Evidence from immunoblotting studies on uncoupling protein that brown adipose tissue is not present in the domestic pig

PAUL TRAYHURN,[1] NORMAN J. TEMPLE, AND JOHNY VAN AERDE

Nutrition and Metabolism Research Group, Departments of Medicine, Pediatrics, and Foods & Nutrition, 536 Newton Research Building, University of Alberta, Edmonton, Alta., Canada T6G 2C2

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Adipose tissues and other tissues of the pig have been examined for the presence of the mitochondrial "uncoupling protein," characteristic of brown adipose tissue, in order to assess whether brown fat is present in this species. Mitochondria were prepared from various tissues and the proteins separated on the basis of molecular weight by sodium dodecyl sulphate – polyacrylamide gel electrophoresis. Immunoblotting procedures were then used to probe for uncoupling protein, employing a rabbit anti-(rat uncoupling protein) serum. Pigs were examined at 4 days, 4 weeks, and 8 weeks of age. No evidence for the presence of uncoupling protein was found at any of these ages. The protein was, however, readily detected in brown adipose tissue from rats, mice, golden hamsters, guinea pigs, Richardson's ground squirrel, and lambs. An additional group of pigs was acclimated to the cold (10°C) for a period of 10 days prior to the examination of tissues, but again uncoupling protein was not detected in any tissue. These results indicate that uncoupling protein is either absent from adipose tissues of the pig or is present at such a low concentration that it is unlikely to support thermogenesis. It is concluded that the pig does not contain adipose tissue that is functionally "brown;" adipose tissues in this species appear to be exclusively "white."

Key words: brown adipose tissue, white adipose tissue, uncoupling protein, thermogenesis, immunoblotting.

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Les tissus adipeux et les autres tissus du porc ont été examinés, afin de mettre en évidence la présence de la protéine découplante mitochondriale caractéristique du tissu adipeux brun. Les mitochondries ont été préparées à partir de plusieurs tissus et les protéines ont été separées selon leur poids moléculaire par électrophorèse sur gel de polyacrylamide avec dodécyl sulfate de sodium. La protéine découplante a été identifiée par immunoblotting en utilisant un anticorps produit chez le lapin, contre la protéine découplante de rat. Les porcs examinés étaient âgés de 4 jours, 4 et 8 semaines. A aucun de ces âgés la protéine découplante n'a été mise en évidence, alors qu'elle a été détectée dans le tissu adipeux brun de rats, souris, hamsters dorés, cobayes, écureuils de Richardson et agneaux. Un groupe supplémentaire de porcs acclimatés au froid (10°C) pendant une période de 10 jours avant l'examen de tissus, a également été examiné. Dans ces conditions, la protéine découplante n'a été détectée dans aucun des tissus examinés. Ces résultats démontrent que la protéine découplante est soit absente du tissu adipeux, soit présence à de si faibles concentrations qu'elle ne joue aucun rôle dans la thermogenèse. Nous concluons que le porc ne contient pas de tissu adipeux fonctionnellement "brun;" chez cette espèce, le tissu adipeux semble exclusivement "blanc."

Introduction

Brown adipose tissue (BAT) is now considered to have several distinct functions in mammals. The most widely recognized role of the tissue is in the generation of thermoregulatory heat by nonshivering thermogenesis, a role which is particularly evident in the newborn and young (see Nedergaard et al. 1986). The tissue is also generally considered to play a role in the regulation of energy balance, at least in rodent species (see Rothwell and Stock 1986; Trayhurn 1986). A further important role for BAT is the provision of triiodothyronine from thyroxine, through the presence of a high activity of a type II deiodinase (Silva and Larsen 1983).

