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Noradrenaline treatment of rats stimulates H_2O_2 generation in liver mitochondria

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1. Treatment of rats with noradrenaline stimulated H_2O_2 generation in liver mitochondria using succinate, choline or glycerol 1-phosphate as substrate. The dehydrogenase activity with either succinate or choline as substrate showed no change, whereas that with glycerol 1-phosphate increased. 2. The effect was obtained with noradrenaline, but not with dihydroxyphenylserine. 3. Phenoxybenzamine and yohimbine, but not propranolol, prevented the response to noradrenaline treatment. 4. Phenylephrine could stimulate H_2O_2 generation, whereas isoprenaline had only a marginal effect. 5. Theophylline treatment slightly decreased the generation of H_2O_2 in liver mitochondria, but treatment with pargyline, Ro4-1284 and dibutyryl cyclic AMP had little effect. 6. These studies showed that noradrenaline might possibly be acting through the α_2-adrenergic system.

A variety of cells and cellular organelles have been shown to generate H_2O_2 in significant amounts (for reviews, see Chance *et al.*, 1979; Ramasarma, 1982). Generation of H_2O_2 in biomembranes is now recognized to be a physiological process and H_2O_2 cannot be dismissed as a mere toxic intermediate in oxidative metabolism. Cellular H_2O_2 has been assigned more roles in phenomena such as phagocytosis (Dri *et al.*, 1979; Chance *et al.*, 1979), synthesis of thyroid hormones (Taurog, 1970) and prostaglandins (Morse *et al.*, 1977; Porter *et al.*, 1977), a possible second messenger role for insulin (May & de Haen, 1979; Mukherjee, 1980) and oxytocin (Mukherjee & Mukherjee, 1982), thermogenesis (Rich *et al.*, 1976; Swaroop & Ramasarma, 1981*a*) and in the parasitism of protozoa [for review, see Weinbach (1981) and Ramasarma (1982)].

Mitochondrial H_2O_2 generation can be supported by various substrates that donate electrons to ubiquinone in the respiratory chain through the respective dehydrogenases but not by ascorbate or xanthine (Boveris *et al.*, 1972; Swaroop & Ramasarma, 1981*b*). In spite of large differences in the rates of dehydrogenase activity, the rate of H_2O_2 generation was the same with succinate, choline, glycerol 1-phosphate or proline as substrates, and with a mixture of these substrates there was no additivity of the rates. These and other properties led to the suggestion that the H_2O_2 generator was a common one and that only a small part of the electron-transport chain was available for this pathway (Boveris *et al.*, 1976). One other property of the H_2O_2 generator system was the requirement of antimycin A and an uncoupler (Boveris & Chance, 1973) and the locus of electron transfer to oxygen seemed to be ubiquinone (Boveris *et al.*, 1976).

We have demonstrated that H_2O_2 generation in mitochondria responds to altered thyroid status and thermogenic conditions (Swaroop & Ramasarma, 1981*a*). In the present paper, we show that treatment of animals with noradrenaline, a thermogenic hormone, stimulated H_2O_2 production in the liver mitochondria. This is the first report showing the hormonal regulation of mitochondrial H_2O_2 generation and the involvement of an α-adrenergic mechanism in effects of catecholamines on liver oxidative metabolism.

Materials and methods

Animals and treatment

Male albino rats of Wistar strain (90–120g) were used throughout the studies. They were fed on standard Hindustan–Lever pellet diet *ad libitum*. All the animals in experimental and control groups were treated identically. The compounds were administered at specified doses intraperitoneally in 0.9% NaCl, either in solution or in suspension and the controls received equivalent amounts of 0.9% NaCl alone. All killings were done between 10:00 and 12:00h. Any effects of stress during treatment are common between the control and the experi-

mental groups, which were simultaneously analysed. Results are means ± S.D. of 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 of <0.05 was considered significant.

Measurement of enzyme activities

Liver mitochondria were obtained by differential centrifugation in 0.25 M-sucrose by the method described by Kurup *et al.* (1970). H_2O_2 generation in mitochondria was measured by the scopoletin/horseradish peroxidase method (Loschen *et al.*, 1971) in phosphate buffer, as described previously (Swaroop & Ramasarma, 1981*b*). Activities of various dehydrogenases were determined by the manometric method using phenazine methosulphate as follows: succinate as substrate (Bernath & Singer, 1962); choline as substrate (Rendina & Singer, 1959); glycerol 1-phosphate as substrate (Ruegamer *et al.*, 1964). Mitochondrial protein was estimated by the biuret method (Gornall *et al.*, 1949) in the presence of 0.1% sodium deoxycholate.

