Oxidations in Kidney Mitochondria of Heat-Exposed Rats: Regulation by Cytochrome *c*

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Abstract

Exposure of rats to higher environmental temperature (36–37°C) decreased the capacity of their kidney mitochondria to oxidize succinate. The decrease was corrected on the addition of exogenous cytochrome *c*. Kidney mitochondria of heat-exposed animals showed decreased rates of H_2O_2 generation when α-glycerophosphate, but not succinate, was used as electron donor. These mitochondria also showed decreased activity of α-glycerophosphate dehydrogenase but not of succinate dehydrogenase. The content of cytochrome *c* in kidney mitochondria of heat-exposed animals was low even though the concentration of the pigment in the whole tissue did not decrease. Starvation as well as administration of an antithyroid agent like propylthiouracil simulated some of the effects of heat exposure on kidney mitochondria, but the cytochrome *c*-dependent reversal of inhibition of oxidation was obtained only in heat exposure.

Introduction

Exposure of endotherms to heat stress necessitates physiological change to counter the adverse effects of elevated environmental temperature. The lack of a positive temperature differential between the body and the environment poses difficulties in the dissipation of metabolic heat by radiation and convection, leaving only evaporation or salivation as the available method for cooling the body. In the case of animals like the rat, which do not possess sweat glands, aspiratory loss of water from the lungs and salivation provide the cooling. Even when water is not limiting, excessive evaporation may tend to

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concentrate body fluids like the blood and urine (Folk, 1966), and this may lead to cellular damage.

Elevation of environmental temperature lowers the basal metabolic rate (BMR) and decreases the energy needs of the body. Consistent with this, decreased rates of oxidation of substrates by mitochondria were reported in heat-acclimated hamsters and deer mice (Cassuto and Chaffee, 1966; Chayoth and Cassuto, 1972; Roberts and Chaffee, 1976). Detailed investigations are required to elucidate how metabolically active organs adjust their oxidative and energy metabolism during acclimation to heat. Recently it was reported from this laboratory that in hepatic mitochondria of heat-exposed rats, substrate oxidation decreased while coupled phosphorylation remained unaffected (Swaroop and Ramasarma, 1982). In this paper we report that in kidney mitochondria, which show the same effect, addition of exogenous cytochrome c reverses the decrease in respiration, indicating, for the first time, regulation of oxidative phosphorylation by cytochrome c under the condition.

Materials and Methods

Animals and Treatments

Male albino rats (110–120 g) of the Wistar strain obtained from the Central Animal Facility of this Institute were maintained on a commercial Hind Lever pellet diet. Animals of the control group were kept at 22–23°C in a ventilated room. Experimental animals were kept in a ventilated chamber whose inside temperature was maintained at 36–37°C. Food and water were given *ad lib.* for both the groups.

Propylthiouracil was fed to the animals in the diet (0.16%, w/w) for the periods indicated. The pellet diet was powdered before the chemical was mixed. Diet was withdrawn from the experimental animals for 8 days in the experiments on starvation. Water was allowed *ad lib.* Vitamins were given twice a week as described earlier (Chhabra *et al.,* 1979).

Isolation of Mitochondria

At the end of the time period indicated, the animals were killed by cervical dislocation, and mitochondria of the kidney cortex were isolated by differential centrifugation of the homogenate prepared in 0.25 M sucrose containing 2 mg of bovine serum albumin per milliliter (Johnson and Lardy, 1967). The mitochondrial pellet was washed once and used with the minimum of delay. An equal number of control and experimental animals were killed at about 10.00 a.m. in order to minimize errors due to variations.