The primary mechanism for the generation of heat in BAT is a proton conductance pathway located in the mitochondria. This pathway leads to a proton short circuit, dissociating substrate oxidation from ATP synthesis (see Nicholls and Locke 1984; Nicholls et al. 1986). The pathway is regulated by a 32 000 M_r mitochondrial protein, termed uncoupling protein or thermogenin. Uncoupling protein, and the proton conductance pathway, appear to be unique to BAT, so that the presence of the protein in an adipose tissue provides a powerful diagnostic tool for differentiating the brown and white forms of the tissue (Cannon et al. 1982; Lean et al. 1983; Hansen et al. 1984).

Current views on the functional importance of BAT have been derived primarily from studies on laboratory rodents. However, it is clearly necessary to establish the species distribution of the tissue, so as to determine the general applicability of present ideas on its significance. One species in which there has been some uncertainty as to whether BAT is present is the domestic pig. The young, though not newborn, pig appears to have some capacity for noradrenaline-stimulated nonshivering thermogenesis (LeBlanc and Mount 1968; Jamieson et al. 1984), and during this form of heat production there is a substantial increase in blood flow to various adipose tissues (Jamieson et al. 1984; Mayfield et al. 1986), consistent with the possibility that these tissues are functionally "brown." While fetal and 6-month-old pigs have been reported not to contain BAT (Dauncey et al. 1981; Hausman and Thomas 1987), the presence of adipose tissue with the general histological appearance of brown fat has been noted in 2 to 3 month-old pigs (Dauncey et al. 1981).The most compelling evidence for BAT in the pig comes from a study in which immunoreactivity consistent with the presence of uncoupling protein was reported in adipose tissue from animals aged 1 week or less (Henningfield and Swick 1987).

In the present study, we set out to systematically examine

[1]Author to whom correspondence may be sent at the following address: Division of Biochemical Sciences, Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, Scotland, U.K.

adipose tissues of the pig for the presence of uncoupling protein, using immunoblotting procedures, with a view to establishing whether BAT is indeed present in this species, and if so, whether the amount of uncoupling protein can be increased by acclimation to the cold. Pigs were examined at three different ages (4 days, 4 weeks, and 8 weeks), and the effects of cold acclimation were investigated in 4-week-old animals. The results provide no evidence for the presence of BAT in the pig.

Materials and methods

Animals

The pigs used in this study were of the PIC (Pig Improvement Canada) breed (Lacombe × Landrace with Large White), from the University of Alberta Research Swine Unit. Both sexes were taken, but the majority of the animals were female. The young were kept with the sow until weaning at 28 days of age. On weaning, the pigs were fed Starter's Feed, containing 22.8% protein (by weight) and with an energy density of 14.7 kJ/g. Ambient temperature was maintained at 25–28°C. In the study on the effects of cold acclimation, pigs were taken from the sow at 28 days of age and the environmental temperature gradually decreased, according to the following protocol: 21°C on day 29, 18°C on day 30, 15°C for days 31 and 32, 12°C for days 33 and 34, and 10°C for days 35–45.

Three pigs were studied in each age-group and the same number was used in the cold-acclimation experiment. The pigs were sedated with halothane and killed by the administration of an overdose of T-61 (Hoescht, Canada).Samples of the following organs and tissues were rapidly removed and placed on ice: heart, liver, kidney (medulla), and adipose tissue from six different sites. These sites were subscapular, axillary, subcutaneous (from the rear back), perirenal, pericardial, and peritoneal. After transport on ice to the laboratory, the tissues were stored at −85°C to await analysis.

Gel electrophoresis

Tissues were thawed and homogenized in a buffer (pH 7.2) containing 250 mM sucrose, 1 mM HEPES, and 0.2 mM EDTA. Mitochondria were then prepared by differential centrifugation, as described previously for BAT (Trayhurn et al. 1987); this procedure was adopted for all tissues, to maximize the chance of obtaining "BAT" mitochondria. Mitochondrial protein was measured using a modified Lowry method, with bovine serum albumin as a standard (Trayhurn et al. 1987).