Chemicals

All the biochemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., unless otherwise mentioned. Other chemicals were obtained as follows: noradrenaline (Koch–Light Laboratories, Colnbrook, Bucks., U.K.); propranolol, phenylephrine and isoprenaline (gifts from Dr. R. M. Marchbanks, Institute of Psychiatry, London S.E.5, U.K.); phenoxybenzamine (Smith, Kline and French, Bangalore, India); pargyline (Abbot Co., Chicago, IL, U.S.A.); Ro4-1284 (Roche Products, Bombay, India). All other common reagents were of analytical grade.

Results

Effect of noradrenaline treatment on H_2O_2 generation

In the first set of experiments, rats were treated with noradrenaline (2 mg/kg body wt., intraperitoneal injection), killed after 4 h and the livers were processed to obtain mitochondria. With succinate, choline or glycerol 1-phosphate as substrate, the rate of H_2O_2 generation in liver mitochondria increased significantly in noradrenaline-treated rats compared with controls (Table 1). The extent of stimulation of H_2O_2 generation was nearly the same with the three substrates. The effect of noradrenaline treatment appears to be on a common component of the H_2O_2-generator system, as this was uniformly affected with all the three substrates. However, the activities of the dehydrogenases, measured by the reduction of the dye phenazine methosulphate, with these substrates behaved differently. Activities of choline dehydrogenase and succinate dehydrogenase did not show significant change, whereas glycerol 1-phosphate dehydrogenase activity increased by 80% in noradrenaline-treated animals (Table 1). In view of this, results with choline or succinate and glycerol 1-phosphate were presented in further experiments.

Time course of stimulation of H_2O_2 generation

Fig. 1 shows the time course of the stimulation of H_2O_2 generation in liver mitochondria from rats treated with a single dose of noradrenaline. Increase in mitochondrial H_2O_2 generation was observed 2 h after the intraperitoneal injection of noradrenaline. A maximum of an approx. 2-fold increase was obtained 4 h after the treatment and it decreased thereafter and reverted to the basal value by 12 h.

Effect of different concentrations of noradrenaline

The stimulation of H_2O_2 generation increased with increasing concentration of noradrenaline and reached a maximum at 2 and 2.5 mg/kg body wt. for succinate and glycerol 1-phosphate as substrates respectively (Fig. 2). The effective concentration of about 2 mg/kg body wt. was in the same range as that obtained in the previous experiments which elicited effects on enzymes (Sitaramam & Ramasarma, 1974; George & Ramasarma, 1977),

Table 1. *Effect of noradrenaline treatment on H_2O_2 generation and dehydrogenase activities in liver mitochondria*
Noradrenaline (2 mg/kg body wt.) was injected intraperitoneally as a solution in 0.9% NaCl to the rats and the animals were killed after 4 h. Control animals received injections of 0.9% NaCl. Units of activity for H_2O_2 generation were nmol of H_2O_2/min per mg of protein and for dehydrogenase were ng-atoms of oxygen/min per mg of protein.

Substrate	Enzyme activity		Change (%)	*P* value
	−Noradrenaline	+Noradrenaline		
H_2O_2 generation				
Succinate	0.37 ± 0.03	0.66 ± 0.10	178	<0.01
Choline	0.44 ± 0.02	0.81 ± 0.04	184	<0.001
Glycerol 1-phosphate	0.40 ± 0.04	0.76 ± 0.06	190	<0.001
Dehydrogenase				
Succinate	307 ± 17	354 ± 81	116	NS
Choline	159 ± 3	184 ± 16	115	NS
Glycerol 1-phosphate	14 ± 1	25 ± 6	178	<0.02