Assays

Polarographic measurements of oxygen uptake were made using a Gilson K-ICT-C oxygraph fitted with a Clark-type oxygen electrode. The reaction system contained 2 mg of bovine serum albumin per milliliter. Other details were the same as described earlier (Krishna Kantha and Kurup, 1973). Respiratory control index (RCI) was calculated as the ratio of the oxidation rates in state 3 (ADP present) and state 4 (ADP exhausted) as described by Chance and Williams (1955). Activities of succinate dehydrogenase [succinate; (acceptor) oxidoreductase, EC 1.3.99.1] and α-glycerophosphate dehydrogenase [L-glycerol-3-phosphate, oxidoreductase, EC 1.1.99.5)] were measured by the reduction of dichlorophenolindophenol as decrease in absorbance at 600 nm in a reaction system containing catalytic amounts of phenazine methosulfate (King, 1967). The rate of generation of H_2O_2 was measured by the decrease in scopoletin fluorescence in the presence of horseradish peroxidase (Loschen *et al.*, 1971) in a Hitachi–Perkin-Elmer spectrofluorimeter.

The concentrations of cytochromes in mitochondria were estimated by difference spectra [(hydrosulfite reduced $-$ $K_3Fe(CN)_6$ oxidized) (Gazzotti *et al.*, 1980)]. The content of cytochrome *c* in tissue homogenates was determined from difference spectra (reduced $-$ oxidized) of the pyridine hemochrome after repeated treatment with acid-acetone (Rieske, 1967).

Protein was measured by the biuret method (Gornall *et al.*, 1949), deoxycholate being used for solubilization. All biochemicals were purchased from Sigma Chemical Co., St. Louis, Missouri. All other chemicals used were of the purest grades available. Solutions were prepared in water, double-distilled in an all-quartz apparatus. All solutions were adjusted to pH 7.4 before use. Sucrose solutions were prepared fresh daily.

Results

Effects of Heat Exposure

Body and Kidney Weights.   Exposure of rats to heat stress (36–37°C) resulted in drastic decrease in gain in body weight when compared to animals kept at ambient temperature. It is interesting to note that there was no weight gain in the kidneys in heat-exposed animals during the entire experimental period of 30 days (Table I). Similar effects were reported in golden hamsters on prolonged heat exposure (Cassuto and Chaffee, 1966; Arine *et al.*, 1973). During the period of the experiment the kidney weight normalized to 100 g body weight remained remarkably constant at 0.9 ± 0.1 g. This value decreased to 0.75 ± 0.07 g at 20 days and beyond of heat exposure. However,

Table I. Effect of Heat Exposure on Oxidative Phosphorylation in Kidney Mitochondria[a]

Period of heat exposure	Test system	Control	Heat-exposed	Percent control
10 days	Body wt. (g)	158 ± 16	123 ± 17	78[c]
	Kidney wt. (g)	1.5 ± 0.2	1.1 ± 0.1	73[b]
	State 3 oxidation	364 ± 65	301 ± 52	83[c]
	Plus cytochrome c	350 ± 49	383 ± 54	109
	RCI	4.0 ± 0.7	3.1 ± 1.6	103
	ADP/O	1.6 ± 0.2	1.4 ± 0.2	88
15 days	Body wt. (g)	165 ± 6	129 ± 10	78[b]
	Kidney wt. (g)	1.1 ± 0.1	0.8 ± 0.06	73[b]
	State 3 oxidation	443 ± 67	370 ± 45	83[b]
	Plus cytochrome c	467 ± 57	473 ± 72	101
	RCI	4.0 ± 0.8	3.7 ± 0.7	93
	ADP/O	1.7 ± 0.2	1.9 ± 0.2	100
20 days	Body wt. (g)	200 ± 12	136 ± 16	68[b]
	Kidney wt. (g)	1.8 ± 0.15	1.0 ± 0.06	56[b]
	State 3 oxidation	443 ± 67	377 ± 52	85[b]
	Plus cytochrome c	467 ± 57	473 ± 115	101
	RCI	4.0 ± 0.8	3.9 ± 1.0	98
	ADP/O	1.7 ± 0.2	1.6 ± 0.4	94
30 days	Body wt. (g)	211 ± 26	149 ± 8	62[b]
	Kidney wt. (g)	1.8 ± 0.2	1.1 ± 0.1	61[b]
	State 3 oxidation	495 ± 62	391 ± 65	79[b]
	Plus cytochrome c	506 ± 78	437 ± 92	86
	RCI	4.2 ± 0.7	3.7 ± 0.7	88
	ADP/O	1.6 ± 0.2	1.6 ± 0.2	100