The mitochondria were solubilized by heating at 70°C for 30 min in a protein dissociation solution containing 1% (w/v) sodium dodecyl sulphate (SDS), 0.1 *M* Tris base, and 1.5% (w/v) dithiothreitol. The proteins were then separated on the basis of molecular weight by SDS–polyacrylamide gel electrophoresis (Neville 1971) using a vertical slab gel system (Tyler Research Instruments Corporation, Edmonton, Alberta). Up to 25 μg of mitochondrial protein from each pig tissue was applied to the gel.

Mitochondria from adipose tissues of several species other than the pig were also prepared and examined. The species and tissues taken were interscapular BAT from rats and mice acclimated at 4°C for 3 weeks, interscapular BAT from guinea pigs and golden hamsters housed at room temperature (21°C), perirenal BAT from newborn lambs, perirenal adipose tissue from adult dogs, and visceral fat from magpies.

A purified uncoupling protein standard was prepared from mitochondria isolated from axillary BAT of Richardson's ground squirrels (*Spermophilus richardsonii*) by the method of Lin and Klingenberg (1980).

Immunoblotting

The separated proteins were transferred to a nitrocellulose membrane by Western blotting (Towbin et al. 1979) with a BioRad Trans-Blot Cell (BioRad Canada Ltd., Mississauga, Ontario). The membranes were probed for the presence of uncoupling protein with a rabbit anti-(rat uncoupling protein) serum, as previously described (Milner et al. 1989). Briefly, spare binding sites on the nitrocellulose membranes were first blocked by incubating with 3% (w/v) gelatin in 20 mM Tris–500 mM NaCl (pH 7.5). Following subsequent incubation with the antiserum, the antigen–antibody complex was identified by a colour reaction, employing goat anti-rabbit IgG alkaline phosphatase conjugate (BioRad, Richmond, California, U.S.A.).

Results

In the main part of this study, a number of tissues were examined for the presence of uncoupling protein in pigs at 4 days and at 4 and 8 weeks of age using immunoblotting for the identification of the protein. Figures 1 and 2 show representative immunoblots obtained at each of the three ages. A purified uncoupling protein standard from Richardson's ground squirrel was included on each immunoblot, and a sharp band of immunoreactivity is clearly evident in the 32 000 dalton (Da) region. Mitochondria from perirenal BAT of lambs were used as a reference and again an immunoreactive band was found with a molecular mass of approximately 32 000 Da.

In contrast, there was no evidence of immunoreactivity at a molecular weight corresponding to uncoupling protein in any of the pig tissues examined at each of the three ages (Figs. 1 and 2). The lack of immunoreactivity in the 32 000 Da region was apparent in all of the six different adipose tissue depots, as well as with the heart, kidney, and liver. Some immunoreactivity was detected, however, in the 50 000 Da region with mitochondria from the liver and kidney. The nature of this material is unclear, but it does not seem to bear any relationship to uncoupling protein; its molecular weight is too low to represent a dimer of the protein. We have occasionally observed similar immunoreactivity with liver mitochondria from rodents (P. Trayhurn and G. Jennings, unpublished observations).

In the second study, 4-week-old pigs were acclimated to the cold (10°C) for 10 days, with a view to increasing nonshivering thermogenesis and thereby maximizing the potential for the development of any BAT. Since the temperature was gradually reduced to 10°C, the animals were 6.5 weeks of age when tissues were sampled. The same series of adipose and nonadipose tissues were examined as before; Fig. 3 shows a representative immunoblot. There was again no evidence in any of the tissues for immunoreactivity at a molecular weight corresponding to uncoupling protein. Immunoreactivity was clearly observed with the purified uncoupling protein from ground squirrels and with the perirenal adipose tissue mitochondria from lambs.