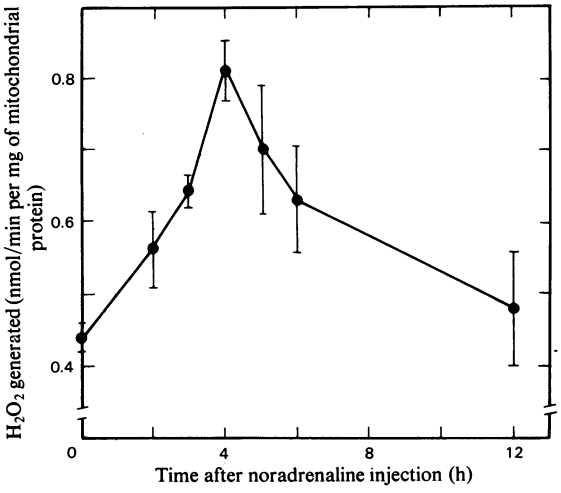

Fig. 1. *Time course of noradrenaline-dependent increase in mitochondrial H_2O_2 generation*
Noradrenaline (2mg/kg body wt.) was injected in 0.9% NaCl intraperitoneally as one dose and the rats were killed at the time intervals indicated. One group of rats given 0.9% NaCl alone served as a control.

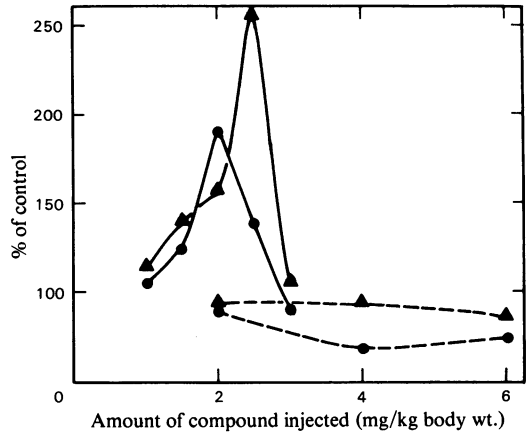

Fig. 2. *Effect of concentration of noradrenaline and dihydroxyphenylserine on mitochondrial H_2O_2 generation*
Noradrenaline (1–3mg/kg body wt.) or dihydroxyphenylserine (2–6mg/kg body wt.) in 0.9% NaCl were injected intraperitoneally as one dose and the rats were killed after 4h. One group of rats given 0.9% NaCl alone served as a control and mean values as a percentage of the value in the control group are plotted. ——, Noradrenaline; – – – –, dihydroxyphenylserine; ●, succinate as substrate; ▲, glycerol 1-phosphate as substrate.

but the lack of effect at high concentration on the H_2O_2 generation remains to be explained.

Pargyline, a monoamine oxidase inhibitor that prevents degradation of noradrenaline (Hellerman & Erwin, 1968), and the drug Ro4-1284, which increases the concentration of noradrenaline at the site by enhancing the rate of its release (Pletscher *et al.*, 1962), were unable to show stimulation of H_2O_2 generation. Thus a critical concentration at the site of action seems to be required for obtaining the effect on H_2O_2 generation.

Effect of 3,4-dihydroxyphenylserine and adrenaline

Treatment of animals with 3,4-dihydroxyphenylserine did not have any affect on H_2O_2 generation in liver mitochondria (Fig. 2). 3,4-Dihydroxyphenylserine is known to bypass the plasma membrane, become decarboxylated by L-amino acid decarboxylase present in liver cytosol (Diarman *et al.*, 1972) and increase the intracellular levels of noradrenaline (McCann *et al.*, 1972). These results showed noradrenaline action to be at the level of plasma membrane, possibly involving specific adrenergic receptors.

Another vasoactive amine adrenaline, which had several common effects with noradrenaline, failed to elicit this response when injected into animals at a dose of 0.25mg/kg body wt. (Table 2). Higher doses of adrenaline were not tested owing to its severe toxic effects.

Liver mitochondria from adrenalectomized rats did not show any change in H_2O_2 generation compared with sham-operated control group (Table 2), suggesting that the basal level of mitochondrial H_2O_2 generation was not dependent on adrenal hormones.

Effect of adrenergic agonists

Phenylephrine and isoprenaline are well-known specific agonists for adrenergic receptors (Schmelck & Hanoune, 1980). Phenylephrine, an α-agonist, increased the generation of H_2O_2 in liver mitochondria in treated rats (Table 2). Isoprenaline, a β-agonist, showed an increase of only 20%, a difference of marginal statistical significance ($P = 0.05$) (Table 2).

Effect of theophylline

Treatment with theophylline, which is known to increase intracellular cyclic AMP levels by inhibiting phosphodiesterase (Wells & Kramer, 1981), showed a small but significant decrease in mitochondrial H_2O_2 generation (Table 2), but this effect may not be due to increased cyclic AMP as administration of dibutyryl cyclic AMP directly elicited little change (Table 2).

