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# Changes in cytochrome oxidase activity in brown adipose tissue during oestrous cycle in the rat

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## Abstract

Cold-acclimated female Wistar rats were killed at a known phase of the oestrous cycle to investigate whether natural fluctuations of female sex steroids during the cycle are mirrored by changes in brown adipose tissue (BAT) activity. Four phases: proestrus, oestrus, dioestrus I and dioestrus II, were identified by the cells types appearing in vaginal smears. BAT was removed and analysed for composition and specific indicators of BAT activity. No changes in BAT mass, protein or mitochondrial content were detected during the cycle. Thermogenesis was also unchanged as judged by both lipid multilocularity and mitochondrial GDP-binding. However, cytochrome c oxidase (COX) activity did cycle with a minimum at proestrus and a maximum at dioestrus I. The involvement of oestradiol in such a cycle is suggested by the fact that oestradiol treatment decreased COX activity to values similar to those found in proestrus. The physiological function of a cyclic oxidative capacity remains to be elucidated.

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## Introduction

In cold-acclimated rodents, thermoregulatory thermogenesis occurs in brown adipose tissue (BAT) depots, which are located adjacent to a number of important internal organs. The high thermogenic capacity of brown fat is due to the presence of an uncoupling protein (UCP-1). This acts as a proton conductance channel which allows protons to re-enter the mitochondrial matrix without ATP synthesis. The increased proton permeability allows an enhanced oxidation of substrates uncoupled from ATP production so that heat is generated at a very high rate (1). The intense substrate oxidation of active BAT has a significant impact on energy balance status. Thus, chronically cold-exposed rats show a reduced rate of body weight gain compared with animals at thermoneutrality, despite an enhanced food intake (2).

Female sex steroids are thought to contribute to the control of BAT function. Brown adipocytes isolated from rats treated with either oestradiol or progesterone show a reduced response to noradrenaline, which suggests a direct effect on brown adipocytes (3, 4). Further, oestradiol administered chronically to cold-acclimated rats inhibits BAT thermogenesis (5) whereas the level of sympathetic activity is not modified (6). The physiological role of such effects is only partially understood. Plasma oestradiol and progesterone levels increase during pregnancy, reaching a maximum in the 3rd week. At that time, there is a reduced BAT activity not only in moderate (7) but also in severe cold (8). Such suppression has been interpreted both as a way of preferentially directing substrates towards foetal growth (9) and as a way of reducing foetal oxygen consumption, thereby reducing maternal and hence foetal deep body temperature (10). Since virgin rats have a natural rhythmic fluctuation in plasma oestradiol and progesterone during the oestrous cycle, with a peak at the time of proestrus (11), we have investigated whether these cyclic fluctuations of sex steroids are accompanied by parallel fluctuations in indices of BAT activity. In addition, in a second study, the effects on these indices of administered oestradiol were investigated.

## Materials and methods

Female Wistar rats of 200–220 g body weight were acclimated to 6 °C for either 6–8 weeks in the oestrous cycle reference measurements or for 3 weeks in the oestradiol-treatment experiment. They were held in individual cages with free access to food and water and a light–darkness schedule of 12 h : 12 h. The food provided was a commercial diet containing 66.7% carbohydrate, 19.3% protein, 3.4% fat, 4.9% cellulose and 5.7% mineral (w/w). Animals were cared for and used in accordance with the principles of The Council of European Communities (86/609 EEC).

**Table 1** Body weight, IBAT mass and protein content (means ± S.E.) of cold-acclimated rats during the oestrous cycle. The number of animals is in parentheses.

| | Days in cold | Body weight (g) | IBAT mass (mg) | Proteins | |
|---|---|---|---|---|---|
| | | | | mg/100 mg IBAT | mg/IBAT |
| Proestrus (8) | 53 ± 1 | 260 ± 6 | 965 ± 60 | 9.7 ± 0.5 | 92.3 ± 5.0 |
| Oestrus (7) | 48 ± 3 | 259 ± 6 | 992 ± 120 | 8.8 ± 0.7 | 84.6 ± 6.8 |
| Dioestrus I (5) | 47 ± 2 | 258 ± 14 | 947 ± 58 | 9.3 ± 1.0 | 86.3 ± 6.5 |
| Dioestrus II (6) | 51 ± 3 | 255 ± 7 | 969 ± 79 | 8.5 ± 0.6 | 81.1 ± 7.1 |

No statistically significant differences were found when comparing values from different phases by one-way ANOVA.

In both experiments, vaginal smears were taken daily, starting 3–4 days prior to the experiment, to determine the phase of the cycle. Smears were stained by dipping into a filtered 0.1% aqueous solution of methylene blue, allowed to dry at room temperature, then examined microscopically to determine from the characteristic pattern of cell types present the stage of the oestrous cycle at death. In the second experiment, cold-acclimated rats were implanted, under ether anaesthesia, with a Silastic capsule either empty or filled with 17$\beta$-oestradiol. Administration of oestradiol lasted 2 weeks.