In an additional experiment, designed in part to further verify the validity of the immunoblotting methodology, BAT mitochondria from several different species were examined for the presence of uncoupling protein. Figure 4 shows the immunoblot obtained. Immunoreactivity in the 32 000 Da region, consistent with the presence of uncoupling protein, was observed with BAT mitochondria from Richardson's ground squirrel, rats, lambs, mice, and golden hamsters. No such immunoreactivity was obtained, however, with mitochondria isolated from pig liver, with perirenal adipose tissue from an adult dog, or with visceral adipose tissue from magpies.

Some small differences between species in the apparent molecular weight of uncoupling protein are evident in Fig. 4, consistent with a previous report (Freeman et al. 1985).

Discussion

The central aim of this study was to establish whether or not BAT is present in the domestic pig. Traditionally, the identifica-

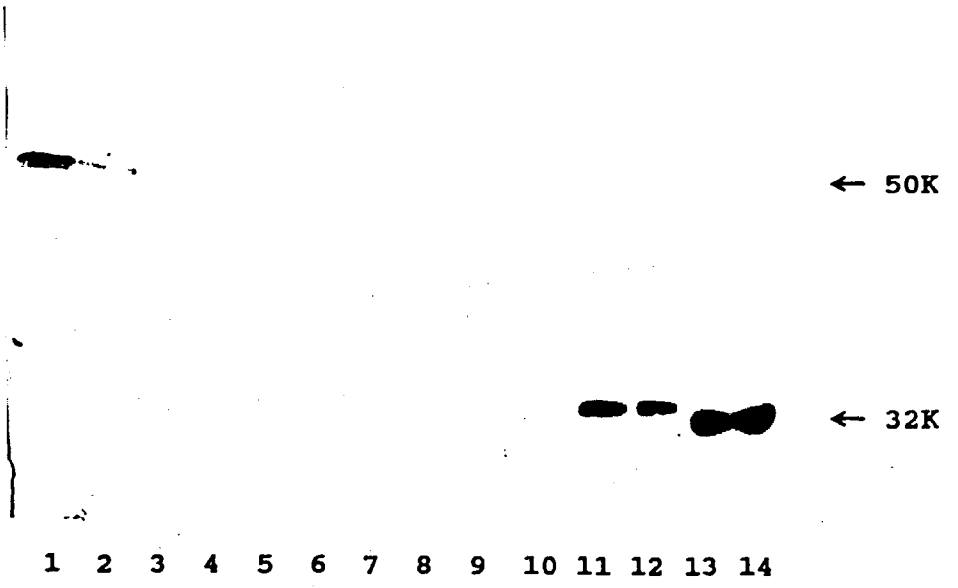

FIG. 1. Immunoblotting for uncoupling protein of tissues of pigs at 4 days of age. Mitochondria were prepared and the immunoblotting procedure was performed as described in Materials and methods. Except where otherwise indicated, approximately 25 μg of mitochondrial protein was used for each sample. 1, liver; 2, kidney; 3, heart; 4, axillary adipose tissue; 5, peritoneal adipose tissue; 7, pericardial adipose tissue; 8, subcutaneous adipose tissue; 9, subscapular adipose tissue; 10, blank; 11 and 12, pure uncoupling protein from ground squirrels (100 ng); 13 and 14, lamb perirenal adipose tissue (5 μg of mitochondrial protein). The arrows indicate the position of molecular weight markers.

tion of the tissue in an animal has been made on the basis of the histological appearance and the reddish-brown colour. The primary histological features generally considered to distinguish BAT from white adipose tissue is the multilocular structure of the stored lipid (unilocular in white adipose tissue) and the presence of large numbers of mitochondria with a well-developed cristae structure (mitochondria are much more sparse in white adipose tissue).These distinctions are far from absolute, however, and in some situations it is not possible to differentiate between the two forms of adipose tissue on a histological basis; for example, in obese animals and in older animals where the requirements for thermogenesis are low. Despite the limitations, there is nonetheless some histological evidence for the presence of BAT in 2- to 3-month-old pigs (Dauncey et al. 1981).

