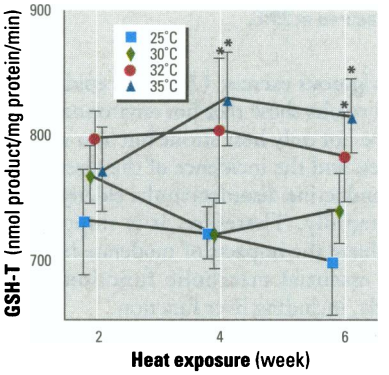

Figure 4. Effect of long-term heat exposure on hepatic glutathione transferase (GSH-T) activities in young rats. Each value represents mean activity ± standard deviation of five rats. *Significantly different from control values measured at 25°C ($p<0.05$).

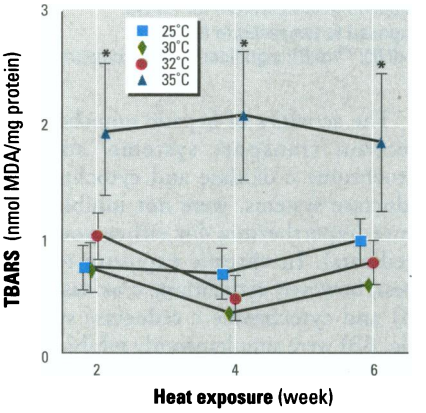

Figure 5. Effect of long-term heat exposure on hepatic thiobarbituric acid reacting substances (TBARS) production in young rats. Each value represents mean malondialdehyde (MDA) production ± standard deviation of five rats. *Significantly different from control values measured at 25°C ($p<0.05$).

Table 1. Effect of environmental temperature on the activities of cytosolic GSH peroxidase, cytosolic GSH transferase, catalase in liver and kidney, and hepatic mitochondrial cytochrome c oxidase and cytochrome c reductase systems in young and aged rats

	7-week-old		12-month-old		17-month-old		25-month-old		
	25°C[a]	35°C[a]	25°C[a]	35°C[a]	25°C[a]	35°C[b]	25°C[a]	35°C[b]	35°C[a]
GSH-peroxidase (nmol NADPH/mg protein/min)	441 ± 33	594 ± 51**	749 ± 29	717 ± 81	1039 ± 173	898 ± 178	1125 ± 170	1041 ± 69	810 ± 66*
GSH-transferase (nmol product/mg protein/min)	738 ± 25	788 ± 38	864 ± 47	695 ± 36**	872 ± 32	858 ± 27	892 ± 58	831 ± 41	784 ± 50
Catalase (μmol/mg protein/min)									
Liver	449 ± 23	421 ± 23	432 ± 6	283 ± 39**	327 ± 23	223 ± 11**	364 ± 20	214 ± 19**	236 ± 34**
Kidney	110 ± 1	102 ± 2	122 ± 6	83 ± 11**	81 ± 8	57 ± 3**	63 ± 4	60 ± 4	56 ± 3
Hepatic mitochondria									
Cytochrome c oxidase (nmol/mg protein/min)	164 ± 11	175 ± 16	171 ± 20	190 ± 26	160 ± 26	166 ± 20	190 ± 17	196 ± 3	153 ± 23
Cytochrome c reductase (nmol/mg protein/min)	51.1 ± 4.3	52.7 ± 5.5	55.5 ± 9.5	74.9 ± 10.8	46.4 ± 12.1	43.7 ± 6.1	44.0 ± 3.7	48.9 ± 3.5	34.7 ± 8.6

GSH-peroxidase, glutathione peroxidase. Each value represents mean activity ± standard deviation of five rats.
[a]Exposed to temperature for 7 days.
[b]Exposed to temperature for 3 days.
*$p<0.05$; **$p<0.01$; significant when compared to controls measured at 25°C.

