Journal of Bioenergetics and Biomembranes, Vol. 19, No. 4, 1987

Generation of Hydrogen Peroxide by Brown Adipose Tissue Mitochondria

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Received October 3, 1986; revised January 15, 1987

Abstract

This is the first report on the generation of H_2O_2 by brown adipose tissue mitochondria. Flavin dehydrogenase-linked substrates like succinate, glycerol-1-phosphate, and fatty acyl CoA were good substrates for the reaction, while NAD^+-linked substrates were less effective. In cold-acclimated animals the activity showed a substantial increase (2.5-fold). The K_m and V_{max} of the reaction were considerably lower than those of the respective dehydrogenase. Metal ions, particularly Cu^{2+} and Fe^{2+} were potent inhibitors of the reaction. Nucleoside diphosphates, which were inhibitors by themselves, potentiated the inhibitory action of Fe^{2+} ions. In most of the properties, the H_2O_2 generator of brown adipose tissue mitochondria resembled that of liver mitochondria.

Key Words: Cold exposure; thermogenesis; BAT mitochondria; H_2O_2 generator; GDP inhibition.

Introduction

Generation of H_2O_2 by mitochondria is increasingly being recognized as a physiologically significant reaction (Chance *et al.*, 1979). Thus, the molecule has been assigned a key role in the alternate oxidase system of higher plants (Rich *et al.*, 1976), in phagocytosis (Iyer and Quastel, 1963), and probably in the mechanism of action of insulin (Mukherjee and Lynn, 1977; May and de Haen, 1979; Mukherjee, 1980). Studies in this laboratory have shown that the generation of H_2O_2 by rat liver mitochondria was subject to the regulatory influence of norepinephrine and thyroxine (Swaroop and Ramasarma, 1981a, 1985; Swaroop *et al.*, 1983). A variety of compounds, like succinate, NAD^+-linked substrates, palmitoyl CoA, octonoate

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0145-479X/87/0800-0397$05.00/0

(Boveris *et al.*, 1972), and glycerol-1-phosphate (Swaroop and Ramasarma, 1981b), could donate electrons for the reaction, which presumably involves a substrate-specific primary dehydrogenase, electron transfer through ubiquinone (Boveris *et al.*, 1976; Turrens *et al.*, 1985; Patole *et al.*, 1986), and an autooxidizable component located on the respiratory chain between rotenone- and antimycin A-sensitive sites (Cadenas *et al.*, 1977). The final step is considered to be the dismutation of superoxide radicals generated by the above process (Loschen *et al.*, 1974).

Recent studies have indicated a possible role for H_2O_2 generation in thermogenesis (Rich *et al.*, 1976; Ramasarma and Sivaramakrishnan, 1978; Swaroop and Ramasarma, 1981a; Ramasarma, 1982). Brown adipose tissue (BAT)[3] is believed to play a significant role in nonshivering thermogenesis. For example, Foster and Frydman (1978) calculated that up to 60% of nonshivering thermogenesis could be accounted for by this tissue and concluded it to be the primary site of heat production. No enzyme generally considered characteristic of mammalian mitochondria appears to be missing from the BAT organelles (Cannon and Lindberg, 1979). In this paper we report for the first time the presence of an active H_2O_2 generator in BAT mitochondria and describe its properties.

Materials and Methods

Chemicals

Biochemicals were purchased from Sigma Chemical Co., St. Louis, Missouri. Phenolic compounds were obtained from Koch-Light Laboratories, Colnbrook, Bucks, U.K. Other chemicals used were of the purest grades available. Solutions were made in deionized water double-distilled in an all-quartz apparatus and adjusted to pH 7.0 before use.

Mitochondria

Male albino rats (100–200 g) of the Wistar strain obtained from the Central Animal Facility of this Institute were maintained on a commercial pellet diet obtained from Lipton Co., Ltd. Animals were exposed to cold (0–4°C) for 4 weeks in a chamber provided with artificial lighting during 9.00–17.00 hr. Animals kept at ambient temperature (22–25°C) served as control. Water and food were supplied *ad libitum*. The animals were killed by stunning and decapitation. Interscapular BAT was freed of adhering skeletal

[3]The following abbreviations are used: BAT, brown adipose tissue; $\phi_{1/2}$, concentration of metal ion for inhibition of 50% of the activity.

muscle and homogenized in ice-cold 0.25 M sucrose containing 2 mg/ml of bovine serum albumin. Mitochondria were sedimented from the homogenate by differential centrifugation in a Sorvall RC-5 refrigerated centrifuge as described by Cannon and Lindberg (1979), washed once with 0.25 M sucrose not containing serum albumin, suspended in sucrose solution, and used with a minimum of delay. About 25 mg protein/g of BAT was recovered in the fraction. Liver mitochondria from the same animals were prepared according to Kurup *et al.* (1970).