It is now clear that thermogenesis in BAT is regulated by the 32 000 M_r uncoupling protein located in the inner mitochondrial membrane (Nicholls and Locke 1984). This protein appears to be unique to BAT. It has not been identified in other tissues (Cannon et al. 1982; Lean et al. 1983; Hansen et al. 1984) and as such its presence in an adipose tissue provides a powerful biochemical criterion for determining whether that tissue is functionally "brown." Immunological procedures provide a potent means of identifying uncoupling protein, and this approach has been used in the present study on the pig. A similar approach has also been employed recently in investigations on BAT in several other species, particularly cattle, sheep, and ground squirrels (Casteilla et al. 1987; Milner et al. 1989).

No evidence was found here for the presence of uncoupling protein in adipose tissues, or nonadipose tissues, of the pig at any of the three ages examined. Nor was the protein detected when pigs were acclimated to the cold in an attempt to maximize nonshivering thermogenesis. In the commonly studied rodent species, acclimation to cold leads to the substantial development of BAT, with a major increase in the amount of uncoupling protein and the thermogenic capacity of the tissue (see Himms-Hagen 1986; Trayhurn et al. 1987). The sensitivity of the immunological procedure that we have employed is such that 50 ng of uncoupling protein can readily be detected. Since up to 25 μg of mitochondrial protein was present in each individual sample, uncoupling protein would in principle be detectable if it were present at, or above, a concentration of 0.2% of mitochondrial protein. In other species, such as rats and mice, the concentration of uncoupling protein in BAT mitochondria has been reported in the range of 5–15% of mitochondrial protein in cold-acclimated animals (Ashwell et al. 1983; Lean et al. 1983; Trayhurn et al. 1987) and down to 0.5% of mitochondrial protein in animals acclimated at a thermoneutral temperature (Trayhurn et al. 1987). Thus if uncoupling protein were present in the pig, its concentration would be somewhat less than that found in rodents acclimated at thermoneutrality, when the requirements for thermogenesis are at a minimum.

It should be emphasized that we have employed an antiserum raised against uncoupling protein isolated from the rat. There is, therefore, the possibility that the failure to detect uncoupling protein in adipose tissues of the pig could be a consequence of poor cross-reactivity between the pig and rat proteins. However, the cross-reactivity of uncoupling protein between species is generally high (Ricquier et al. 1983; Hansen et al. 1984; Henningfield and Swick 1987), as illustrated in Fig. 4 where the anti-(rat uncoupling protein) serum readily detected the protein in BAT mitochondria from mice, guinea pigs, golden hamsters, ground squirrels, and lambs, as well as from rats. In addition, previous studies have indicated that there is also a high degree of cross-reactivity between uncoupling protein from species as divergent as rats and humans (Lean et al. 1986).

We have also performed immunoblotting with an antiserum raised against uncoupling protein from the guinea pig, and again no evidence for the presence of the protein in pigs was found (A. Howe and P. Trayhurn, unpublished observations). It therefore

