Nonshivering Thermogenesis in a Marsupial (the Tasmanian Bettong Bettongia gaimardi) Is Not Attributable to Brown Adipose Tissue

Randy W. Rose, Adrian K. West, Ji-Ming Ye, Graeme H. McCormack, Eric Q. Colquhoun
1School of Zoology, University of Tasmania, Hobart, Tasmania 7001, Australia; 2Division of Biochemistry, University of Tasmania, Hobart, Tasmania 7001, Australia

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ABSTRACT

The Tasmanian bettong (Bettongia gaimardi, a marsupial) is a rat-kangaroo that increases nonshivering thermogenesis (NST) in response to norepinephrine (NE). This study attempted to assess whether brown adipose tissue (BAT), a specialized thermogenic effector, is involved in NST in the bettong. Regulatory NST, indicated by resting oxygen consumption (V\textsubscript{O\textsubscript{2}}) of the whole body, was measured under conscious conditions at 20°C with various stimuli: cold (4°C ± 5°C) or warm (23°C) acclimation, NE injection, and the \(\beta_3\)-adrenoceptor agonist (BRL) 37344. In line with the functional studies in vivo, the presence of BAT was evaluated by examining the expression of the uncoupling protein 1 (UCP1) with both rat cDNA and oligonucleotide probes. Both NE and BRL 37344 significantly stimulated NST in the bettong. After cold acclimation of the animals (at 4°C ± 5°C for 2 wk), the resting V\textsubscript{O\textsubscript{2}} was increased by 15% and the thermogenic effect of NE was enhanced; warm-acclimated animals showed a slightly depressed response. However, no expression of UCP1 was detected in bettongs either before or after cold exposure (2 wk). These data suggest that the observed NST in the marsupial bettong is not attributable to BAT.

Introduction

Mammals are able to maintain a relatively constant body temperature with a daily rhythm over a wide range of environmental temperatures. When exposed to cold, a mammal adopts two strategies to raise heat production: shivering thermogenesis and nonshivering thermogenesis (NST). Whereas shivering is an acute response derived from stratified muscle contraction, this response is gradually replaced by NST when the animal is acclimated to the cold. In small or newborn eutherian mammals (e.g., rodents and rabbits), much of the NST is thought to occur in brown adipose tissue (BAT) where the uncoupling protein (UCP) works as a proton-conductance pathway to dissipate the proton gradient across the mitochondrial inner membrane to generate heat rather than adenosine triphosphate (ATP; Bouillaud et al. 1985; Himms-Hagen 1990; Trayhurn 1994). This thermogenic process is controlled by the sympathetic nervous system, primarily via \(\beta_3\)-adrenoceptors. Consequently, norepinephrine (NE) secretion is increased in response to cold (Himms-Hagen 1990; Arch and Kaumann 1993; Lowell 1996).

Recently, several other UCPs have been identified. A novel UCP, designated UCP\(_2\), is widely expressed in various tissues, including muscle and adipose tissues in humans and mice (Fleury et al. 1997). A third member of the UCP family, UCP\(_3\), has also been characterized to be more specific to skeletal muscle (Boss et al. 1997; Gimeno et al. 1997). These newly discovered UCPs share relatively low homology in their amino acid sequences with the UCP found exclusively in BAT (Boss et al. 1997; Fleury et al. 1997; Gimeno et al. 1997). To distinguish it from the newly identified UCPs, the traditional UCP is now named as UCP\(_1\). The tissue specificity of UCP\(_1\) in BAT has been widely used as a sensitive marker to identify whether BAT is present or absent in a species (Brander et al. 1993; Trayhurn 1994).

Marsupials are a diverse group of mammals with well-developed thermoregulation (Dawson and Dawson 1982; Rose et al. 1990; Körtner and Geiser 1996). Several marsupials increase their NST in response to NE. For instance, NE elicits a significant increment in V\textsubscript{O\textsubscript{2}} in the potoroo (Potorous tridactylus; Nicol 1978) and in pouch young Bennett’s wallabies (Loudon et al. 1985). The Tasmanian bettong (Bettongia gaimardi) is a rat-kangaroo (body mass = 1.6–2.2 kg) closely related to potoroos, wallabies, and large kangaroos (Rose et al. 1990). In this species, NE induces NST (in pouch young and adults) via \(\alpha_1\)- and \(\beta\)-adrenoceptors (Ye et al. 1996; Rose et al. 1998).
Because this thermogenic response is not entirely blocked by phentolamine plus propranolol (Ye et al. 1996), β-adrenoceptors or atypical β-adrenoceptors may also be involved.