[a]Succinate oxidation was determined by polarography (state 3 oxidation). Horse heart cytochrome c (36 μM) was added where indicated. The rate of state 3 oxidation is expressed as ng atoms O/min/mg mitochondrial protein. The rats were exposed to the heat stress for the stated period, and the mitochondria from the kidney were prepared as described in Materials and Methods. The values are mean ± S.D. of 10–16 independent estimations (animals) in each group.
[b]P values <0.01 (significance—heat-exposed vs. control).
[c]P values <0.05.

the protein content (mg/g tissue) did not show any change. It remains to be seen whether these changes reflect decreased need of kidney tissue (and functions) in heat exposure.

Mitochondrial Respiration. A change in energy demands of the cell should be reflected in the oxidative activity of the organelles. Consistent with the lowered BMR in heat exposure (Cassuto, 1968), active oxidation by liver mitochondria was shown to decrease significantly (Swaroop and Ramasarma, 1982). The results in Table I show that state 3 oxidation of succinate by kidney mitochondria was significantly decreased on exposure of the animal to heat. Exposure up to 30 days did not result in recouping of the lost activity.

Neither RCI nor ADP/O was affected by the treatment, indicating the intactness of the membrane as well as the conservation of the phosphorylation mechanism.

The most significant finding in this investigation was the correction on addition of exogenous cytochrome c of the decrease in oxidative activity suffered by kidney mitochondria (Table I). This reversal effect was complete up to 20 days of exposure and was less effective at 30 days (Table I).

H_2O_2 Generation. In recent years there have been increasing attempts to assign a key role for the mitochondrial H_2O_2 generator in thermogenesis (Rich *et al.,* 1976; Ramasarma and Sivaramakrishnan, 1978; Swaroop and Ramasarma, 1981; Ramasarma, 1982). Kidney mitochondria isolated from heat-exposed animals did not show any change in succinate-dependent H_2O_2 generation up to 20 days of exposure but decreased at 30 days of exposure (Table II). In contrast, when α-glycerophosphate was used as electron donor, kidney mitochondria of animals exposed for only 10 days showed a decrease in H_2O_2 generation. The loss of activity increased up to 70% with continued exposure (Table II).

Dehydrogenase Activities. The H_2O_2 generator is presumed to involve the dehydrogenase flavoprotein and the ubiquinone segment of the respiratory chain (Boveris and Chance, 1973; Turrens and Boveris, 1980). The effect of exposure to heat on the activities of the two dehydrogenases was tested. The data in Table III reveal that while succinate dehydrogenase activity of kidney mitochondria was relatively unaffected by exposure of the animal to heat, α-glycerophosphate dehydrogenase activity decreased by 47% in 10 days and by 62% in 30 days.

Table II. Effect of Heat Exposure on H_2O_2 Generation in Kidney Mitochondria[a]

Period of exposure	Substrate	pmol H_2O_2/min/mg protein		Percent control
		Control	Heat exposed	
10 days	Succinate	277 ± 48	276 ± 64	99
	α-Glycerophosphate	183 ± 37	109 ± 14	60[b]
15 days	Succinate	358 ± 77	350 ± 90	98
	α-Glycerophosphate	200 ± 28	78 ± 15	39
20 days	Succinate	270 ± 36	254 ± 14	94
	α-Glycerophosphate	252 ± 14	80 ± 16	32[b]
30 days	Succinate	300 ± 21	172 ± 44	57[b]
	α-Glycerophosphate	197 ± 45	55 ± 19	28[b]

[a]Treatment of animals was the same as given under Table I. Freshly prepared mitochondria (0.4–1.0 mg/protein) used for measuring H_2O_2 generation by fluorimetry. The values given are mean $\pm$ S.D. of 8–12 independent estimations (animals).
[b]Significance—heat exposed vs. control: P values <0.01.