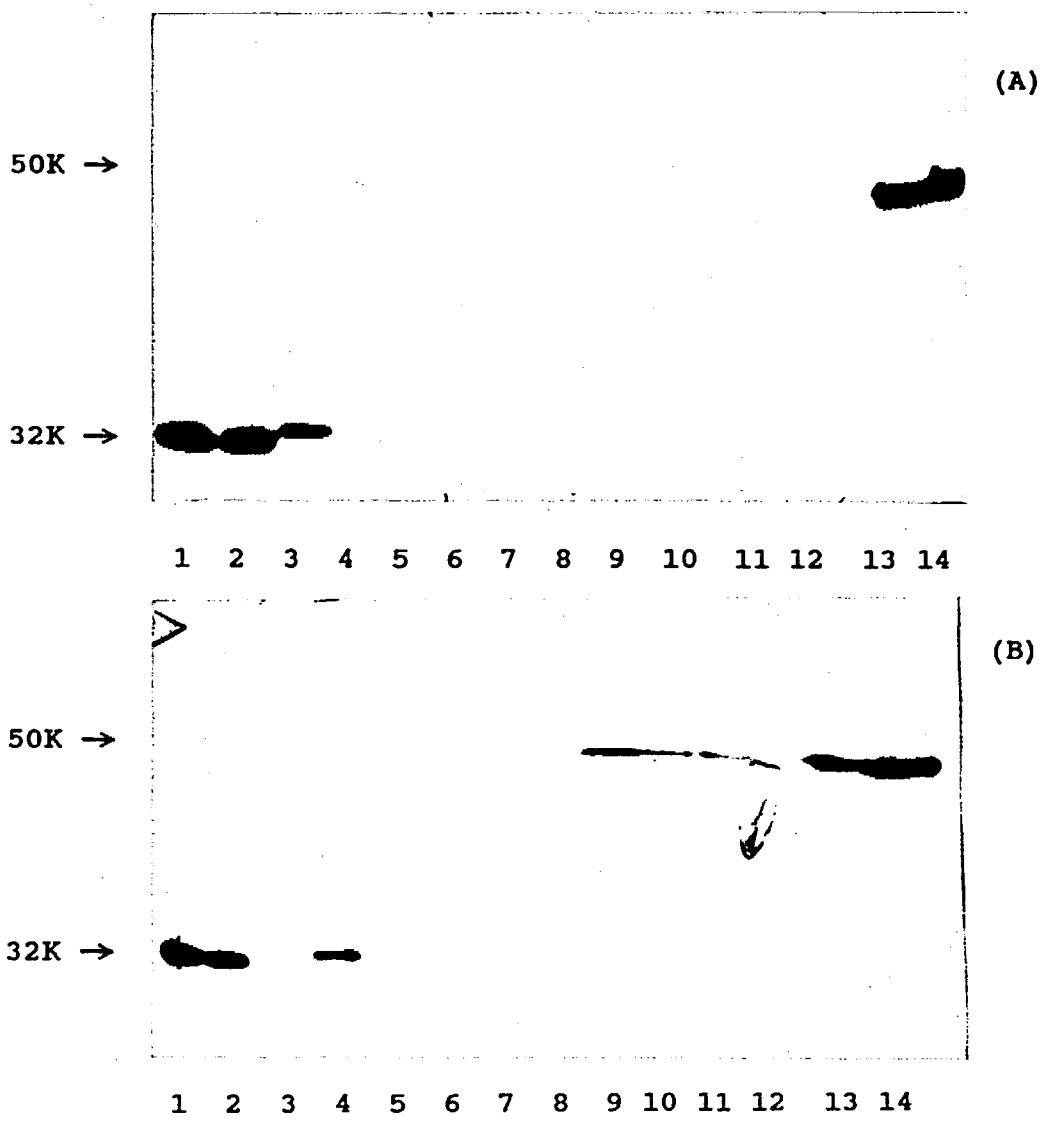

FIG. 2. Immunoblotting for uncoupling protein of pig tissues at 4 weeks (A) and 8 weeks (B) of age. 1 and 2, lamb perirenal adipose tissue; 3 and 4, purified uncoupling protein from ground squirrels; 5, blank; 6, subscapular adipose tissue; 7, subcutaneous adipose tissue; 8, pericardial adipose tissue; 9, perirenal adipose tissue; 10, peritoneal adipose tissue; 11, axillary adipose tissue; 12, heart; 13, liver; 14, kidney. For other details see legend to Fig. 1.

seems unlikely that the failure to detect uncoupling protein in the pig is due to poor cross-reactivity. Other factors that need to be considered include the question of the breed or strain of pigs. This is particularly important in view of the report that immunoreactive material with a molecular weight characteristic of uncoupling protein was detected in inguinal and perinephric adipose tissue from pigs aged 1 week or less (Henningfield and Swick 1987). Unfortunately, the breed investigated in this previous study is not given, but it is likely to be different from that used here (Pig Improvement Canada).

