

Exclusive occurrence of thermogenin antigen in brown adipose tissue

Barbara Cannon, Anders Hedin* and Jan Nedergaard

*Departments of Metabolic Research and *Immunology, The Wenner-Gren Institute, University of Stockholm, Norrtullsgatan 16, 113 45 Stockholm, Sweden*

Received 25 October 1982

Thermogenin is the purine-nucleotide binding polypeptide in brown adipose tissue mitochondria (M_r 32000) which confers upon these mitochondria the ability to produce heat. An enzyme-linked immunosorbent assay (ELISA) has been developed to demonstrate and quantitate the occurrence of thermogenin antigen in small amounts of tissue, and thus to characterize different depots of fat tissue as white or brown. The extreme sensitivity of the method allows determination of thermogenin in samples equivalent to <1 mg tissue. The results indicate that thermogenin seems to be exclusively localised in brown fat mitochondria (as compared to white fat, liver or heart muscle mitochondria), and thermogenin antigen could only be found in brown adipocytes (as compared to white adipocytes). Thus, brown and white adipose tissue are probably ontogenetically different

<i>Brown adipose tissue</i>	<i>ELISA</i>	<i>Mitochondria</i>	<i>Nonshivering thermogenesis</i>	<i>Thermogenin</i>
		<i>White adipose tissue</i>		

1. INTRODUCTION

The demonstration that the thermogenic function of brown adipose tissue is directly related to the presence in the mitochondria of a specific polypeptide – thermogenin – with a subunit M_r 32000 has made it possible to study thermogenesis at the molecular level [1,2], (reviews [3–5]).

Thermogenin binds purine nucleotides (e.g., GDP) with a high affinity [6] and the estimation of this binding has become the parameter most widely used to quantitate the state of the tissue [7].

However, there are indications that thermogenin may appear in an inactive form which may, e.g., be activated by norepinephrine [8]. Further, the possibility exists that thermogenin may occur in small amounts or in an inactive form in tissues other than brown fat where, under certain conditions, some characteristics resembling those of brown fat mitochondria can be evoked [9].

Particularly, in the case of the distinction between brown and white adipose tissue, it has been discussed whether brown fat is but an 'immature'

form of white adipose tissue, or whether it should be considered as a bona fide organ in itself, with its own ontogeny [10].

The present interest in the connection between the activity of brown adipose tissue and the tendency to evolve obesity in animals and man (review [11]) has made it necessary to be able to characterize different depots of fat tissue in terms of being white or brown.

We therefore found it necessary to develop a technique which could identify thermogenin even in an inactive form, and which would allow quantification of thermogenin in small amounts of tissue.

We present here results obtained with an enzyme-linked immunosorbent assay system (ELISA) which show that it is possible to quantitate the amount of thermogenin in small samples. The results indicate that thermogenin antigen is not found in tissues other than brown adipose tissue, not even in white, and thus brown adipose tissue must be considered to be a distinct tissue, and probably the sole site where facultative non-shivering thermogenesis occurs in the mammal.

2. MATERIALS AND METHODS

2.1. Isolation of mitochondria

Brown fat mitochondria were prepared from the pooled brown adipose tissue of cold-acclimated golden hamsters as in [12]. They were stored in 0.25 M sucrose.

White fat mitochondria were prepared from the epididymal fat pads of cold-acclimated golden hamsters as in [13]. They were stored in 0.25 M sucrose, 10 mM Tris-HCl (pH 7).

Liver mitochondria were prepared from the livers of cold-acclimated golden hamsters and from rats living at 21°C as in [14,15] and stored in 0.25 M sucrose.

Heart mitochondria were prepared from ox hearts obtained from a local slaughterhouse as in [16] and stored in 0.25 M sucrose.

2.2. Isolation of cells

Isolated brown fat cells from the pooled brown adipose tissue of golden hamsters living at 21°C were prepared as in [5] by the use of a collagenase digestion method.

Isolated white fat cells from the omental and mesenteric white adipose tissue of golden hamsters living at 21°C were prepared as in [17] by the use of a collagenase digestion method.

