MITOCHONDRIA OF BROWN FAT: OXIDATIVE PHOSPHORYLATION SENSITIVE TO 2,4,-DINITROPHENOL

Cliffe D. Joel, William B. Neaves and James M. Rabb

Departments of Biological Chemistry and Psychiatry, Harvard Medical School, and Massachusetts Mental Health Center, Boston, Massachusetts 02115

Received October 9, 1967

Brown adipose tissue performs a physiological role as a quantitatively important specialized site of heat production in adult animals during the frequent arousals from hibernation that occur throughout the winter (Joel, 1965) and in newborn animals during cold-stress (Hull, 1966). The detailed mechanism of this heat production is not clear. Two groups of workers have recently reported experiments in which mitochondria isolated from brown adipose tissue failed to exhibit oxidative phosphorylation sensitive to DNP[1] (Smith, Roberts and Hittelman, 1966; Lindberg, dePierre, Rylander and Afzelius, 1967). Hittelman, Fairhurst and Smith (1967) have reported that the mitochondria of brown adipose tissue, in contrast to those of other tissues, failed to exhibit Ca^{++} uptake driven by ATP in the absence of oxidizable substrate. These findings were interpreted in favor of the hypothesis that brown adipose tissue mitochondria inherently lack the normal coupling between the electron transfer system and ATP synthesis. By this hypothesis, substrates would be oxidized at a high rate by mitochondria with the energy liberated directly as heat rather than partially conserved in the high energy bonds of ATP. These biochemical data acquired additional interest with the report that the inner membrane stalked subunits, recently associated with certain factors in oxidative phosphorylation (Kagawa and Racker,

[1]Abbreviations used: DNP, 2,4-dinitrophenol; ATP, adenosine triphosphate; EDTA, ethylenediamine tetraacetate.

1966) could not be demonstrated in brown adipose tissue mitochondria by the usual techniques of negative staining (Afzelius, 1966; Lindberg et al., 1967). An alternative interpretation of these reports is that the coupling apparatus of brown adipose tissue mitochondria is unusually labile and is not as easily demonstrable in vitro as that of many other tissues. The present report shows that mitochondria isolated under proper conditions from the brown adipose tissue of cold-adapted rats do indeed exhibit oxidative phosphorylation that can be uncoupled by DNP.

METHODS---Male Sprague-Dawley rats weighing 100 g were cold-adapted by leaving them in a 4° C room for 4-8 weeks in order to increase the amount of brown adipose tissue available (Pagé and Babineau, 1950; Smith et al., 1966). Mitochondria were prepared from the interscapular brown adipose tissue by a modification of the method of Kennedy and Lehninger (1949). Caution was taken during removal of the brown adipose tissue from the animal in order to avoid contamination by skeletal muscle or white adipose tissue. The tissue from several rats was collected in 0.88 M sucrose containing 5×10^{-3} M sodium EDTA and 1 percent bovine serum albumin (Mann Research Laboratories). The albumin contained less than 0.03 moles of FFA per mole of protein and was employed in order to prevent uncoupling of oxidative phosphorylation by FFA (Björntorp, Ellis and Bradford, 1966). For the tissue from each rat (approximately 400 mg), 2 ml of this solution was used. The temperature was maintained at 0-4° C throughout the entire preparative procedure. After homogenization with a teflon pestle, the suspension was transferred to a plastic centrifuge tube and spun at 15,000 x g for 10 minutes. The floating lipid layer and the supernatant fluid were discarded and the walls of the tube were wiped free of fat. The pellet was resuspended in the original volume of the sucrose-EDTA-albumin solution and again homogenized with a teflon pestle. The suspension was centrifuged at 1200 x g for 3 minutes and the pellet was discarded. The supernatant was trans-

ferred to another plastic tube containing an amount of 1 percent albumin in 1.5 M NaCl that was equal to 10 percent of the volume of supernatant, and the suspension was immediately mixed thoroughly and centrifuged at 10,000 x g for 10 minutes. The mitochondrial pellet was homogenized with 1 percent albumin in 0.15 M NaCl, using 0.8-2.5 ml of this solution per g of brown adipose tissue, and this final suspension was used for measurements of oxidative phosphorylation.