The report that uncoupling protein is present in the pig was based on the presence of *two* immunoreactive bands in the 32 000 M_r region rather than a single band. Two bands were also noted in the same study with mitochondria from sheep adipose tissue (Henningfield and Swick 1987), although only one was evident in the present work. It should be noted that immunoreactivity was reported in adipose tissue mitochondria of the pig with 50 μg of mitochondrial protein loaded onto the gel, which was up to 10 times the amount of protein used with the other species examined (Henningfield and Swick 1987).The sensitivity of the immunological procedure employed in the earlier report was similar to the present study in that 50 ng of uncoupling protein could be detected. It seems possible that the two immunoreactive bands observed previously in the pig with large amounts of mitochondrial protein applied to a gel may represent a nonspecific reaction. Alternatively, if one of the bands does indeed represent uncoupling protein, then the protein would seem to be present at such a low concentration that it is unlikely to be able to support thermogenesis.

Although the possibility of strain or breed differences cannot be entirely discounted, it appears from the results reported here that BAT is absent from the pig, using the criterion of the

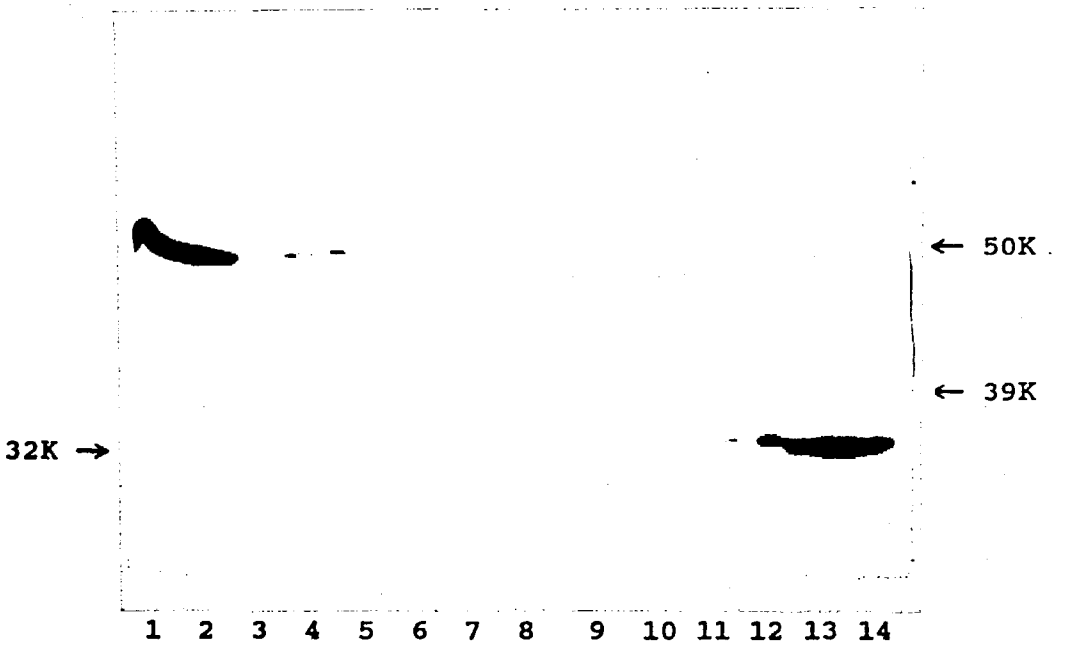

FIG. 3. Immunoblotting for uncoupling protein of tissues following acclimation of pigs to the cold (10° for 10 days). 1, liver; 2, kidney; 3, heart; 4, axillary adipose tissue; 5, peritoneal adipose tissue; 6, perirenal adipose tissue; 7, pericardial adipose tissue; 8, subcutaneous adipose tissue; 9, subscapular adipose tissue; 10, blank; 11 and 12, purified uncoupling protein from ground squirrels; 13 and 14, lamb perirenal adipose tissue. For other details see the legend to Fig. 1.