Table III. Effect of Heat Exposure on the Dehydrogenase Activities in Kidney Mitochondria[a]

Period of exposure	Substrate	nmol/min/mg protein		Percent control
		Control	Heat exposed	
10 days	Succinate	283 ± 52	227 ± 21	80
	α-Glycerophosphate	12.9 ± 1.9	6.8 ± 1.4	53[b]
20 days	Succinate	241 ± 15	236 ± 25	98
	α-Glycerophosphate	13.1 ± 2.0	6.7 ± 1.1	51[b]
30 days	Succinate	241 ± 15	291 ± 17	121
	α-Glycerophosphate	11.4 ± 0.5	4.3 ± 0.1	38[b]

[a]Treatment of the animals was the same as given under Table I. The dehydrogenase activities were determined spectrophotometrically. The values given are mean ± S.D. of 8–12 independent estimations (animals).
[b]Significance—heat exposed vs. control: P values <0.01.

Propylthiouracil Treatment

The results in Tables II and III indicate that the changes in rate of generation of H_2O_2 paralleled those of α-glycerophosphate dehydrogenase. It is well known that this enzyme is regulated by thyroxine (Lee et al; 1959; Lee and Lardy, 1965). Treatment of animals with the antithyroid agent, 6-propyl-2-thiouracil, decreased the level of thyroxine in circulation by interfer-

Table IV. Effect of Administration of Propylthiouracil on Oxidation in Kidney Mitochondria[a]

Period of feeding	Test system	Control	Experimental	Percent control
20 days	Body wt. (g)	180 ± 9	145 ± 5	70[c]
	Kidney wt. (g)	1.4 ± 0.1	1.1 ± 0.1	79[c]
	State 3 oxidation	476 ± 43	367 ± 14	77[c]
	Plus cytochrome c	484 ± 68	394 ± 14	81
	H_2O_2 generation	289 ± 29	138 ± 33	48[b]
	α-Glycerophosphate dehydrogenase	19.2 ± 1.2	8.7 ± 1.3	45[b]
30 days	Body wt. (g)	210 ± 23	150 ± 10	71[b]
	Kidney wt. (g)	1.5 ± 0.1	0.9 ± 0.1	60[b]
	State 3 oxidation	405 ± 49	276 ± 44	68[c]
	Plus cytochrome c	415 ± 52	342 ± 47	82
	H_2O_2 generation	140 ± 22	51 ± 16	36[b]
	α-Glycerophosphate dehydrogenase	15.7 ± 2.7	4.8 ± 0.3	31[b]

[a]Experimental animals were fed with propylthiouracil (0.16%, w/w) in the diet for the indicated periods. Succinate was used as the substrate for measuring the rate of state 3 oxidation (ng-atoms O/min/mg protein). α-Glycerophosphate was used as the substrate for measuring the dehydrogenase activity (nmol/min/mg protein) and H_2O_2 generation (pmol/min/mg protein). The values given are mean ± S.D. of three independent estimations (animals).
[b]P values <0.01 (significance—experimental vs. control).
[c]P values <0.05.

ing with iodination of thyroglobulins (Burgi *et al.*, 1976; Taurog, 1976; Davidson *et al.*, 1976). Animals fed with propylthiouracil showed decrease in body and kidney weights and also the activities of α-glycerophosphate-dependent H_2O_2 generation and dehydrogenase (Table IV). It may be noted that oxidation of succinate (measured under phosphorylating conditions) was lowered significantly on treatment with propylthiouracil. However, the reversal achieved on addition of cytochrome c was only partial unlike that obtained in heat stress (compare Tables I and IV).

Starvation Experiments

Decrease in the oxidative activity of liver mitochondria during starvation could be corrected by the addition of exogenous cytochrome c (Rasheed *et al.*, 1980). Since the food intake of animals also decreased in heat exposure (Swaroop and Ramasarma 1982), the possibility existed that the decrease in oxidation and its reversal by cytochrome c observed in heat-exposed animals (Table I) could really be effects of partial starvation. Therefore experiments on effects of starvation of animals on kidney mitochondria were carried out.

Starvation of the animals caused drastic decreases in body and kidney weights similar to heat-exposed animals. However, the decrease in weight of the body was much more than that of the kidney. Kidney weight when expressed per 100 g body weight therefore increased in starvation, in contrast to its decrease observed in the hypothyroid states of heat exposure and propylthiouracil treatment.