Oxygen consumption was measured in the Warburg apparatus in an atmosphere of air. The incubation medium contained, in micromoles per vessel, pyruvate, 24; malate, 24;, ATP, 4.8; diphosphopyridine nucleotide, 0.72; cytochrome c, 0.03; KF, 60; glucose, 120; $MgCl_2$, 12; and 2.4 mg hexokinase (Sigma Type III, 145 K.M. units per mg). The buffer was potassium phosphate, 24 micromoles per vessel; tris-(hydroxymethyl) aminomethane, 60; HCl, 50; pH 7.4. Some vessels contained 0.12 micromoles of DNP (Smith et al., 1966). The final pH was adjusted to 7.4 with KOH. The final volume after addition of the mitochondrial suspension was 2.8 ml, and the total content of albumin in this volume was 36 mg. The center well contained 0.2 ml of 5 percent aqueous KOH and a folded wick of filter paper. The vessels containing incubation medium were kept at 0° C until addition of mitochondrial suspension to the sidearm. The vessels were promptly attached to the manometers and placed in the water bath at 30° C. After one minute of shaking, the contents of the sidearm were tipped into the main chamber and oxygen consumption readings were begun 3-5 minutes later. Oxygen consumption was essentially linear throughout the incubation. The incubation was terminated by addition of 9.6 ml of 5 percent trichloroacetic acid solution, and the content of inorganic phosphate in the incubation medium was determined by the method of Gomori (1942). Controls were run with heat-inactivated mitochondria in order to determine the initial concentration of inorganic phosphate in the incubation mixture.

RESULTS AND DISCUSSION---The results are shown in Table I. The mean P:O ratio for the 7 vessels incubated in the absence of DNP was 1.08, and the addition of DNP to the 7 paired vessels diminished the P:O ratio by 0.54 ± 0.14 (mean ± standard error; $P = 0.007$ by the paired t-test). For the experiments shown in Table I, the oxygen consumption was 25 ± 10 percent higher in the presence of DNP, but this increase was of only borderline statistical significance ($P = 0.05$ by the paired t-test). Five other vessels not containing DNP were run simultaneously with the

TABLE I

Effect of DNP on Oxidative Phosphorylation in Mitochondria from Brown Adipose Tissue

	Without DNP			With DNP		
Experiment	Oxygen uptake	Phosphate uptake	P:O ratio	Oxygen uptake	Phosphate uptake	P:O ratio
1	6.4	5.9	0.92	6.9	2.9	0.42
2	2.4	2.3	0.96	3.1	2.3	0.74
"	2.2	3.5	1.59	3.1	0.8	0.26
"	2.7	2.8	1.04	1.9	1.2	0.63
3	12.8	12.9	1.01	17.9	10.0	0.56
"	12.0	12.9	1.08	16.3	9.7	0.60
"	12.5	12.3	0.98	18.8	10.5	0.56

Data for oxygen uptake and phosphate esterification are expressed as microatoms per vessel for the entire incubation period. The period of incubation between the tipping of the mitochondrial suspension from the sidearm and termination of activity by addition of trichloroacetic acid was 8 minutes for Experiment 1, 18.5 for 2, and 15.5 for 3. The total oxygen consumption reflected variations in incubation time and mitochondrial concentration, and it is noteworthy that the P:O ratio was not greatly influenced by these two vriables. The total mitochondrial nitrogen content per vessel was measured to be 7.45 mg in Experiment 3.