Enzyme Assays

The rate of generation of H_2O_2 was measured by the decrease in scopoletin fluorescence in a system containing 150 mM potassium phosphate buffer, pH 7.6, 3.3 mM EDTA, 1.67 μM scopoletin, 330 nM horseradish peroxidase, 660 nM antimycin A, and 500–750 μg of mitochondrial protein in a total reaction volume of 3 ml (Loschen *et al.*, 1971). After incubation for 10 min at 30°C to allow for the oxidation of endogenous substrates, the reaction was started by the addition of substrate (concentrations indicated), and the rate of decrease of scopoletin fluorescence was recorded in a Hitachi–Perkin-Elmer spectrofluorimeter with excitation and emission at 360 nm and 465 nm, respectively. Under the experimental conditions employed, a 1 : 1 stoichiometry was established between H_2O_2 and scopoletin.

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 and the decrease in absorbance at 600 nm in a reaction system containing catalytic amounts of phenazine methosulfate (Nair and Kurup, 1986). Palmitoyl CoA dehydrogenase [Acyl CoA: (acceptor) oxidoreductase, EC 1.3.99.3] activity was measured according to Beinert (1962). The reaction system contained, in 1 ml, 15 mM potassium phosphate buffer, pH 7.6, 1 mg bovine serum albumin, 30 μM 2,6-dichlorophenolindophenol, 60 μM FAD, 50 μM phenazine methosulfate, and 50 μg of mitochondrial protein. The reaction was started by the addition of palmitoyl CoA and the decrease in absorbance at 600 nm recorded in a Shimadzu UV 200S spectrophotometer (E_{mM} = 16.9). The reaction was linear for 2–3 min, yielding an absorbance decrease of 0.2 at 40 μM substrate concentration.

Care was taken to verify that compounds added to the H_2O_2 generating system did not interfere directly with scopoletin fluorescence. Mitochondrial protein was estimated by the biuret method using bovine serum albumin as standard and deoxycholate for solubilization (Gornall *et al.*, 1949). Least-squares analysis and determination of correlation coefficients for

Lineweaver–Burk and Dixon plot fits were carried out using a Hewlett-Packard model 1000 computer.

Results

Effect of Phosphate Ions and Antimycin

The rate of generation of H_2O_2 by BAT mitochondria (216 pmol/min per mg protein) was twice as high as that by hepatic mitochondria in the absence of PO_4^{3-} ions, but was stimulated by about 75% on the addition of 150 mM phosphate which was optimum. In contrast, the generation of H_2O_2 by rat liver mitochondria was stimulated 6-fold on the addition of PO_4^{3-} ions, the optimum concentration being 400 mM (Swaroop and Ramasarma, 1981b). Similarly, antimycin A, which stimulated the reaction about 7-fold in rat liver mitochondria (Boveris and Chance, 1973), increased the activity of BAT mitochondria by only a maximum of 50% at 100 nM concentration. However, in view of the reported higher requirement of the antibiotic to inhibit BAT mitochondrial respiratory activity (Cannon and Nedergaard, 1985), the concentration of antimycin A in the reaction system was kept at 660 nM.

Rates with Different Electron Donors

In the normal adult animal the content of interscapular BAT is only about 100 mg. There is a large increase in the mass of the tissue (about 1 g) on cold acclimation. Therefore, the experiments reported in this paper were performed with mitochondria isolated from the tissue of cold-acclimated animals. However, for purpose of comparison, the rates of generation of H_2O_2 by BAT mitochondria isolated from control and cold-acclimated animals are depicted in Table I.

Flavin dehydrogenase-linked substrates like succinate and glycerol-1-phosphate could support H_2O_2 generation in BAT mitochondria while NAD^+-linked substrates were less effective. No detectable H_2O_2 was produced when NADH was used as electron donor. In these properties the BAT mitochondria resembled hepatic mitochondria. However, while choline was a good substrate for the hepatic mitochondrial H_2O_2 generator (Swaroop and Ramasarma, 1981b), it was ineffective with BAT mitochondria.