The results in Table V clearly show that starvation caused a drastic decrease in the capacity of kidney mitochondria to oxidize succinate. However, addition of exogenous cytochrome c did not effectively reverse this loss in activity. As in heat exposure, both activities of α-glycerophosphate-dependent H_2O_2 generation and dehydrogenase were decreased drastically on starvation. These changes were limited to α-glycerophosphate as the substrate since it

Table V. Effect of Starvation on the Oxidative Activities in Kidney Mitochondria[a]

Test system	Control	Starved	Percent control
Body wt. (g)	184 ± 12	109 ± 16	59[b]
Kidney wt. (g)	1.5 ± 0.1	1.0 ± 0.1	67[b]
State 3 oxidation	415 ± 28	134 ± 33	32[b]
Plus cytochrome c	450 ± 21	179 ± 30	40[b]
H_2O_2 generation	248 ± 67	36 ± 16	15[b]
α-Glycerophosphate dehydrogenase	19.9 ± 2.7	3.1 ± 0.8	16[b]

[a] The experimental animals were starved for 8 days. Succinate was used as the substrate for measuring the rate of state 3 oxidation (ng-atoms O/min/mg protein). α-Glycerophosphate was used as the substrate for measuring the dehydrogenase activity (nmol/min/mg protein). The values given are mean ± S.D. of three independent estimations (animals).
[b] Significance—starved vs. control: P value <0.01.

was found that neither succinate dehydrogenase nor succinate-dependent H_2O_2 generation showed any significant change in starvation (data not given). The results described above support the view that the cytochrome c-dependent reversal of the inhibition of oxidation in kidney mitochondria is specific to heat exposure.

Cytochrome Concentration

The need for addition of exogenous cytochrome c for realizing full activity of state 3 oxidation indicated the possibility that kidney mitochondria in heat-exposed rats are deficient in cytochrome c. It has been reported (Rasheed et al., 1980) that hepatic mitochondria in starved rats became deficient in cytochrome c even though the concentration of the pigment in the whole liver tissue did not decrease. The data presented in Table VI reveal that exposure to heat stress caused a significant decrease in the content of cytochrome c in kidney mitochondria (34–44%). A decrease of a similar order was registered in starvation as well as propylthiouracil feeding. The concentration of cytochrome $a \cdot a_3$ did not change significantly in any of the treatment. It may be mentioned that the content of cytochrome c in kidney cortex did not decrease on exposure of the animals to heat stress. Thus, control and 20-day exposed animals contained 24.9 ± 0.9 and 24.6 ± 0.4 nmol of cytochrome c, respectively, per gram of kidney. Cytochrome c depletion from mitochondria seems, therefore, a common feature in heat exposure, propylthiouracil treatment, and starvation.

Table VI. Effect of Heat Exposure, Propylthiouracil Treatment, and Starvation on Cytochrome Concentration of Kidney Mitochondria[a]

Treatment of animals	Cytochrome	pmol/mg mitochondrial protein		Percent control
		Control	Experimental	
Heat exposed	c	588 ± 24	389 ± 22	66[b]
(20 days)	$a \cdot a_3$	243 ± 28	238 ± 44	96
Heat exposed	c	600 ± 64	338 ± 10	56[b]
(30 days)	$a \cdot a_3$	287 ± 21	302 ± 10	105
Propylthiouracil	c	550 ± 29	389 ± 33	71[b]
(fed 20 days)	$a \cdot a_3$	263 ± 27	253 ± 28	93
Propylthiouracil	c	535 ± 49	357 ± 15	67[b]
(fed 30 days)	$a \cdot a_3$	267 ± 35	212 ± 16	79
Starvation	c	450 ± 33	254 ± 47	56[b]
(8 days)	$a \cdot a_3$	232 ± 39	208 ± 31	90

[a]The values given are mean ± S.D. of 3–6 independent estimations (animals). The concentrations of the cytochromes were calculated from difference spectra.
[b]Significance—experimental vs. control: P value <0.01.

