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Heat exposure and hypothyroid conditions decrease hydrogen peroxide generation in liver mitochondria

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1. Exposure of rats to heat (39 ± 1°C) decreased H_2O_2 generation in mitochondria of the liver, but not of the kidney or the heart. 2. The effect was obtained with three substrates, succinate, glycerol 1-phosphate and choline, with a decrease to 50% in the first 2–3 days of exposure, and a further decrease on longer exposure. 3. The dehydrogenase activity with only glycerol 1-phosphate decreased, which is indicative of the hypothyroid condition, whereas choline dehydrogenase activity remained unchanged and that of succinate dehydrogenase decreased on long exposure. 4. The serum concentration of thyroxine decreased in heat-exposed rats. 5. Thyroxine treatment of rats increased H_2O_2 generation. 6. Hypothyroid conditions obtained by treatment with propylthiouracil or thyroidectomy caused a decrease in H_2O_2 generation and changes in dehydrogenase activities similar to those with heat exposure. 7. Treatment of heat-exposed or thyroidectomized rats with thyroxine stimulated H_2O_2 generation by a mechanism apparently involving fresh protein synthesis. 8. The results indicate that H_2O_2 generation in mitochondria of heat-exposed animals is determined by thyroid status.

Exposure of animals to temperature above the thermoneutral zone would be expected to depress the thermogenic mechanisms, as the animals no longer need to generate heat for the purpose of maintaining body temperature. Cellular thermogenesis in endotherms is considered to be under the control of thyroxine and noradrenaline. A major role had been proposed for thyroxine in temperature adaptation and for noradrenaline in non-shivering thermogenesis. [for reviews see Jansky (1973) and Himms-Hagen (1976)]. On exposure of animals to heat stress, thyroid activity (Yousef & Johnson, 1968) and noradrenaline synthesis (Shum *et al.*, 1969) were found to decrease. Thus several of the responses to heat exposure may be obtained indirectly through these hormones. The biochemical changes occurring in response to heat stress have been studied to understand the process of adaptation. In men exercising at a higher ambient temperature, Francesconi & Mager (1978) reported that plasma concentrations of creatine kinase increased, whereas lactate dehydrogenase and serum transaminases were unaffected. In immature chicks exposed to heat both ATP citrate lyase and phosphofructokinase increased (Moss & Balnave, 1978). In deer mice subjected to heat, activities of serine dehydratase and threonine dehydratase decreased (Roberts & Chaffee, 1976). Heat-exposed hamsters showed decreased activities in kidney of glycerol phosphate dehydrogenase (Arine *et al.*, 1973). In liver, rates of oxidation of succinate, glutamate and β-hydroxybutyrate (Cassuto & Chaffee, 1966; Roberts & Chaffee, 1976) and of glycogen breakdown, glucose production and glucose-6-phosphatase (Chayoth & Cassuto, 1971, 1972) decreased. In a comprehensive study on the changes in mitochondrial oxidative metabolism in livers of rats under conditions of heat stress, Swaroop & Ramasarma (1982) showed that rates of oxidation of succinate and glutamate (+ malate) decreased without affecting respiratory control index and ADP/O ratio, and also uncoupler-stimulated ATPase activity. In such animals the concentrations of hepatic mitochondrial cytochrome *c* and cytochrome $a + a_3$ and of ubiquinone in the liver tissue were lower compared with controls at ambient temperature. Work in our laboratory had shown that alternative shunt pathways of electron transport, represented by ubiquinone-dependent succinate–neotetrazolium reductase (Aithal & Ramasarma, 1971) responded to temperature stress with remarkable correlation with needs of thermogenesis (Ramasarma & Sivaramakrishnan, 1978). Mitochondrial H_2O_2

generation supported by various substrates that donate electrons to ubiquinone through their respective dehydrogenases (Boveris *et al.*, 1972; Swaroop & Ramasarma, 1981*a*) is another ubiquinone-dependent (Boveris *et al.*, 1976) shunt pathway [for reviews see Chance *et al.* (1979) and Ramasarma (1982)]. Treatment of rats with noradrenaline stimulated H_2O_2 generation, and the hormone was shown to act through the α_2-adrenergic system (Swaroop *et al.*, 1983). In a preliminary study we found that choline-dependent H_2O_2 generation decreased, but not choline dehydrogenase activity, in animals under heat exposure (Swaroop & Ramasarma, 1981*b*). In the present paper, the results of the detailed investigation on the response of H_2O_2 generation to heat exposure and altered thyroid status of rats are given.