The observation that fatty acyl CoA is as good a substrate as Krebs' cycle intermediates assumes importance in view of the postulate that, in BAT, heat production is largely contributed by the oxidation of fatty acids (Cannon and Johansson, 1980). The increase in the specific activity (about 2-fold) on acclimation to cold is also consistent with the postulated function of the tissue as a generator of heat. It may be mentioned that palmitoyl

Table I. Effect of Cold Exposure on the Rate of Generation of H_2O_2 by BAT Mitochondria[a]

Substrate	Concentration (mM)	pmol H_2O_2/min per mg protein	
		Control	Cold-exposed
Palmitoyl CoA	0.02	160 ± 5.0	400 ± 24
Glycerol-1-phosphate	1.00	143 ± 37	357 ± 25
Succinate	0.05	114 ± 22	199 ± 4
Glutamate	1.00		
+	+		
Malate	1.00	49	74

[a]In the case of control, BAT obtained from a number of animals kept at room temperature were pooled for the preparation of mitochondria and the values are the mean ± SD of three independent samples. Animals were exposed to 0–4°C for 30 days for the preparation of "cold-exposed" samples. The rates are the mean ± SD of four or more independent samples. The values at optimum concentration of substrates as indicated are given. Addition of carnitine and malate along with palmitoyl CoA did not cause any significant stimulation in the rate of the reaction.

CoA-supported H_2O_2 generation by BAT mitochondria is totally inhibited by myxothiazole (0.5 μg/ml), eliminating any possible involvement of peroxisomes in this reaction.

Assayed under the same conditions, the specific activity of liver mitochondria isolated from the same cold-exposed (30 days) animals was twice that of BAT mitochondria with NAD^+-linked substrates, equal with succinate, and one-half with glycerol-1-phosphate or palmitoyl CoA (data not given).

Dehydrogenase Activities

The generation of H_2O_2 is presumed to involve the dehydrogenase flavoprotein and the ubiquinone segment of the mitochondrial respiratory chain (Boveris *et al.*, 1976; Turrens *et al.*, 1985; Patole *et al.*, 1986). In the case of liver mitochondria it has been reported that the K_m and V_{max} of H_2O_2 generation were only a small fraction of those of the dehydrogenase activity (Swaroop and Ramasarma, 1981b). The data in Table II show that a similar relationship holds for BAT mitochondria also. The K_m values for dehydrogenase activities agree well with those reported in the literature (Cannon and Lindberg, 1979). For H_2O_2 generation, the K_m of BAT mitochondria is the same as that of rat liver mitochondria, when succinate is used as electron donor. However, the affinity of BAT mitochondria for glycerophosphate is more than 10-fold higher than that of hepatic mitochondria. It is interesting to note that the succinate dehydrogenase activity of the BAT organelles is 3 times as high as that of rat liver mitochondria while the glycerophosphate dehydrogenase activity is 30 times as high (Swaroop and Ramasarma, 1981b).

Table II. Comparison of the K_m and V_{max} of H_2O_2 Generator and Primary Dehydrogenases of BAT Mitochondria[a]

Substrate	K_m (μM)		V_{max} (nmol/min per mg protein)	
	H_2O_2 generator	Dehydrogenase	H_2O_2 generator	Dehydrogenase
Palmitoyl CoA	9	75	0.59	84
Succinate	10	3570	0.25	1307
Glycerol-1-phosphate	170	8200	0.38	260

[a]The constants were calculated from *X*- and *Y*-intercepts of double reciprocal plots fitted by least-squares analysis using a HP 1000 computer. The V_{max} represents nmol H_2O_2 or dye reduced (dehydrogenase) per min/mg protein. The correlation coefficients of regression ranged between 0.97 and 0.99 ($p < 0.001$).

Inhibiton by Metal Ions

The requirement of EDTA in the reaction system for the realization of optimum activity indicated that metal ions might be inhibitory to the reaction. The data presented in Table III reveal the sensitivity of the reaction to divalent metal ions. The extreme sensitivity to inhibition by copper even in the presence of EDTA is particularly noteworthy. Generation of H_2O_2 by liver mitochondria has been reported to be sensitive to Cu^{2+} ions (Swaroop and Ramasarma, 1981b).

Inhibition by Radical Scavengers

The involvement of oxygen radicals as intermediates in the generation of H_2O_2 was tested by the addition of reagents which rapidly react with them.