Table 2. Effect of environmental temperature on thiobarbituric acid reacting substances (TBARS) production in homogenate, microsomes, and mitochondria in the liver and homogenate in the kidney in young and aged rats

	7-week-old		12-month-old		17-month-old		25-month-old		
TBARS (nmol MDA/mg protein)	25°C[a]	35°C[a]	25°C[a]	35°C[a]	25°C[a]	35°C[b]	25°C[a]	35°C[b]	35°C[a]
Liver homogenate	0.75 ± 0.19	3.40 ± 0.57**	3.30 ± 0.15	12.83 ± 5.45**	5.13 ± 1.49	14.20 ± 1.80**	0.42 ± 0.14	13.52 ± 3.96**	14.39 ± 4.14**
Liver microsome	0.72 ± 0.12	0.57 ± 0.09	0.80 ± 0.11	2.08 ± 0.30**	1.36 ± 0.01	1.72 ± 0.28*	0.65 ± 0.15	1.49 ± 0.29**	1.65 ± 0.11**
Liver mitochondria	0.55 ± 0.08	0.43 ± 0.06	0.70 ± 0.10	1.72 ± 0.63*	0.86 ± 0.16	1.56 ± 0.21**	0.44 ± 0.08	1.31 ± 0.16**	1.51 ± 0.32**
Kidney homogenate	0.45 ± 0.04	0.55 ± 0.05	1.03 ± 0.10	1.46 ± 0.23*	1.04 ± 0.08	1.45 ± 0.22*	0.69 ± 0.15	1.95 ± 0.66*	1.53 ± 0.20**

Each value represents the MDA producton ± standard deviation in five rats.
[a]Exposed to temperature for 7 days.
[b]Exposed to temperature for 3 days.
*$p<0.05$; **<0.01; significant when compared to controls measured at 25°C.

Table 3. Effect of environmental temperature on the content of cytochrome P450 and cytochrome b_5, and the activities of aminopyrine *N*-demethylase (AP-demethylase), tetramethylparaphenylenediamine, (TMPD)-peroxidase, NADPH-cytochrome P450 reductase, and NADH-cytochrome b_5 reductase in hepatic microsomes

	7-week-old		12-month-old		17-month-old		25-month-old		
	25°C[a]	35°C[a]	25°C[a]	35°C[a]	25°C[a]	35°C[b]	25°C[a]	35°C[b]	35°C[a]
Cytochrome P450 (nmol/mg protein/min)	0.490 ± 0.021	0.479 ± 0.020	0.477 ± 0.041	0.437 ± 0.069	0.438 ± 0.023	0.458 ± 0.023	0.334 ± 0.009	0.338 ± 0.046	0.407 ± 0.058
Cytochrome b_5 (nmol/mg protein/min)	0.354 ± 0.009	0.395 ± 0.006	0.399 ± 0.041	0.392 ± 0.013	0.359 ± 0.041	0.388 ± 0.026	0.271 ± 0.029	0.281 ± 0.035	0.273 ± 0.022
AP-demethylase (nmol HCHO/mg protein/min)	9.13 ± 0.72	10.64 ± 0.99	8.72 ± 0.77	3.87 ± 1.04**	8.14 ± 0.62	6.72 ± 0.12**	4.3 ± 0.6	3.4 ± 0.9	3.3 ± 0.3*
TMPD-peroxidase (nmol oxidized/mg protein/min)	92.2 ± 7.1	98.3 ± 7.2	81.7 ± 10.0	47.3 ± 10.3**	72.3 ± 6.2	56.6 ± 3.7*	25.3 ± 2.6	26.6 ± 3.9	27.6 ± 3.6
NADPH P450 reductase (nmol/mg protein/min)	56.4 ± 4.7	55.0 ± 3.3	56.0 ± 1.6	34.3 ± 4.1**	49.9 ± 2.8	48.5 ± 0.5	38.2 ± 4.0	37.7 ± 6.6	30.5 ± 2.6*
NADH b_5 reductase (μmol/mg protein/min)	3.99 ± 0.12	4.15 ± 0.02	4.12 ± 0.32	4.15 ± 0.17	4.17 ± 0.02	3.97 ± 0.20	4.05 ± 0.35	3.76 ± 0.15	2.94 ± 0.24**

Each value represents the MDA production ± standard deviation in five rats.
[a]Exposed to temperature for 7 days.
[b]Exposed to temperature for 3 days.
*$p<0.05$; **<0.01; significant when compared to controls measured at 25°C.