Materials and methods

Materials

Thyroxine was obtained from BDH Chemicals, Poole, Dorset, U.K. Scopoletin, antimycin A, horseradish peroxidase (type II), 6-propyl-2-thiouracil and other biochemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. The radioimmunoassay kit for thyroxine was obtained from Shabha Atomic Research Centre, Bombay, India. Deionized, double-quartz-distilled water was used for all solutions.

Animals, treatments and enzyme assays

Male albino rats of the Wistar strain (90–120g) were used. They were fed on standard Hindustan–Lever pellet diet *ad libitum*. All the animals in experimental and control groups were treated identically. Any effects of stress during treatment are common between the control and the experimental groups, which were simultaneously analysed. Results are means ± S.D. for independent analyses of four to six rats in each group. The level of significance was calculated by Student's *t* test, and a *P* value less than 0.05 was considered significant.

Animals were exposed to heat stress in a ventilated heat chamber maintained at 39 ± 1°C. At this temperature rats were alive even after 2 months of exposure. Control rats were kept at ambient temperature (day 20–25°C, night 10–15°C).

Thyroxine (1 mg/kg body wt.) was injected into rats intraperitoneally as a solution in 0.9% NaCl containing 0.005M-KOH, and control rats received only 0.9% NaCl. In the experiments in Table 2, one group of rats was killed 12h after a single dose and another after receiving one dose/day for 3 days and 24h after the last dose. In the experiments in Table 3, one single dose was given 12h before killing in the experiments at ambient temperature, and in heat-exposure experiments one dose/day was given for 3 days and the last one 24h before killing.

Propylthiouracil (100mg/day per kg body wt.) was injected into rats intraperitoneally in 0.5% (w/v) gelatin for 7 days, and controls received only gelatin. The rats were killed 24h after the last dose.

Thyroidectomy was performed by standard surgical procedures, and sham-operated rats were used as controls. In the experiments in Table 2, groups of rats were killed at 7 or 27 days after thyroidectomy. In the experiments in Table 3, groups of rats were used at 18 and 12 days after thyroidectomy for experiments at ambient temperature and with heat exposure, respectively.

Cycloheximide (2mg/kg body wt.) was injected to rats intraperitoneally. Two doses were given, at 12h and 6h before killing.

The rats were killed between 10:00 and 11:00h by cervical dislocation, and the livers (and other tissues where mentioned) were processed for preparation of mitochondria as described by Kurup *et al.* (1970). The following enzyme activities were measured in mitochondrial samples: H_2O_2 generation with three substrates (0.05mM-succinate, 10mM-glycerol 1-phosphate or 0.13mM-choline), succinate dehydrogenase (Bernath & Singer, 1962), glycerol-1-phosphate dehydrogenase (Ruegamer *et al.*, 1964) and choline dehydrogenase (Rendina & Singer, 1959). Mitochondrial protein was measured by the biuret method (Gornall *et al.*, 1949) in the presence of 0.1% sodium deoxycholate.

Thyroxine radioimmunoassay

Radioimmunoassay of thyroxine was carried out as described by Abraham (1977). All the freeze-dried preparations in the kit were reconstituted in 0.08M-barbitone buffer (pH 7.6) containing NaN_3 (0.02%, w/v) and/or bovine serum albumin. The protein-bound thyroxine was released by adding anilinonaphthalenesulphonic acid, and this was allowed to compete with labelled antigen to bind the specific antibody. All the assays were done in duplicate.

Results

Effect of heat exposure on mitochondrial H_2O_2 generation

Rats exposed to heat stress were killed after a period of 5 days, and mitochondria were prepared from liver, kidney and heart. The rates of H_2O_2 generation measured with succinate as the substrates are given in Table 1; only liver mitochondria showed a significant decrease in H_2O_2 generation at the time period tested.