Ascites cells were kindly provided by Dr Bertil Pettersson and washed thoroughly by centrifugation before use.

2.3. Isolation of thermogenin

Thermogenin was isolated from the brown fat mitochondria of 6 cold-acclimated golden hamsters as in [18], except that in the final centrifugation the detergent was exchanged for 0.1% Tween 20 and 5 mM mercaptoethanol was added. The isolated protein was stored at 10–20 µg/ml at –20°C and used to prepare antiserum and in the immunological assays.

2.4. Preparation of anti-thermogenin antiserum

Rabbits were injected with ~15 µg thermogenin antigen mixed with Freund's complete adjuvant in the thermogenin isolation buffer. Booster injections were given after 3 and 4 weeks and the rabbits were bled from the ear vein 1 week later. Thereafter booster injections were given every 6 weeks. Serum was obtained by centrifugation after the

blood had coagulated overnight at 5°C.

Control serum was obtained from rabbits which had only been injected with Freund's complete adjuvant in buffer.

Serum was stored at –20°C until use.

2.5. Indirect enzyme-linked immunosorbent assay (ELISA) for demonstration of thermogenin

An indirect ELISA was performed as in [19] using as conjugate sheep anti-rabbit immunoglobulin G conjugated to alkaline phosphatase. Thermogenin, solubilised mitochondria or solubilised cells (in 0.05% Tween 20 and 5 mM mercaptoethanol) were allowed to coat plastic micro-ELISA plates and these were evaporated to dryness. After washing, the rabbit antiserum was added, the plates incubated and washed, and thereafter incubated with the conjugate. After further washing, the substrate for alkaline phosphatase, *p*-nitrophenylphosphate, was added and the plates incubated until a satisfactory extinction was measured at 405 nm (routinely 30–45 min).

In some cases a competitive indirect ELISA was also performed. In these cases, thermogenin-coated plates were incubated with rabbit antiserum containing known amounts of thermogenin, solubilised mitochondria or solubilised cells, and thereafter the assay was performed as above.

2.6. Protein

Protein was determined by the biuret method (mitochondria) or by the Lowry method [20].

2.7. Sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was run according to Weber and Osborne [21] or Laemmli [22] in 10% polyacrylamide. The gels were stained with Coomassie blue.

3. RESULTS AND DISCUSSION

3.1. Purity of the antigen

Thermogenin, prepared by the hydroxyapatite method [18], even in heavily overloaded SDS–PAGE gels showed only one band (not shown), indicating that this is the only major protein in the preparation. Although the presence of very minor, highly antigenic contaminants cannot be entirely excluded, their presence seems unlikely.

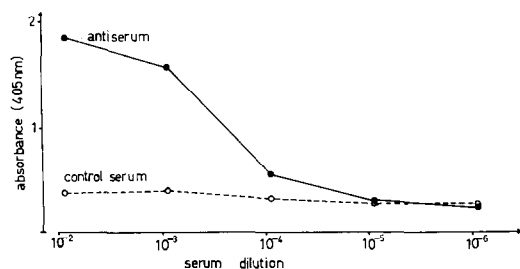


Fig. 1. Dose-response curve of anti-thermogenin antiserum in indirect ELISA. Micro-ELISA plates were coated with 25–50 ng thermogenin/hole, incubated for 3 h at 37°C, dried and further coated with 1% bovine serum albumin. After washing, the plates were incubated with serially diluted serum, followed by conjugate as in section 2. Incubation with substrate was for 30 min at room temperature.

3.2. Titration of anti-thermogenin antiserum against thermogenin in indirect ELISA

Fig. 1 demonstrates the reactive strength of the anti-thermogenin antiserum against thermogenin in indirect ELISA. High activity was found at serum dilutions of 1:1000 and no activity was found with control serum.

3.3. Demonstration of thermogenin antigen in brown fat mitochondria

In fig. 2 the results of competitive indirect ELISA are shown, clearly demonstrating the presence of the antigen in brown fat mitochondria but its absence in white fat mitochondria and liver mitochondria from hamster. At high concentrations, white fat mitochondria gave a slight reaction which is probably unspecific, but at least indicates that the amount of thermogenin is 100–1000 times lower in white fat mitochondria than in brown fat mitochondria.