The activities of hepatic mitochondrial electron transport systems, such as cytochrome c oxidase and cytochrome c reductase systems, were not inhibited by acute hyperthermia (in either young or aged rats). In chronic exposure to heat stress, however, cytochrome c oxidase (Fig. 6A) and cytochrome c reductase systems (Fig. 6B) were simultaneously inhibited.

To detect the heat shock response, isolated hepatic cells and PMNs in rats were labeled with [^{35}S]methionine and analyzed by SDS-PAGE. Analysis of the labeled cells by SDS-PAGE showed the induced synthesis of the protein band corresponding to the 70-kDa protein. In isolated hepatic cells and PMNs, the 70-kDa heat shock induced proteins were expressed immediately by heat stress as shown in Figure 7A.

Discussion

Typical hyperthermia sometimes occurs during severe heat waves in summer and during hard exercise. Classic heat stroke commonly occurs in temperate large cities during extreme heat stress associated with an increased heat island effect. The incidence of heat-related morbidity and mortality increases in persons, especially aged persons, because of extremely hot temperatures in summer (*2–4,39–42*). On the other hand, exertion-induced heat exhaustion in young persons and aged persons occurs in both hot and cool environments as a result of vigorous exercise (*10*). Our epidemiological results show that hot environments produce not only heat stroke but also some diseases, and the incidence of the diseases such as endocrine disorders in the elderly increases rapidly. Therefore, it is important to evaluate the impacts of moderate heat stress on essential metabolic functions in the body, including liver function.

After exposure to a hot environment at 35°C, moderate passive hyperthermia was observed and rectal temperature immediately increased approximately 1.2°C. Rectal temperature of young and aged rats exhibited thermal homeostasis to hot environments. The results confirm the evidence that thermoregulatory function in rats is maintained during aging (*43*). Still in aged animals, the hyperthermic damage by heat stress was more serious than that in young animals. Thus, cellular and intracellular membrane damage and denaturation of enzymes might be important in the pathogenesis of passive hyperthermia caused by hot environments (*11*).

In animals and humans, some physiological and biochemical adaptations could occur to protect essential cell functions against increased temperatures and to permit a rapid recovery from heat stress (*5,6,44*). Because the adaptive response to heat stress depends on animal species and aging, animals and humans have different sensitivities for withstanding thermal injury (*8–10*).

In young rats, hepatic cytosolic GSH peroxidase activities, especially selenium GSH peroxidase activities, were greatly induced in response to increased environmental temperatures. Induction of GSH peroxidase activities in liver continued during a long-term heat exposure period of 6 weeks. In young rat liver, GSH peroxidase activities were induced not only in cytosols but also in mitochondria.

In young rats, hepatic catalase activities were not affected by hot environments, whereas in aged rats, hepatic catalase activities were seriously decreased. Catalase activities in the kidney of aged rats were also reduced by hot environments.

Many biological processes, such as xanthine oxidase, myeloperoxidase, and an uncoupling reaction of electron transport systems in mitochondria and microsomes, produce oxygen radicals from molecular oxygen (*12,13*). Peroxidative damage of macromolecules, such as 8-hydroxy-2′-deoxyguanosine of DNA, accumulates in the animal body during aging (*45*), and the activities of protective enzymes metabolizing the oxidative products are significantly related to aging (*46*).

Under normal environmental conditions, hepatic cytosolic GSH peroxidase activities in aged rats were markedly higher than those activities in younger rats. In aged rats, GSH peroxidase activity was not induced, but was actually decreased by heat