Table 1. *Effect of heat exposure on mitochondrial H_2O_2 generation in liver, kidney and heart*
Groups of rats were exposed to heat for 5 days. Mitochondria isolated from liver, kidney and heart tissues were tested for generation of H_2O_2 with succinate as the substrate. Control rats kept at ambient temperature were simultaneously tested. ‡$P<0.05$.

Tissue	H_2O_2 generation (nmol/min per mg of mitochondrial protein)	
	Control	Heat-exposed
Liver	0.31 ± 0.03	0.13 ± 0.04‡
Kidney	0.76 ± 0.06	0.81 ± 0.12
Heart	0.93 ± 0.03	0.96 ± 0.17

In the second set of experiments, this effect in liver mitochondria was shown to be present even after 2 days of heat exposure. Similar decreases were found with two other substrates, glycerol 1-phosphate and choline. The corresponding dehydrogenase activity for each of the three substrates was also measured in these mitochondria. Choline dehydrogenase remained unchanged, succinate dehydrogenase decreased by 36% by 6 days and glycerol-1-phosphate dehydrogenase showed a very large decrease of 67% in 6 days (Table 2*a*). It is well known that glycerol-phosphate dehydrogenase is dependent on thyroxine status, and the observed decrease is consistent with the hypothyroid condition obtained in heat stress.

A progressive decrease of H_2O_2 generation with all the three substrates was obtained on continuous exposure to heat for a period of 12 days (Fig. 1). These results also reveal a trend in the changes: a decrease by about 50% in the first 2–3 days, and a further decrease after 5–6 days of exposure. Even after prolonged exposure for over 40 days, the activity remained at about 30% and was never abolished (results not shown).

Effect on serum thyroxine concentration

Decreased thyroid activity (Yousef & Johnson, 1968) and thyroxine excretion (Hutchins, 1969) had been reported in rats exposed to heat, but these conclusions were based on estimation of protein-bound iron or clearance of [^{131}I]thyroxine. Concentrations of serum thyroxine measured by radioimmunoassay were found to decrease by 30–50% of the control values of $6.4 \pm 0.2\,\mu g/100\,ml$ during heat exposure for 1–3 days. This is the first direct demonstration of decreased circulating thyroxine concentration in heat exposure.

Effect of thyroxine treatment

It was decided to test the effect of treatment of normal intact animals with thyroxine. Generation

Table 2. *Effect of heat exposure, thyroxine treatment, propylthiouracil treatment and thyroidectomy on H_2O_2 generation and dehydrogenase activities in liver mitochondria*
Details of treatment and assays with the three substrates are given in the Materials and methods section. *$P<0.01$, ‡$P<0.05$.

	Experiment	Period of treatment (days)	Dehydrogenase activity (ng-atoms of O/min per mg of protein)			H_2O_2 generation (nmol of H_2O_2/min per mg of protein)		
			Succinate	Glycerol 1-phosphate	Choline	Succinate	Glycerol 1-phosphate	Choline
(*a*)	Control	–	222 ± 12	14.0 ± 1.0	116 ± 10	0.46 ± 0.04	0.49 ± 0.03	0.42 ± 0.02
	Heat exposure	2	196 ± 8	10.6 ± 0.4*	105 ± 16	0.25 ± 0.04*	0.28 ± 0.05*	0.24 ± 0.03*
		6	141 ± 42‡	4.6 ± 0.8*	125 ± 6	0.23 ± 0.05*	0.21 ± 0.04*	0.14 ± 0.01*
(*b*)	Control	–	202 ± 14	14.8 ± 1.2	110 ± 18	0.43 ± 0.04	0.45 ± 0.03	0.48 ± 0.02
	Thyroxine treatment	0.5	258 ± 44	22.4 ± 5.4‡	126 ± 10	0.55 ± 0.01‡	0.64 ± 0.09‡	0.58 ± 0.02‡
		4	320 ± 38‡	74.2 ± 3.2*	108 ± 14	0.92 ± 0.09‡	0.75 ± 0.04*	0.69 ± 0.02*
(*c*)	Control	–	202 ± 18	13.2 ± 0.6	110 ± 8	0.46 ± 0.05	0.40 ± 0.04	0.44 ± 0.02
	Propylthiouracil treatment	7	180 ± 32	7.0 ± 0.4*	120 ± 22	0.17 ± 0.13*	0.09 ± 0.02*	0.26 ± 0.08
(*d*)	Control	–	248 ± 19	15.2 ± 1.0	98 ± 8	0.39 ± 0.03	0.40 ± 0.04	0.44 ± 0.02
	Thyroidectomy	7	236 ± 20	3.0 ± 1.6*	98 ± 8	0.23 ± 0.03*	0.14 ± 0.03*	0.26 ± 0.03*
		27	208 ± 18	2.8 ± 0.4*	101 ± 20	0.18 ± 0.06*	0.08 ± 0.01*	0.17 ± 0.03*