Table 2. *Effect of adrenergic agonists and cyclic AMP*

Groups of rats were given various compounds as a single dose by intraperitoneal injection in 0.9% NaCl. Control groups given 0.9% NaCl were simultaneously tested. Animals were killed 4 h after the injection. The results are also expressed as percentages of control group in each experiment. Adrenalectomy was performed by standard surgical procedure and rats were maintained for 7 days on 0.9% NaCl instead of water before the experiment. Abbreviation used: NS, not significant.

Treatment of animals		H_2O_2 generation (nmol/min per mg of mitochondrial protein)			
Compound injected	Dose (mg/kg body wt.)	Control	Experimental	Change (%)	*P* value
Choline as substrate					
Adrenaline	0.25	0.48 ± 0.02	0.50 ± 0.04	104	NS
Adrenalectomy	–	0.48 ± 0.02	0.51 ± 0.06	106	NS
Phenylephrine	5	0.38 ± 0.04	0.65 ± 0.03	171	<0.001
Isoprenaline	40	0.49 ± 0.02	0.60 ± 0.05	122	0.05
Pargyline	25	0.30 ± 0.07	0.33 ± 0.10	110	NS
Ro4-1284	10	0.30 ± 0.07	0.25 ± 0.05	83	NS
Theophyline	50	0.47 ± 0.01	0.37 ± 0.02	79	<0.001
Dibutyryl cyclic AMP	15	0.30 ± 0.07	0.29 ± 0.06	97	NS
Glycerol 1-phosphate as substrate					
Adrenaline	0.25	0.51 ± 0.05	0.56 ± 0.05	110	NS
Adrenalectomy	–	0.51 ± 0.05	0.48 ± 0.05	94	NS
Phenylephrine	5	0.37 ± 0.02	0.53 ± 0.04	143	<0.002
Isoprenaline	40	0.46 ± 0.04	0.51 ± 0.02	111	NS
Pargyline	25	0.35 ± 0.10	0.37 ± 0.08	106	NS
Ro4-1284	10	0.35 ± 0.10	0.35 ± 0.08	100	NS
Theophyline	50	0.42 ± 0.02	0.32 ± 0.02	76	<0.002
Dibutyryl cyclic AMP	15	0.35 ± 0.10	0.34 ± 0.09	97	NS

Table 3. *Effect of treatment with adrenergic blockers and cyclohexamide*

Phenoxybenzamine (20 mg/kg body wt.), propranolol (25 mg/kg body wt.) and yohimbine (2.5 mg/kg body wt.) were given separately as one dose intraperitoneally 20 min before the injection of noradrenaline (2 mg/kg body wt.). Animals were killed 4 h after the noradrenaline treatment. Control rats received a 0.9% NaCl solution.

Treatment of animals		H_2O_2 generation (nmol/min per mg of mitochondrial protein)			
Compound injected	Dose (mg/kg body wt.)	−Noradrenaline	+Noradrenaline	Change (%)	*P* value
Choline as substrate					
0.9% NaCl		0.36 ± 0.03	0.57 ± 0.02	158	<0.001
Phenoxybenzamine	20	0.40 ± 0.03	0.35 ± 0.02	88	NS
0.9% NaCl		0.51 ± 0.02	0.73 ± 0.02	143	<0.001
Propranolol	25	0.57 ± 0.01	0.87 ± 0.06	153	<0.001
0.9% NaCl		0.42 ± 0.04	0.75 ± 0.09	178	<0.001
Yohimbine	2	0.47 ± 0.03	0.57 ± 0.04	121	<0.05
0.9% NaCl		0.33 ± 0.03	0.66 ± 0.01	200	<0.002
Cycloheximide	2	0.40 ± 0.05	0.56 ± 0.02	140	<0.002
Glycerol 1-phosphate as substrate					
0.9% NaCl		0.35 ± 0.04	0.51 ± 0.02	146	<0.002
Phenoxybenzamine	20	0.36 ± 0.01	0.39 ± 0.02	108	NS
0.9% NaCl		0.39 ± 0.02	0.68 ± 0.05	174	<0.002
Propranolol	25	0.46 ± 0.03	0.76 ± 0.03	165	<0.002
0.9% NaCl		0.51 ± 0.05	1.08 ± 0.21	212	<0.002
Yohimbine	2	0.59 ± 0.17	0.71 ± 0.12	120	NS
0.9% NaCl		0.35 ± 0.04	0.59 ± 0.01	169	<0.002
Cycloheximide	2	0.36 ± 0.03	0.49 ± 0.02	136	<0.002