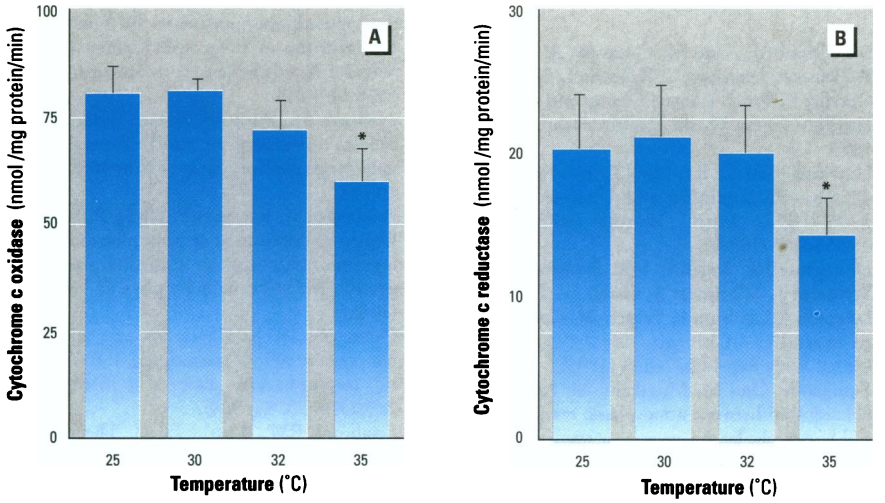

Figure 6. Effect of 6 weeks of exposure to various temperatures on cytochrome c oxidase (A) and cytochrome c reductase systems (B) in hepatic mitochondria in young rats. Each value represents mean activity ± standard deviation of five rats.
*Significantly different from control values measured at 25°C ($p<0.05$).

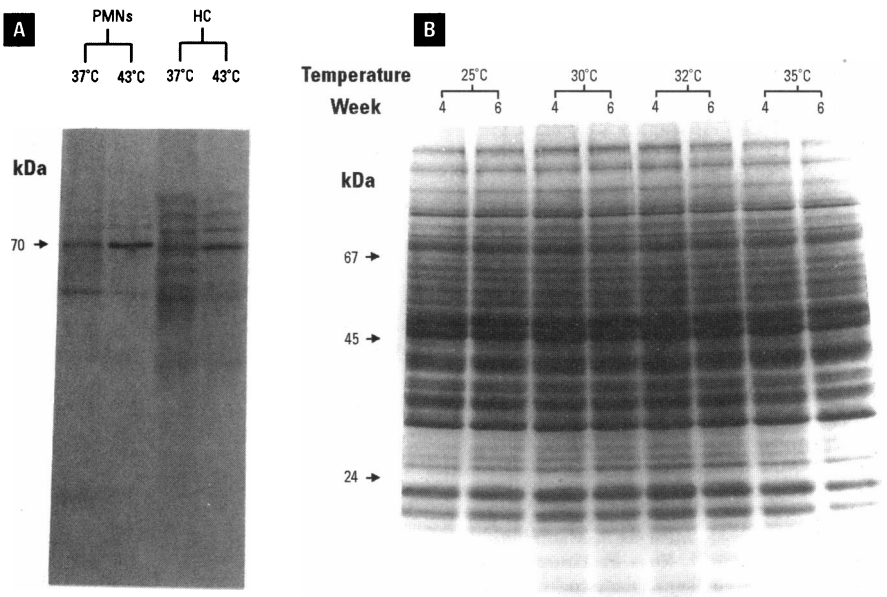

Figure 7. Effect of heat stress on heat shock response in (A) polymorphonuclear leukocytes (PMNs) and isolated hepatic cells (HC) and (B) hepatic cytosol *in vivo*. Analysis of the [^{35}S]methionine-labeled cells by SDS-PAGE showed the induced synthesis of the protein band corresponding to the 70-kDa protein, indicated by an arrow in Figure 7A.

stress. From this evidence, it is necessary to clarify the biochemical relationship between the adaptable induction of the enzyme and peroxides production in the animal body during aging.

Furthermore, selenium GSH peroxidase activities in human liver are very low in comparison with those in rat liver (*14,16,47*). Therefore, it is important to consider the age-related induction of GSH peroxidase when the effects of acute heat stress on human health are evaluated.

In aged rats, other protective enzymes for oxygen radicals such as catalase were also markedly decreased after heat exposure; therefore, lipid peroxidation in liver was seriously induced in aged rats in hyperthermia. Lipid peroxidation was greatly induced not only in liver homogenate but also in intracellular structures such as mitochondria and microsomes in aged animals.

It is well known that heat shock response induces cellular SOD in mammalian cell culture (*48*). In this *in vivo* experiment, cytosolic SOD was not induced by heat stress. Cofactors of antioxidant enzymes, such as GSH and GSSG, also were not affected by heat stress in this experiment.