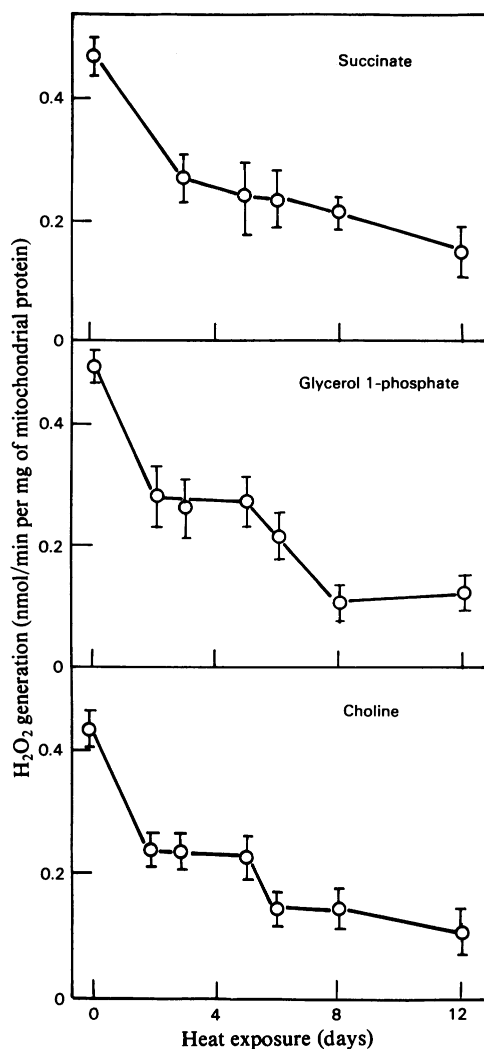

Fig. 1. *Effect of period of heat exposure on H_2O_2 generation by mitochondria*

Groups of rats were exposed to heat for the indicated periods up to 12 days. Liver mitochondria were used for measuring H_2O_2 generation with succinate, glycerol 1-phosphate or choline as the substrate. At all time intervals tested the decreases were significant ($P < 0.01$).

of H_2O_2 with the three substrates tested increased significantly at 12h after a single dose of thyroxine (Table 2*b*). On long-term treatment with repeated doses for 4 days the effect was more marked. In contrast, the dehydrogenase activity was unaffected with choline as the substrate, increased at 4 days with succinate as the substrate, and increased significantly at both time intervals with glycerol 1-phosphate as the substrate. The last observation confirms the hyperthyroid status of the animals. Also that the change in H_2O_2 generation was not due to a change in the dehydrogenase activity is implicit from these results.

Effect of hypothyroid conditions

It followed that the rate of H_2O_2 generation may directly correlate with thyroid status, and the decrease obtained in heat exposure was due to the hypothyroid status of the animals. This is confirmed under two other hypothyroid conditions produced by propyluracil treatment or thyroidectomy.

A large decrease in H_2O_2 generation was found in animals treated with propyluracil for 7 days with all the substrates tested. As expected, only the dehydrogenase activity with glycerol-1-phosphate decreased, but not that with succinate or choline (Table 2*c*). Similar changes were observed in thyroidectomized animals tested 7 and 27 days after the operation. The sham-operated rats did not show any changes (Table 2*d*). These results indicate that the decrease in H_2O_2 generation follows the decrease in thyroid activity in heat exposure.