Ox heart mitochondria and rat liver mitochondria gave entirely negative results (not shown).

Determination of the concentration of thermogenin antigen in brown fat mitochondria by comparison with isolated thermogenin revealed 50–100 μg thermogenin/mg mitochondrial protein. This value should be compared with the one obtained from a calculation based upon a dimeric M_r of 64 000 [23] and a nucleotide-binding value of 0.8 nmol/mg protein in these mitochondria [24]. Through this a concentration of $\sim 55 \mu\text{g}/\text{mg}$ is ob-

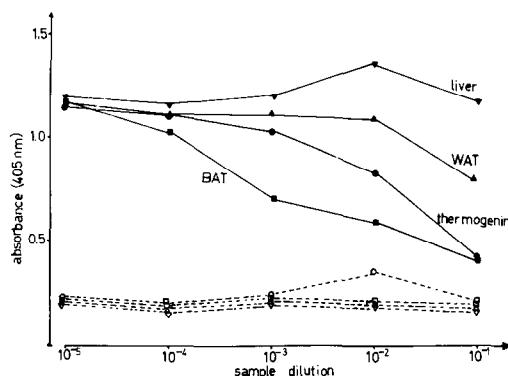


Fig. 2. Inhibition of thermogenin/anti-thermogenin ELISA by varying concentrations of isolated mitochondria from different tissues or by thermogenin. Micro-ELISA plates were coated with thermogenin as above. Antiserum and control serum were used at a dilution of 1:1000, and mixed with varying concentrations of mitochondria and thermogenin. Thermogenin was here initially at 10 $\mu\text{g}/\text{ml}$, and the mitochondria at 1.5 mg/ml. The mitochondria were solubilised in 0.9% NaCl, 0.05% Tween 20, 5 mM mercaptoethanol. Incubation with substrate was for 45 min at room temperature. WAT and BAT indicate mitochondria from white and brown adipose tissue, respectively; (—) incubations with anti-thermogenin antiserum; (---) incubations with control serum.

tained; thus the values obtained here are in good agreement with those from other studies, indicating that this method can be used as a sensitive assay for the presence of thermogenin antigen (<100 ng protein is required for quantitative results).

3.4. Demonstration of thermogenin antigen in brown fat cells

Isolated brown fat cells also reacted well with anti-thermogenin antiserum as shown in fig. 3. Here an equivalent number of white fat cells did not give any specific response. Ascites cells were also completely without effect (not shown).

The results above indicate a concentration of thermogenin in the cells of $\sim 2\text{--}10 \mu\text{g}/10^6$ cells. This can be compared with an estimated presence of $\sim 1 \text{ mg mitochondria}/10^6$ cells [25] and a nucleotide-binding value of $\sim 0.4 \text{ nmol}$ in brown fat mitochondria from control hamsters [24], resulting in an expected concentration of $25 \mu\text{g}/10^6$ cells. It should be noted that with this system the presence

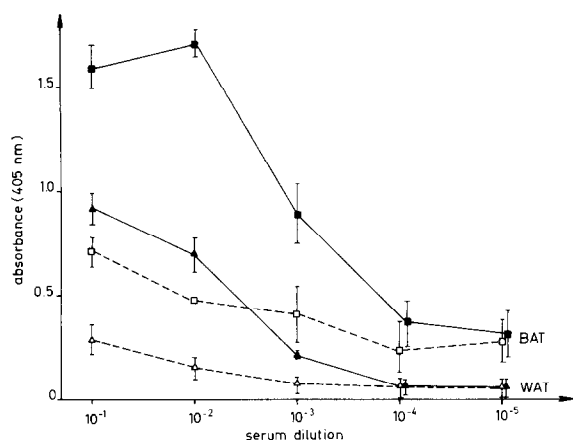


Fig. 3. Indirect ELISA of brown and white fat cells from hamster. 20000 solubilised cells were used/hole and were incubated and dried as thermogenin in fig. 1, and the ELISA carried out as in that figure. Incubation with substrate was for 30 min at room temperature. WAT and BAT indicate cells from white and brown adipose tissue, respectively; (—) incubations with anti-thermogenin antiserum; (---) incubations with control serum. The points shown are means \pm SE from 3 expt on as many different preparations, and each performed in duplicate.

of thermogenin can be demonstrated with <100000 cells (equivalent to <1 mg tissue).