However, the role of BAT in marsupial thermogenesis is still debated. So far, most histologic examinations have failed to show this tissue (Nicol 1978; Hayward and Lisson 1992). On the basis of a thorough and systemic survey, Hayward and Lisson (1992) have concluded that marsupials, including the bettong, do not possess histologic BAT. However, a recent report on the use of an antibody to UCP1, has suggested the presence of BAT in a small (8–10 g) insectivorous marsupial (Sminthopsis crassicaudata) in response to cold exposure (Hope et al. 1997). Although histologic experiments have been unable to identify BAT in the bettong (Hayward and Lisson 1992), this result has not been confirmed by molecular biology studies.

In this study, we further examined thermogenic responses of the bettong to various treatments that are known to elicit NST attributed to BAT in other small mammals. In conjunction with these functional experiments, we attempted to assess whether BAT tissue could be identified on the basis of UCP1 expression at the mRNA level.

Material and Methods

Animals

Thirteen Tasmanian bettongs (Bettongia gaimardi, 1.6–2.2 kg) (eight males and five females) were obtained from the breeding colony of the School of Zoology, University of Tasmania, Hobart (R. W. Rose). Initially, the animals were housed in an animal compound in a natural day/night cycle with free access to food and water. They were accustomed to human handling since birth. Diet was a choice of apples, bread, and Pal dog pellets (Uncle Ben’s of Australia), containing 15% crude protein and 10% fat. During the period of experimentation, the ambient temperature ranged between 15° and 10°C. During the period of experimentation, the ambient temperature ranged between 15° and 10°C.

Purpos.

Temperature Acclimation

Experiments involving cold or warm acclimation were performed in another five bettongs (three males and two females). Before the acclimation, both BMR and NE-elicited VO2 were measured at 20°C. NE or saline (0.1 mL) was injected as a bolus (0.4 mg kg⁻¹ intramuscular) as in our previous study (Rose et al. 1998). Metabolic readings were obtained 15 min after injection to avoid influences of the handling. The warm exposure was carried out in a temperature-controlled room at 25°C for 2 wk with 12L : 12D day/night cycle. This temperature is 4°C above the normal mean maximum temperature for the hottest month of the year in Hobart. The cold exposure was carried out in a cold room at 4°–5°C for 2 wk with a 12L : 12D cycle. This temperature is slightly lower than the mean minimum temperature during winter in Hobart. After the cold acclimation, both basal and NE-elicited VO2 were measured at 20°C (as in Ye et al. 1996; Rose et al. 1998). The animals were then killed with an overdose of pentobarbital and tissues of interest taken for the analysis of UCP1 mRNA expression.

Determination of UCP1 mRNA

Northern blotting was used to detect expression of UCP1 mRNA in the bettong. Fat depots from interscapular and peri-aortic sites were taken from bettongs (normal [N = 2], and cold exposed at 4°–5°C for 24 h [N = 2], and acclimated for 14 d [N = 4]). Total RNA was isolated and northern blotted using standard techniques (Hannan and West 1991). Liver and interscapular BAT tissues from normal (20°C) and cold-acclimated (4°–5°C) rats (280–300 g) were used as respective negative and positive controls. Northern blots were probed with a cDNA clone of rat UCP1 (generously provided by D. Ricquier [Bouillaud et al. 1985]) under conditions of reduced stringency. After the results were obtained, the cDNA probe was stripped off the membrane, and a 27-mer oligonucleotide homologous to rat UCP1 mRNA, as described by Brander et al. (1993), was used to reprobe the membrane. The cDNA was hybridized as described at 37°C, while the UCP1 oligonucleotide was hybridized at 35°C. Finally, an oligonucleotide homologous to 18S rRNA was used to indicate the relative amounts of RNA in each track.