Table III. Constants for the Inhibition of H_2O_2 Generation in BAT Mitochondria by Metal Ions[a]

Metal ion	K_i (μM)		$\phi_{1/2}$ (μM)	
	−EDTA	+EDTA	−EDTA	+EDTA
Cu^{2+}	1.0	0.5	1.2	8.3
Fe^{2+}	16.9	7.6	9.3	21.2
Mn^{2+}	6.3	39.4	15.6	13.7
Fe^{3+}	40.1	67.8	26.2	43.8

[a]Inhibition of H_2O_2 generation by BAT mitochondria was tested in the absence and presence of 3.3 mM EDTA in the reaction mixture. The concentration of metal ion to inhibit 50% of the activity ($\phi_{1/2}$) and K_i were obtained from Dixon plots obtained by least-squares analysis. The correlation coefficients ranged from 0.93 to 0.99 ($p < 0.001$). The rates of H_2O_2 generation (using glycerol-1-phosphate as substrate) in the absence and presence of EDTA were 280 and 400 pmol per min/mg protein respectively.

Table IV. Effect of Radical Scavengers on the Rate of Generation of H_2O_2 by BAT Mitochondria[a]

Addition	Concentration	Inhibition (%)
Superoxide dismutase	5 μg/ml	9
Benzoate	2000 μM	39
Histidine	600 μM	43
Nitroblue tetrazolium	40 μM	56
Ferricytochrome *c*	5 μM	65
Ascorbate	10 μM	100

[a]Radical scavengers were added at the time of preincubation. The concentrations indicated are optimum. Glycerol-1-phosphate was used as substrate. The system produced 344 pmol H_2O_2 per min/mg protein in the absence of any inhibitor.

The sensitivity of the reaction to inhibition by relatively low concentrations of ascorbate, cytochrome *c*, and nitroblue tetrazolium indicates the involvement of superoxide anion in the reaction (Nishikimi, 1975; Torres *et al.*, 1979; Swaroop and Ramasarma, 1981b). The failure of exogenously added superoxide dismutase to enhance the reaction rate would indicate that an adequate level of the enzyme is available in BAT mitochondria. In rat liver mitochondria, addition of the dismutase stimulated the rate of generation of H_2O_2 (Nohl and Hegner, 1978; Swaroop and Ramasarma, 1981b). Benzoate, which reacts with $OH^{\cdot}$ in preference to $O_2^{-\cdot}$ or H_2O_2 (Torres *et al.*, 1979), and histidine, a quencher of singlet oxygen (Lynch and Fridovich, 1979), were inhibitory but at high concentrations. This would indicate that dismutation of superoxide anion was the major pathway for the generation of H_2O_2 by BAT mitochondria (Table IV).

Effect of Phenolic Compounds

Compounds containing one or more phenolic hydroxyl groups have been shown to be potent inhibitors of H_2O_2 generation by liver and brain mitochondria, without affecting the activity of either the dehydrogenase or superoxide dismutase (Swaroop and Ramasarma, 1981b; Patole *et al.*, 1986). Hydrogen peroxide generator of BAT mitochondria also showed a high sensitivity to inhibition by phenolic compounds (Table V). Protocatechuic acid and ferulic acid showed greater inhibitory potency.

Effect of Nucleoside Diphosphates

Adenine nucleotides (ADP and ATP) have been reported to inhibit the H_2O_2 generator of rat liver mitochondria (Swaroop and Ramasarma, 1981b). The generation of H_2O_2 by pigeon heart mitochondria was sensitive to ADP

Table V. Effect of Phenolic Compounds on the Rate of Generation of H_2O_2 by BAT Mitochondria[a]

Addition	Concentration (μM)	Activity (%)
None	–	100
Tyrosine	200	75
Gentisic acid	20	72
p-Hydroxybenzoic acid	40	47
Anisic acid	20	47
Resorcinol	20	36
p-Coumaric acid	20	36
Protocatechuic acid	10	25
Ferulic acid	8	18
Isoferulic acid	8	11

[a]Glycerol-1-phosphate was used as the substrate. The inhibitors were added to the reaction system at the time of preincubation. The concentrations indicated are optimum. The percent values are expressed taking the activity in the absence of any phenolic compound (330 pmol/min per mg protein) to be 100.