Discussion

The drastic decrease in state 3 oxidation suffered by kidney mitochondria is consistent with the lowering of BMR in heat exposure, starvation, and hypothyrodism. These treatments lower the content of cytochrome c in the mitochondrial membrane, practically to the same extent. However, the fall in oxidation is corrected by exogenous cytochrome c only in the case of heat exposure. This brings to light the diversity of mechanisms that operate in the regulation of mitochondrial electron transport. Apparently, in the case of heat exposure, regulation of respiration is achieved entirely by the dissociation of a mobile respiratory component (cytochrome c) while with the other two treatments additional regulatory mechanisms are brought into play.

The differential effects of heat exposure on kidney and liver mitochondria illustrate this further. Heat exposure lowers the rate of state 3 oxidation in liver mitochondria as well (Swaroop and Ramasarma, 1982). The decrease in the content of mitochondrial cytochrome c also follows the same pattern in the two organs. However, the fall in oxidation is corrected by exogenous cytochrome c only in kidney mitochondria; added cytochrome c does not stimulate respiration in mitochondria from liver of these animals (unpublished observations). In contrast, the decrease in the respiratory rate shown by liver mitochondria of starved animals is corrected by exogenous cytochrome c (Rasheed et al., 1980). However, the pigment fails to stimulate the lowered respiratory rate of kidney mitochondria of starved animals (this paper) even though in both organelles there is a drastic decrease in the content of the cytochrome. These stress-specific and organ-specific effects merit detailed investigation.

The depletion of cytochrome c from the mitochondria of heat-exposed animals could not be a result of greater membrane damage during isolation, because the treatment did not decrease respiratory control which is a good index of membrane integrity. This was further confirmed by the assay of the intermembrane enzyme, adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3). The activities of this enzyme in mitochondria of kidneys isolated from control and heat-exposed animals, respectively, were nearly the same: fresh preparations, 94 and 85 nmol of ATP generated per minute per milligram protein; and frozen–thawed preparations, 162 and 186. Therefore no damage of membranes is indicated.

A regulatory role for cytochrome c in mitochondrial oxidative phosphorylation under stress conditions is also indicated by these results. The location of cytochrome c on the cytosolic side of the inner membrane as an extrinsic protein and its ready solubility in aqueous systems (Racker, 1970), its easy displacement from the membrane by changes in pH or ionic strength (Jacobs and Sanadi, 1960), and the susceptibility of its amino acid residues, particularly of lysine, to modification with consequent changes in binding and redox

properties (Dickerson and Timkovich, 1975) and of basic amino acids (Vijaya and Ramasarma, 1983) make the pigment an ideal candidate for a regulatory role. The tissue-specific requirement of cytochrome in these stress conditions gives selectivity for this regulation.

A lowering of mitochondrial α-glycerophosphate dehydrogenase in the three stress conditions of heat stress, propylthiouracil treatment, and starvation reconfirms its unique relationship to hypothyroid status. These studies prove that kidney responds similarly to the liver in these cases wherein a decrease in BMR and lowered circulating thyroid hormone level are well documented. With all this excellent correlation it remains to be seen how this enzyme, known for its function of transferring reducing equivalents from cytosol into mitochondria (Greville, 1969), can play a role in regulating thermogenesis. The generation of H_2O_2 in liver mitochondria decreased in hypothyroid conditions independent of the dehydrogenase activities of the respective substrates (Swaroop and Ramasarma, 1981). These and other evidence led to the hypothesis of an H_2O_2-generator system, common to all substrate dehydrogenases (Boveris et al., 1976), that responds directly to activation of α-adrenergic receptors (Swaroop et al., 1983). In light of this, the present finding of selective decrease of α-glycerophosphate-dependent H_2O_2 generation in kidney in heat stress suggests that other modifications, possibly of interaction between the dehydrogenase and the H_2O_2 generator, are also to be considered. It will be of interest to study these stress-dependent changes in animals which will hopefully lead to an understanding of the adaptive mechanisms and interrelationships of mitochondrial oxidation, thermogenesis, and BMR.

Acknowledgments

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