Effects of BRL 37344

Five bettongs from the university’s breeding colony, maintained in outdoor enclosures, were used to assess the effects of BRL 37344 infused from the tail vein via an indwelling catheter during spring (8°–17°C, photoperiod approximately
12L: 12D). The drug was freshly made in sterile 0.9% NaCl solution and infused at a flow rate of 0.02 mL kg$^{-1}$ min$^{-1}$. The NaCl solution was continuously infused to keep the line open before and after BRL 37344 administration. Our previous study showed that infusion of 0.9% NaCl does not affect the metabolic rate of the bettong (Ye et al. 1996). Usually, animals settled down within 15 min with regard to their resting metabolic rate, but 30 min was allowed to elapse before collecting data. $V_O_2$ was measured at 20°C with a modified O$_2$/CO$_2$ gas analyzer (Datex, Labtech, Helsinki).

**Agents and Chemicals**

The [−]-Norepinephrine bitartrate and phenolamine-HCl, [+−]-propranolol-HCl were obtained from Sigma (Castle Hill, N.S.W.). BRL 37344 was a gift from SmithKline Beecham. Oxidized (β)nicotinamide adenine dinucleotide, lactate dehydrogenase, and bovine serum albumin (Fraction V) were purchased from Boehringer Mannheim Corp. (Indianapolis). Other chemicals were analytical grade from Ajax Chemicals (Seven Hills, N.S.W.).

**Statistical Analysis**

Data are presented as mean ± standard error (SE). Student's $t$-tests or ANOVA were used for the statistical analyses. $P<0.05$ was regarded as statistically significant.

**Results**

**Whole-Body Metabolic Response to Cold/Warm Exposure**

Although the difference was not significant ($P=0.78$), four of five animals from the cold-acclimated group lost body mass (Table 1). A two-factor repeated measurement ANOVA showed significant differences between groups ($df=2,14$, $F=7.14$, $P=0.009$) and treatment (NE injection; $df=2,14$, $F=131.66$, $P=0.0001$) in relation to heat production. Post hoc Fisher PSLD tests showed that there were significant differences in the metabolic rate of the cold-acclimated group compared to the other two groups after temperature acclimation. All groups showed a significant increase in mass-specific metabolic rate after injection of NE, but this was significantly greater in the cold-acclimated group of bettongs than in the other two groups ($df=2,14$, $F=7.36$, $P<0.01$). Table 1 also illustrates the percentage increase in metabolic rate after injection of NE; the rate in the cold-acclimated group increased by approximately 71% compared with 47% in the control group and 38% in the warm-acclimated bettongs. This difference was significant ($df=2,14$, $F=4.54$, $P<0.003$). The mass loss in the cold-acclimated group may have compromised the results, as they were calculated as mass-specific values. Reanalyzing the data by using total oxygen consumption and not mass-specific values, we found that the oxygen-consumption values after acclimation were not significant ($P>0.8$), but the effect of NE was significant ($df=2,14$, $F=56.13$, $P<0.001$). The increase in the cold-acclimated group was again significantly greater than in the other two groups ($df=2,14$, $F=4.08$, $P<0.05$), and the percentage change remained the same.

**Whole-Body Thermogenesis Induced by BRL 37344**

As shown in Figure 1, infusion of BRL 37344 at a rate of 20 μg kg$^{-1}$ min$^{-1}$ significantly increased $V_O_2$ by 31% (0.54 ± 0.06 vs. 0.68 ± 0.09 mL g$^{-1}$ h$^{-1}$, $P<0.01$, $N=5$). At the same time, the respiratory coefficient was reduced from 0.86 ± 0.03 to 0.76 ± 0.02 ($P<0.05$). These changes were not abolished by a subsequent coinfusion of propranolol (40 μg kg$^{-1}$ min$^{-1}$) for 40 min.

**Expression of UCP1 mRNA**

Figure 2 shows results from a typical experiment. RNA from the interscapular BAT or liver of two untreated rats, and from the liver and two fat deposits of four bettongs that had been exposed to 4°C–5°C for 15 d, was hybridized to the cDNA probe. Whereas UCP1 mRNA is clearly detected in rat IBAT (lanes 1, 15) but not in rat liver (lanes 2, 16), there is no evidence for analogous signals in any of the bettong samples. Likewise, no UCP1 candidate transcripts were detected in the bettongs undergoing different cold stress treatments (e.g., warm acclimated and then cold exposed for 24 h), or by using the alternative technique.

| Table 1: Effect of temperature acclimation on bettong whole-body metabolic rate |