---

various experiments shown in Table 1 but were not paired with vessels containing DNP. The mean P:O ratio of all 12 vessels run in the absence of DNP was 1.00 ± 0.06, and that of the 7 vessels run in the presence of

DNP was 0.54 ± 0.06 ($P < 0.001$ by the t-test, comparing groups). The residual P:O ratio of 0.54 with DNP is presumably due to phosphorylation at the substrate level (Smith et al., 1966).

The demonstration of the coupling between the electron transfer system and ATP synthesis in brown adipose tissue described here has recently been confirmed by other workers (R. T. Guillory and E. Racker, personal communication).

It appears that the coupling apparatus of brown adipose tissue is more labile than that of many other tissues and that special precautions must be taken in order to demonstrate it in vitro. The conditions of preparation and incubation of mitochondria in the present experiments differed in a number of respects from those of other workers who have previously attempted to demonstrate oxidative phosphorylation in brown adipose tissue (Smith et al., 1966; Lindberg et al., 1967), and it is not clear which of these differences account for the present results. It is noteworthy that the albumin preparation used in the present experiments had such a low content of FFA that all of the binding sites were available for removal of FFA during preparation and incubation of the mitochondria.

The P:O ratio in the present experiments was low, but the fact that a marked uncoupling effect was exhibited by DNP provides a clear indication of the presence of oxidative phosphorylation coupled to the cytochrome chain. The present results do not support the hypothesis that heat production in brown adipose tissue is mediated by an inherent lack of coupling factors in the mitochondria. Instead, it seems likely that the high rate of oxygen consumption and consequent heat production in brown adipose tissue is made possible either by an ATP-ase system (Ball and Jungas, 1961) or by an uncoupling action (Ball, 1965; Björntorp et al., 1966) of the FFA that increase in concentration in brown adipose tissue during the action of norepinephrine (Joel, 1966). Norepinephrine

appears to be the physiological stimulant for heat production by this tissue (Joel, 1965; Hull, 1966; Joel, 1966). Perhaps the mitochondria of brown adipose tissue are fully coupled during resting conditions but become free of respiratory control by adenine nucleotides while the tissue is acitve in its role as a specialized site of heat production.

ACKNOWLEDGEMENT---This work was supported by U. S. P. H. S. Grants AM-05480 from the National Institute of Arthritis and Metabolic Diseases and NB-05481 from the National Institute of Neurological Diseases and Blindness.

REFERENCES

Afzelius, B. A., in Uyeda, R., Ed., Electron Microscopy, 1966, Vol. 2, Maruzen, Tokyo, p. 359 (1966).
Ball, E. G., Ann. N. Y. Acad. Sci., 131, 225 (1965).
Ball, E. G., and Jungas, R. L., Proc. Nat. Acad. Sci. U. S., 47, 932 (1961).
Björntorp, P., Ellis, H. A., and Bradford, R. H., J. Biol. Chem., 239, 339 (1966).
Gomori, G. A., J. Lab. Clin. Med., 27, 955 (1942).
Hittelman, K.J., Fairhurst, A. S., and Smith, R. E., Proc. Nat. Acad. Sci. U. S., 58, 697 (1967).
Hull, D., Brit. Med. Bull., 22, 92 (1966).
Joel, C. D., in Renold, A. E., and Cahill, G. F., Eds., Handbook of Physiology, Section 5, Adipose Tissue, American Physiological Society, Washington, D. C., p. 59 (1965).
Joel, C. D., J. Biol. Chem., 241, 814 (1966).
Kagawa, Y., and Racker, E., J. Biol. Chem., 241, 2475 (1966).
Kennedy, E. P., and Lehninger, A. L., J. Biol. Chem., 179, 958 (1949).
Lindberg, O., de Pierre, J., Rylander, E., and Afzelius, B. A., J. Cell Biol., 34, 293 (1967).
Pagé, E., and Babineau, L.-M., Rev. Can. Biol., 9, 202 (1950).
Smith, R. E., Roberts, J. C., and Hittelman, K. J., Science, 154, 653 (1966).