Effect of adrenergic-blocking agents

To investigate further the mechanism of stimulation of mitochondrial H_2O_2 generation, two specific antagonists of α- and β-adrenergic receptors, namely phenoxybenzamine and propanolol respectively, were tested. These were given to the animals at appropriate doses 20 min before noradrenaline

treatment. When injected alone to rats (controls), the two adrenergic blocking agents had little effect on H_2O_2 generation in liver mitochondria (Table 3). The noradrenaline-stimulated H_2O_2 generation in liver mitochondria was blocked by phenoxybenzamine, but not by propranolol (Table 3). These results indicated the participation of an α-adrenergic system in noradrenaline action.

Yohimbine, a very potent blocking agent of the α_2-adrenergic mechanism (Schmelck & Hanoune, 1980), also prevented to a large extent the noradrenaline-stimulated generation of H_2O_2 in liver mitochondria (Table 3). Yohimbine given alone to the animals did not show any change. Higher doses of yohimbine were not used because of its toxicity.

Cycloheximide (2mg/kg body wt.), when injected intraperitoneally to the animals 15min before noradrenaline treatment, did not prevent the noradrenaline-stimulated H_2O_2 generation (Table 3). It appeared, therefore, that fresh protein synthesis might not be involved in producing the effect.

Discussion

The results reported in the present study demonstrate that liver mitochondrial H_2O_2 generation is stimulated by treatment of rats with noradrenaline. In all the experiments the concentrations of the compounds chosen for intraperitoneal injection were based on previous enzyme studies in this and other laboratories (Grovier *et al.*, 1969; Sitaramam *et al.*, 1979; George & Ramasarma, 1977). In the case of cholesterol biogenesis and hydroxymethylglutaryl-CoA reductase, stimulation was concentration-dependent and increased up to about 3mg/kg body wt. The requirement of a critical concentration of about 2mg/kg body wt. in the present experiments with decrease in stimulation at high concentration is puzzling. The relationship of the injected noradrenaline and its effective concentration or any of its metabolites in the tissue remains to be clarified. The results with dihydroxyphenylserine, theophylline and Ro4-1284 also indicate that intracellular tissue concentration of noradrenaline may not be involved in the process. This was suggestive of a membrane level of action, which is further supported by the sensitivity of the noradrenaline effect to α-adrenergic blocking agents. The prevention of cortisol induction of tryptophan pyrrolase by treatment of animals with noradrenaline is also shown in this laboratory to be dependent on the α-adrenergic system (Sitaramam *et al.*, 1979). In rat liver parenchymal cells, activation of phosphorylase and gluconeogenesis and inactivation of glycogen synthetase were shown to depend on activation of the α-adrenergic system without involvement of cyclic AMP (Hutson *et al.*, 1976).

Noradrenaline is a thermogenic hormone, known to play an important role in non-shivering thermogenesis (Jansky, 1973; Himms-Hagen, 1976). Also α-receptors have been implicated in the thermogenic process (Zeisberger & Bruck, 1976). Results reported in the present paper and our previous studies (Swaroop & Ramasarma, 1981*a*) show a high correlation between mitochondrial H_2O_2 generation and thermogenesis, with respect to noradrenaline treatment, α-receptor involvement, altered thyroid status and environmental temperature stress. Exposure of animals to conditions of heat stress decreased H_2O_2 generation in liver mitochondria, and exposure to cold stress increased (Swaroop & Ramasarma, 1981*a*). Administration of thyroxine or noradrenaline stimulated, and hypothyroid conditions decreased H_2O_2 generation. In all cases, H_2O_2 generation was measured in mitochondria from livers of treated animals and therefore the changes represent stable, altered activity. Interestingly, the changes in H_2O_2 generation were similar with choline, succinate or glycerol 1-phosphate as substrates. On the other hand, the activities of the corresponding dehydrogenases were unresponsive, with the exception of glycerol 1-phosphate dehydrogenase, which is known to be regulated by thyroid status. Perhaps this is the first report that noradrenaline treatment stimulates glycerol 1-phosphate dehydrogenase activity. However, the results suggest that the action on the H_2O_2-generation system must be independent of the dehydrogenase and seems to be localized to a common component similar to the phenolic acid-sensitive site near ubiquinone (Swaroop & Ramasarma, 1981*b*).

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