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body Mass (kg ± SEM)</th>
<th>$V_O_2$ ± SEM (mL g$^{-1}$ h$^{-1}$ after Saline)</th>
<th>$V_O_2$ NE ± SEM (mL g$^{-1}$ h$^{-1}$)</th>
<th>Increase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.07 ± .20</td>
<td>.46 ± .02</td>
<td>.67 ± .04</td>
<td>47.37 ± 7.82</td>
</tr>
<tr>
<td>Warm acclimated at 25°C</td>
<td>2.03 ± .17</td>
<td>.47 ± .02</td>
<td>.66 ± .06</td>
<td>38.34 ± 21.9</td>
</tr>
<tr>
<td>Cold acclimated at 5°C</td>
<td>1.88 ± .22</td>
<td>.53 ± .02</td>
<td>.90 ± .07</td>
<td>70.90 ± 12.1</td>
</tr>
</tbody>
</table>

Note. The metabolic rate, expressed as $V_O_2$ (mL O$_2$ g$^{-1}$ h$^{-1}$), was measured by using an indirect calorimetry system in a well-ventilated sealed respiratory chamber at 20°C before and after norepinephrine (NE). NE was injected as a bolus (0.4 mg kg$^{-1}$) intramuscularly. Peak values taken from an average of 5 min were used. Five animals were used in each experiment.
Figure 1. Effect of BRL 37344 on whole-body metabolic rate of normal bettongs. BRL 37344 was infused via the tail vein through an indwelling catheter at a rate of 20 μg kg⁻¹ min⁻¹ for 40 min (filled bar). Basal values (open bar) were obtained during the infusion period of 0.9% NaCl at the same rate. Five experiments were conducted. Data are expressed as means (SE and paired t-test was used for the statistical analysis). P<0.01 versus the basal value.

oligonucleotide probe (data not shown). Hybridization to an 18S oligonucleotide is shown to indicate relative amounts of RNA loaded in each lane.

Discussion

NST could be defined as heat production, other than that caused by shivering or locomotion, often (but not exclusively) in response to increased NE secretion or short photoperiod acclimation. This has been shown to occur in some marsupials only, including the bettong in this study. However, for this to be physiologically significant, it is necessary to also show that this response is augmented by cold acclimation. For the first time in a marsupial, this study has shown that to occur. We have shown that heat production was increased by 15% after cold acclimation (and not at all after warm acclimation), and also that the response to NE was augmented after cold acclimation (from 47% to 71%). This augmented response did not occur in the warm-acclimated bettongs. We have yet to determine whether NE secretion is increased in response to cold acclimation.

Marsupials have a well-regulated thermoregulation (Nicol 1978; Rose et al. 1990; Körtner and Geiser 1996; Song et al. 1996). For instance, the change of metabolic scope in small dasyurid marsupials (<100 g) has been shown to be greater than that of rodents of similar body size (Dawson and Dawson 1982). In some small insectivorous marsupials (<150 g), hibernation (Körtner and Geiser 1996) and torpor (Song et al. 1996) have also been described in response to low ambient temperature. In the bettong, exogenous catecholamines, such as NE, isoproterenol, and phenylephrine, significantly stimulate NST (Ye et al. 1996; Rose et al. 1998).

In our previous study (Ye et al. 1996), NE-induced bettong thermogenesis was not entirely blocked by a combination of phentolamine and propranolol, implying a possible involvement of β₂- or atypical β-adrenoceptors. The stimulation of VO₂ by BRL 37344 in this study further supports this possibility. BRL 37344 is a known β₁-adrenoceptor agonist that stimulates BAT thermogenesis in vitro and increases thermogenesis in animals with BAT (Arch and Kaumann 1993; Summers et al. 1995; Liu et al. 1996). The effect of β₂-adrenoceptor agonists on thermogenesis in possums and wallabies has recently been examined, but no positive results were found (Nicol et al. 1997). Hence, the finding of β₁-agonist-stimulated thermogenesis in a marsupial is novel.

The reason for the disagreement in these two studies is not known but may be because of the species differences within marsupials. For example, the lack of NE-induced thermogenesis was also reported in the Brazilian possum (Monodelphis domestica, 70 g) and the brush tail possum (Trichosurus vulpecula, 3 kg; Nicol et al. 1997). Conversely, the bettong (Ye et al. 1996; Rose et al. 1998), potoroo (1 kg; Nicol 1978), and juvenile wallaby (>1 kg; Loudon et al. 1985) are thermogenically responsive to NE administration. The three species that showed such a response to NE are all Tasmanian members of the Macropodidae. The other marsupials failing to respond to NE belong to non-Macropodid families. These findings led to the suggestion that this phenomenon may be a phylogenetic dif-
ference between Macropodid and non-Macropodid marsupials (Nicol et al. 1997; Rose et al. 1998).