Aged rats have more basal GSH-peroxidase activity than young rats but also have less basal catalase activity; therefore, 12-month-old and 17-month-old rats have more basal lipid peroxides than young rats. At the end of their life span, 25-month-old rats have more basal cytochrome c oxidase activity and less basal cytochrome c reductase activity than young rats. The activity difference between oxidase and reductase may be an important uncoupling reaction of electron transport systems in mitochondria. From the aspect of geriatric research, it is necessary to clarify the relationship between lipid peroxide generation and enzyme activities during aging.

The induction of lipid peroxidation in liver was shown to be a very sensitive biochemical indicator in hyperthermia. The induced formation of TBARS in liver progressively increased in passive hyperthermia resulting from hot environments. It was determined that cellular and intracellular membrane damage and denaturation of enzymes was important in the pathogenesis of heat injury (*11*). Oxygen free radical damage of biological membranes and high molecules is a destructive phenomenon that is associated with many types of cellular damage (*12*). Because peroxidation of lipids in biological membranes is a destructive phenomenon that is associated with many types of cellular damage, hyperthermia appears to have been responsible for greatly increased development of hypertrophy and vacuolated degeneration in hepatic cells.

It has been reported that peroxidation of lipids in biological membranes can damage the cellular redox state, intracellular structure, and some membrane-bound enzymes (*12,49–51*). Because the endoplasmic reticulum of hepatic cells in aged rats showed distorted shapes in response to a hot environment, microsomal electron transport systems, such as cytochrome P450 monooxygenase activities, were seriously affected in hyperthermia.

Because liver should be one of the target organs of heat stress, the biochemical impacts of heat stress on liver functions need to be evaluated. In this study, heat stress seriously injured hepatic endoplasmic reticulum and inhibited some microsomal monooxygenase activities in aged rats. Hepatic microsomal monooxygenase is vital for the metabolism of endogenous and exogenous lipophilic substrates such as steroids and xenobiotics (*52*). Because hot environments greatly damage hepatic microsomal electron transport systems in aged rats, it seems reasonable that heat stress has the potential to cause peroxidative damage in both aged animals and aged humans.

In young and aged rats, distortion of hepatic mitochondria was observed in response to a hot environment; still the activities of hepatic mitochondrial electron transport systems, such as cytochrome c oxidase and cytochrome c reductase systems, were not inhibited by acute hyperthermia. In chronic exposure to heat stress, however, cytochrome c oxidase and cytochrome c reductase systems were simultaneously inhibited.

Because hepatic GSH-peroxidase activities were also not induced in the guinea pig, lipid peroxidation was greatly induced not only in liver homogenate but also in intracellular structures such as mitochondria and microsomes. Therefore, the activities of hepatic mitochondrial electron transport systems were simultaneously inhibited in hyperthermic guinea pigs (*11*).

In aged rats, GSH peroxidase in the kidney was not affected by hot environments, whereas catalase activities were markedly decreased. Therefore, lipid peroxidation in the kidney of aged rats was also significantly induced by heat stress.

It is well known that heat shock response activates heat shock proteins that protect cellular function from destabilization (*53–57*). From *in vivo* experiments, 90-kDa heat shock-inducible proteins were markedly expressed in the guinea pig liver in hyperthermia (*11*). In this study, 70-kDa heat shock inducible protein synthesis was markedly enhanced in isolated hepatic cells and in PMNs.

In conclusion, heat stress-inducible oxygen radical damage becomes more severe according to the age of rats. Aged rats have several times more basal lipid peroxides in liver than young rats. Furthermore, in hyperthermia aged rats also have several times more lipid peroxides than at normal temperature. Therefore, aging and hyperthermia have a synergistic effect on lipid peroxidation. The same result was reported in gerbils: one of the important biological oxygen radical formations, ischemia/repurfusion insult, was more lethal to old gerbils than young gerbils (*58*).

Progress of aging has a potent induction of oxygen free radical formation; therefore, protective enzyme activities for oxygen free radicals may be essential for surviving and recovering from thermal injury in aged persons. Further study is necessary to clarify the biochemical relationship between antioxidant defense systems and the adaptability to oxygen free radical damage in hyperthermia in aged humans and animals.

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