4. CONCLUSIONS

Thermogenin seems to be exclusively localised in brown fat mitochondria. There is no evidence of any latent form of the antigen in any other tissue.

The sensitive ELISA technique can be used to identify and quantify the thermogenin antigen in small samples.

The absence of any thermogenin antigen in epididymal, omental or mesenteric white adipose tissue would support the contention that white adipose tissue and brown adipose tissue are separate organs with different ontogeny.

ACKNOWLEDGEMENTS

The authors thank Agneta Bergström and Barbro Svensson for technical assistance. This work was supported by a grant from the Swedish Natural Science Research Council.

REFERENCES

- [1] Ricquier, D. and Kader, J.-C. (1976) *Biochem. Biophys. Res. Commun.* 73, 577–583.
- [2] Heaton, G.M., Wagenvoort, R.J., Kemp, A. jr and Nicholls, D.G. (1978) *Eur. J. Biochem.* 82, 515–521.
- [3] Nicholls, D.G. (1979) *Biochim. Biophys. Acta* 549, 1–29.
- [4] Cannon, B., Nedergaard, J. and Sundin, U. (1981) in: *Survival in the Cold* (Musacchia, X.J. and Janský, L., eds) pp. 99–120, Elsevier Biomedical, Amsterdam, New York.
- [5] Nedergaard, J. and Lindberg, O. (1982) *Int. Rev. Cytol.* 74, 187–286.
- [6] Nicholls, D.G. (1976) *Eur. J. Biochem.* 62, 223–228.
- [7] Cannon, B. and Nedergaard, J. (1983) *J. Thermal Biol.* in press.
- [8] Desautels, M. and Himms-Hagen, J. (1979) *Can. J. Biochem.* 57, 968–976.
- [9] Selwyn, M.J., Dawson, A.P. and Fulton, D.V. (1979) *Biochem. Soc. Trans.* 7, 216–219.
- [10] Hahn, P. and Novak, M. (1975) *J. Lipid Res.* 16, 79–91.
- [11] Himms-Hagen, J. (1979) *Can. Med. Ass. J.* 121, 1361–1364.
- [12] Cannon, B. and Lindberg, O. (1979) *Methods Enzymol.* 55, 65–78.
- [13] Martin, B.R. and Denton, R.M. (1970) *Biochem. J.* 117, 861–877.
- [14] Hogeboom, G.H., Schneider, W.C. and Palade, G.H. (1948) *J. Biol. Chem.* 172, 619–635.
- [15] Nedergaard, J. and Cannon, B. (1979) *Methods Enzymol.* 55, 1–28.
- [16] Löw, H. and Vallin, I. (1963) *Biochim. Biophys. Acta* 69, 361–374.
- [17] Fain, J.N. (1975) *Methods Enzymol.* 35, 555–561.
- [18] Lin, C.S. and Klingenberg, M. (1980) *FEBS Lett.* 113, 299–303.
- [19] Engvall, E. and Perlmann, P. (1972) *J. Immunol.* 109, 129–135.
- [20] Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* 193, 265–275.
- [21] Weber, K. and Osborne, M. (1969) *J. Biol. Chem.* 244, 4406–4412.
- [22] Laemmli, U.K. (1970) *Nature* 227, 680–685.
- [23] Lin, C.S., Hackenberg, H. and Klingenberg, M. (1980) *FEBS Lett.* 113, 304–306.
- [24] Sundin, U., Moore, G. and Cannon, B. (1982) submitted.
- [25] Lindberg, O., Bieber, L.L. and Houšťek (1976) in: *Regulation of Depressed Metabolism and Thermogenesis* (Janský, L. and Musacchia, X.J. eds) pp. 117–136, Thomas, Springfield OH.