In most small mammals (such as rodents) and hibernating animals, BAT is a major tissue for NST in response to cold exposure or the stimulation of NE or β3-adrenoceptor agonists (Himms-Hagen 1990; Jansky 1995; Lowell 1996). Thus, we used two probes specific for UCP, mRNA to search for the expression of UCP1, to examine the presence or absence of BAT in the bettong. As expected, UCP1 mRNA was easily detected with either rat cDNA or oligonucleotide probes specific for UCP1 in the interscapular fat depot from rats. However, neither the cDNA nor the oligonucleotide probe could detect bands corresponding to UCP1 mRNA in normal or cold-exposed bettongs after hybridization even at reduced stringency. These results suggest that either the bettong does not have ratlike UCP1 mRNA expression or the expressed mRNA has very low homology to the rat UCP1 mRNA. However, because the oligonucleotide probe hybridizes well with a wide range of different species (Brander et al. 1993; Trayhurn 1994), its negative results do not appear to support the latter possibility. This interpretation is consistent with the histologic results in the bettong (Hayward and Lisson 1992).

In the absence of BAT, how does NST occur in the bettong in response to catecholamine stimulation or cold exposure? One possible explanation is alluded to above in relation to the “burning” of white adipose tissue. However, one bettong lost no weight after cold exposure yet showed the greatest enhancement (93%) of the effect of NE. Another explanation that we favor lies in skeletal muscle. As reviewed by Jansky (1995), a number of studies have indicated that skeletal muscle in a whole contributes substantially to the resting body metabolic rate in humans and rats. The evidence for NE-induced Vo2 has been obtained from isolated perfused muscle preparations across a wide range of species including the rat (Clark et al. 1995; Jansky 1995; Ye et al. 1995a), the chicken (Eldershaw et al. 1997), and even the cane toad (Ye 1995). Consistent with its effect in the whole body (Ye et al. 1996), exogenous NE is able to elicit a substantial increment in Vo2 in the perfused bettong hindlimb (Ye et al. 1995b). As BRL 37344 resulted in a marked increase in whole-body Vo2 (Fig. 2) that cannot be attributed to BAT as discussed earlier, we postulated that this effect may relate to its action on muscle based on our earlier observations with NE. In this species (Ye et al. 1995b) and BRL 35135A on the constant pressure and perfused rat hindlimb (Ye et al. 1995a). The stimulation by BRL 37344 of hindlimb Vo2 perfused at constant-flow rates without recirculation does appear to support this hypothesis. In radioligand binding studies, atypical β-adrenoceptors are abundant in muscle with affinity for both NE and BRL 37344 (Summers et al. 1995), despite their elusive nature in molecular biology in this tissue (Summers et al. 1995; Liu et al. 1996; Eldershaw et al. 1997). The role of atypical β-adrenoceptors in muscle thermogenesis is also implicated by the observation that BRL 37344 and CL 316243 (another β3-adrenoceptor agonist) stimulate the uptake and oxidation of glucose in the incubated rat skeletal muscles (Liu et al. 1996).

Several lines of evidence in the literature support an enhanced thermogenic role of muscle NST after cold acclimation in general. In the Muscovy duckling, an animal devoid of BAT, the activity of cytochrome oxidase is increased by 34% after cold acclimation at 4°C for 4 wk (Duchamp and Barré 1993). In rats, muscle thermogenesis is significantly augmented in response to catecholamine stimulation after cold acclimation (Shiota and Masumi 1988; Van Hardeveld et al. 1999). Thus, it appears to be reasonable to speculate that similar changes of muscle may occur in the bettong after cold acclimation.

In conclusion, our results indicate that marsupial bettongs can increase thermogenesis in response to catecholamine stimulation or cold exposure. This response is not attributable to euthermic mammal-like BAT because UCP1 expression is undetectable. Because skeletal muscle responds to NE (Ye et al. 1995b) and BRL 37344 by increasing its Vo2 similarly to that in the whole body, it may play an important role in bettong NST.

Acknowledgments

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