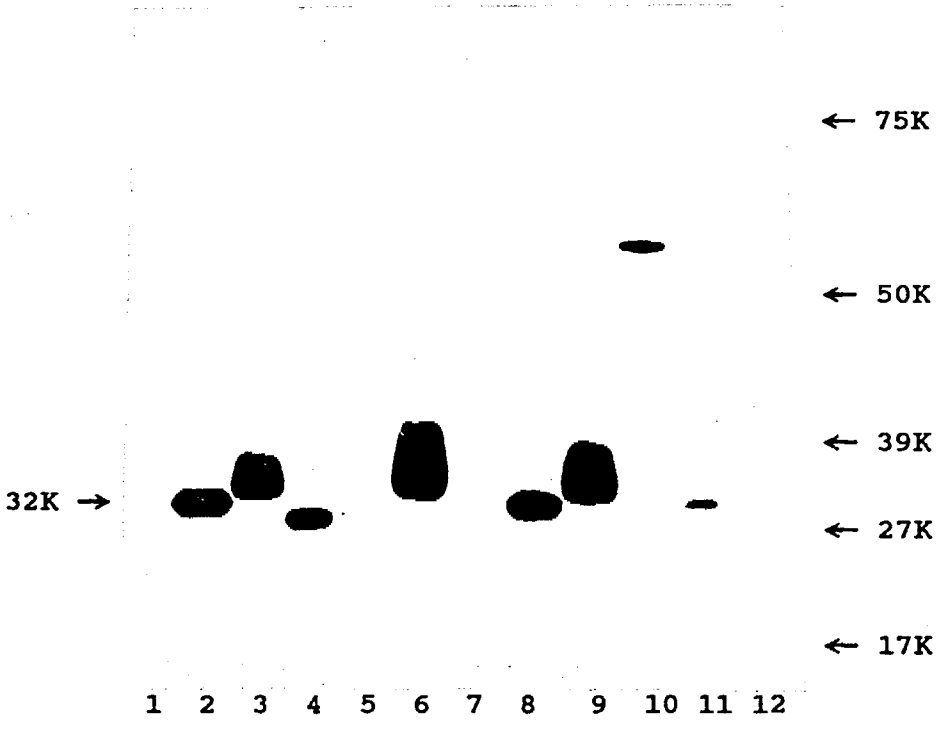

FIG. 4. Immunoblotting for uncoupling protein in tissues from different species. Mitochondria were prepared and examined as described in the Materials and methods. Each sample contained 5 μg of mitochondrial protein. 1, purified uncoupling protein from ground squirrels (100 ng); 2, rat interscapular BAT; 3, guinea pig interscapular BAT; 4, lamb perirenal BAT; 5, dog perirenal adipose tissue; 6, golden hamster interscapular BAT; 7, magpie visceral adipose tissue; 8, ground squirrel axillary BAT; 9, mouse interscapular BAT; 10, pig liver; 11, purified uncoupling protein from ground squirrels (100 ng); 12, blank.

identification of uncoupling protein as the critical diagnostic feature of the tissue. A corollary of this conclusion is that all adipose tissues in the pig should be regarded as functionally "white." Thus nonshivering thermogenesis in pigs must involve mechanisms other than those associated with BAT. The apparent absence of BAT from the pig also has important implications for studies on energy metabolism and whole-body energetics, in that the species can provide an experimental model for investigating energy balance and its regulation where BAT is *not* a factor. Finally, the evolutionary significance of BAT being absent from the pig needs some consideration. It is, however, difficult to provide a satisfactory evolutionary perspective, although it may be relevant that the domestic pig has been subject, over a long period, to intensive selection for agricultural purposes.

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