but not to ATP (Boveris and Chance, 1973). In BAT mitochondria, nucleoside diphosphates assume special significance in view of the postulate that the thermogenic function of these organelles resides in an M_r 32,000 protein called "uncoupling protein" or "thermogenin" which can discharge transmembrane proton gradient and thus prevent ATP synthesis. Nucleoside diphosphates, particularly GDP, bind to this protein and inhibit its proton-translocating activity (Ricquier *et al.*, 1979; Lin and Klingenberg, 1982; Nedergaard and Cannon, 1985). The effect of ADP and GDP on H_2O_2 generation which also takes up protons was tested in the presence and absence of Fe^{2+} ions. The results presented in Table VI show that both nucleotides at 250 μM concentration inhibited the activity by about 25–35%. This concentration is only a quarter of that required to inhibit thermogenin function in intact mitochondria (Cannon and Lindberg, 1979). In the light of

Table VI. Effect of Nucleoside Diphosphates on the Rate of Generation of H_2O_2 by BAT Mitochondria[a]

Nucleoside diphosphate (250 μM)	pmol H_2O_2/min per mg protein	
	$-Fe^{2+}$	$+Fe^{2+}$ (10 μM)
None	200	140
GDP	130	60
ADP	150	80

[a]The standard reaction system without EDTA was used. After preincubation, the reaction was started by the addition of the substrate (glycerol-1-phosphate); the inhibitors were added after the addition of the substrate.

this observation a direct role for thermogenin in H_2O_2 generation cannot be ruled out.

The ability of ADP and GDP to potentiate the inhibitory action of Fe^{2+} is interesting. No difference in the extent of inhibition was observed when the nucleotide was added before or after the addition of Fe^{2+} ions.

Discussion

Ever since the discovery (Jensen, 1966; Hinkle *et al.*, 1967) that mitochondria produce H_2O_2, the bioenergetic and physiological significance of the reaction has been an open question. Exhaustive studies in Chance's laboratory have identified the involvement of the respiratory chain, particularly that of ubiquinone, in the generation of H_2O_2 (Chance *et al.*, 1979). Recent studies indicate that electron transport from ubisemiquinone to the Fe-S center is essential for the reaction (Turrens *et al.*, 1985). This and the fact that H_2O_2 is produced by the organelles isolated from different tissues (Chance *et al.*, 1979; Swaroop and Ramasarma, 1985), including the brain (Patole *et al.*, 1986) and BAT (this paper), would justify the conclusion that generation of H_2O_2 is an integral respiratory chain-linked reaction with probable physiological connotations.

The possible mechanisms of H_2O_2 formation in liver mitochondria with respect to inhibition by metal ion redox couples and phenolic compounds have been amply discussed (Swaroop and Ramasarma, 1981b). The data presented in this paper reveal that the properties of BAT mitochondrial H_2O_2 generator are in most respects similar to those of liver mitochondria. However, the synergistic action of nucleoside diphosphate and Fe^{2+} ions in the inhibition of the reaction merits special mention. Since nucleotides are known to promote lipid peroxidation in the presence of low concentrations of Fe^{2+} (Hochstein and Ernster, 1963), the inhibition of H_2O_2 generation may reflect utilization of the oxygen radical intermediates ($O_2^{-\cdot}$ for example) or H_2O_2 itself for lipid peroxidation. Another possibility is that peroxidation of mitochondrial lipids may effect the reaction rate directly due to changes in bilayer assembly.

The observation that BAT mitochondria produce H_2O_2 and that too at a rate faster than that of liver mitochondria is consistent with the widely held notion that brown adipose is an important organ of thermogenesis (Nedergaard and Lindberg, 1982). The fact that palmitoyl CoA is an efficient electron donor for the reaction echoes the belief that fatty acids are the major fuels for heat production by this tissue (Cannon and Lindberg, 1979). Our finding that the tissue on cold acclimation of animals shows enhanced rates of H_2O_2 generation is in agreement with the thermogenic role ascribed to the

tissue and to the reaction. In effect, because of the increase in size (5- to 6-fold) of the tissue and the rate of the reaction, the generation of H_2O_2 increases more than 10-fold on cold exposure. It may be pertinent to mention in this context that cold exposure does not elicit a similar increase in the specific activity of respiratory chain-linked electron transport in BAT mitochondria (Sundin and Cannon, 1980). Finally, it must be pointed out that the rates of H_2O_2 generation are still too low to account for the overall increase in heat production. However, it may be of interest to investigate the cellular role of increased H_2O_2 production for amplification of the thermogenic signal.

Acknowledgment

Financial assistance from the Department of Science and Technology, Government of India, New Delhi, for the research project "Biological Role of Hydrogen Peroxide" is acknowledged.

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