Effect of thyroxine treatment of thyroidectomized or heat-exposed animals

In the next set of experiments, the effect of thyroxine treatment of animals that were rendered hypothyroid either by thyroidectomy and/or by heat exposure was tested to see if thyroxine alone was capable of restoring the decreased H_2O_2 generation. In all these experiments a single dose of thyroxine 12h before killing was able to increase H_2O_2 generation by 2–3-fold. The actual rate of H_2O_2 generation with either choline or glycerol 1-phosphate as the substrate was the same as in intact animals on thyroxine treatment in all the heat-exposed animals, whereas in thyroidectomized animals the values of ambient controls (about 0.4nmol/min per mg of protein) were reached (Table 3). In one set of animals, treatment with cycloheximide along with thyroxine prevented the restoration of H_2O_2 generation (glycerol 1-phosphate as the substrate) in thyroidectomized animals (Table 3). This result indicated that fresh protein synthesis is involved in the thyroxine-dependent increase in H_2O_2 generation.

Discussion

A significant observation made in this study is the selective response of mitochondrial H_2O_2 generation in the liver tissue in animals exposed to heat or with altered thyroid status. The response was independent of the effect on respiratory-chain dehydrogenases, which is most significant. Glycerol-1-phosphate dehydrogenase concentration was

Table 3. *Effect of thyroxine on H_2O_2 generation in liver mitochondria of thyroidectomized and heat-exposed rats*
Details of treatment are given in the Materials and methods section. The period of thyroxine treatment was 12h in the experiments at ambient temperature, and 3 days (3 doses), the last one 24h before killing, in heat-exposure experiments. Number of days after thyroidectomy or of heat exposure are shown in parentheses. *$P<0.01$.

	H_2O_2 generation (nmol/min per mg of protein)			
	Choline		Glycerol 1-phosphate	
Treatment of animals	Control	Thyroxine treated	Control	Thyroxine treated
Intact	0.04±0.02	0.64±0.07*	0.42±0.04	0.75±0.04*
Thyroidectomized (18 days)	0.21±0.03	0.41±0.03*	0.18±0.05	0.43±0.03*
Thyroidectomized (18 days) +cycloheximide	–	–	0.28±0.04	0.21±0.09
Heat exposure (3 days)	0.22±0.04	0.66±0.05*	0.23±0.05	0.68±0.06*
Heat exposure (3 days) of thyroidectomized (12 days)	0.22±0.04	0.79±0.04*	0.13±0.03	0.74±0.08*

altered in the present experiments, as expected from a changed thyroid status (Lee *et al.*, 1959). Our results also establish that thyroxine is responsible for maintaining mitochondrial H_2O_2 generation, and the thyroid status of the animal determines this activity.

Thyroxine added in the assay system actually inhibited H_2O_2 generation (Swaroop & Ramasarma, 1981*a*). Also the effects observed in the treated animals were seen in isolated mitochondria and therefore represent stable altered activities, and cannot be due to a possible increase in concentration of thyroxine in liver cells or form a part of the proposed direct action of thyroxine on mitochondrial membrane receptors (Sterling *et al.*, 1977), which itself was in doubt (Wahl *et al.*, 1977). Thyroid hormones were found to increase protein synthesis in liver, including mitochondria, by stimulating total RNA synthesis (Tata, 1967; Eberhardt *et al.*, 1980). The thyroxine effect on H_2O_2 generation may include one of these proteins, and its sensitivity to the protein-synthesis inhibitor cycloheximide is suggestive of extramitochondrial action. The cycloheximide effect also indicates that a specific protein in the H_2O_2-generator system will be the target in these thyroxine-stimulated changes, independent of dehydrogenases.

The effects of thyroxine on H_2O_2 generation are parallel to those obtained with the other thermogenic hormone, noradrenaline (Swaroop *et al.*, 1983). The specific effect on H_2O_2 generation, with the three substrates succinate, glycerol 1-phosphate and choline, and the lack of effect on the dehydrogenase activity with either choline or succinate and the increase with glycerol 1-phosphate, were all similar. Cycloheximide treatment prevented thyroxine-stimulated activity, whereas a decrease, but not prevention, was found in the noradrenaline effect. Adrenergic receptors of the α-type were implicated in the noradrenaline effect. It is possible that the two hormones may have independent actions, albeit on the same target, and it remains to be seen if they act through a common factor.

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