On the day of the experiment, the rats were killed by decapitation. Interscapular BAT (IBAT) was quickly removed, cleaned of adherent white adipose tissue (WAT) and muscle, weighed and immediately processed for cytochrome c oxidase (COX) and GDP-binding measurements. At this time, a small piece of BAT was fixed in formalin (100 ml formaldehyde; 900 ml distilled water; 4 g $NaH_2PO_4$. $H_2O$; 6.5 g $Na_2HPO_4$) for morphological analysis. The fixed sample was dehydrated in ascending graded alcohols, cleared in chloroform and impregnated in molten paraffin wax (melting point 56–58 °C). Sections were cut at 5 $\mu$m and were stained using Shorr's formulation (12). In a double-blind test, sections (three from each animal) were projected onto a video screen and were independently graded (by five persons) for degree of multilocularity on a scale from 0–4 (where 0 represents WAT, and 4 BAT fully acclimated for maximum thermogenesis). Mean scores/grades were calculated. These gave an indirect index of thermogenic capacity of IBAT from the sampled animal (13).

GDP-binding was measured as previously described with minor modifications (5). Briefly, mitochondria from each interscapular BAT depot were isolated by several centrifugations at 12 000 ***g***. Protein and COX activity were measured in the initial tissue homogenate and in the final mitochondrial suspension using the procedures of Lowry *et al.* (14) and Yonetani & Ray (15) respectively. Mitochondria were incubated in a medium containing [$^3$H]GDP and trapped under suction in a cellulose membrane. Membranes were disintegrated in scintillation liquid, d.p.m. being measured in a scintillation counter. [$^{14}$C]Sucrose was used as an extramitochondrial marker of the medium trapped in the membrane.

Statistical analysis was carried out using a one-way ANOVA with Duncan's test being used post hoc (SPSS software).

## Results

The animals in the first experiment had spent about 50 days in the cold when they were killed at known phases of the oestrous cycle. We did not find statistical differences between groups either in body weight, IBAT mass or tissue protein content (Table 1). Therefore, the oestrous cycle does not cause a major effect on IBAT mass and gross composition. The level of multilocularity was also unchanged during the oestrous cycle. Thus, Table 2 shows the results of the histological scoring of IBAT samples for multilocularity, related to the stage of the oestrous cycle. IBAT scores remained unchanged during the oestrous cycle thus suggesting no major metabolic changes in the tissue. However, since mitochondria are the intracellular thermogenic machinery, we measured metabolically relevant variables in these organelles. As shown in Table 3, the mitochondrial content of IBAT was unaltered during the oestrous cycle, as well as the activity of UCP-1, assessed by its GDP-binding, which was unaltered during the different phases of the cycle (Table 3). However, Fig. 1 shows that a cyclic fluctuation was evident in the COX activity per mg mitochondrial

**Table 2** Degree of multilocularity (means ± S.E.) of brown adipocytes of cold-acclimated rats during the oestrous cycle. Values are scores on a scale from 0 to 4. The number of animals is in parentheses.

| | Score |
|---|---|
| Proestrus | 3.54 ± 0.17 (8) |
| Oestrus | 3.43 ± 0.41 (7) |
| Dioestrus I | 3.40 ± 0.14 (2) |
| Dioestrus II | 3.40 ± 0.23 (5) |

No statistically significant differences were found when comparing values from different phases by one-way ANOVA.

**Table 3.** Mitochondrial content and thermogenic activity (means ± S.E.) of IBAT of cold-acclimated rats during the oestrous cycle. The number of animals is in parentheses, except for GDP-binding measurements in oestrus (***n*** = **6**) and in dioestrus II (***n*** = **4**).

| | Mitochondrial protein | | GDP-binding | |
|---|---|---|---|---|
| | mg/100 mg IBAT | mg/IBAT | nmol GDP/mg mitochondrial protein | nmol GDP/IBAT |
| Proestrus (8) | 4.7 ± 0.5 | 44.5 ± 4.0 | 1.22 ± 0.18 | 58.5 ± 11.6 |
| Oestrus (7) | 4.4 ± 0.7 | 39.8 ± 3.9 | 1.21 ± 0.04 | 48.2 ± 5.3 |
| Dioestrus I (5) | 3.9 ± 0.4 | 36.0 ± 3.5 | 1.26 ± 0.11 | 44.6 ± 3.3 |
| Dioestrus II (6) | 3.8 ± 0.4 | 37.3 ± 4.4 | 1.34 ± 0.18 | 56.7 ± 5.1 |

No statistically significant differences were found when comparing values from different phases by one-way ANOVA.

protein. Thus, COX activity values found in proestrus were only 58% of those found in dioestrus I. Since IBAT mass was unchanged during the cycle, a cyclic fluctuation was also evident whether COX activity was calculated either per 100 mg tissue or per IBAT depot (Table 4).

Figure 2 depicts COX activity in cold-acclimated control rats at proestrus, at dioestrus I and after oestradiol treatment (experiment 2). Values reached after hormone treatment are similar to those in proestrus.

## Discussion

Earlier studies showed that female sex steroids have a suppressive effect upon BAT function; however, these were done on rats experimentally treated to increase their plasma levels of either oestradiol or progesterone (3, 4, 5, 16). During the oestrous cycle there is a continuous natural change in plasma oestradiol and progesterone levels (11). Thus, a cycling female rat is an excellent physiological model to study the effects on BAT function of short-term cyclic changes in plasma sex steroid levels. We expected this study to show BAT function to be suppressed in naturally cycling virgin rats at the time of proestrus, since this is the time when the levels of oestradiol and progesterone are highest. Cold-acclimated rats were chosen as the experimental model since it is in such animals that BAT activity is at its highest and therefore where it should be easiest to detect suppressive effects of sex hormones.

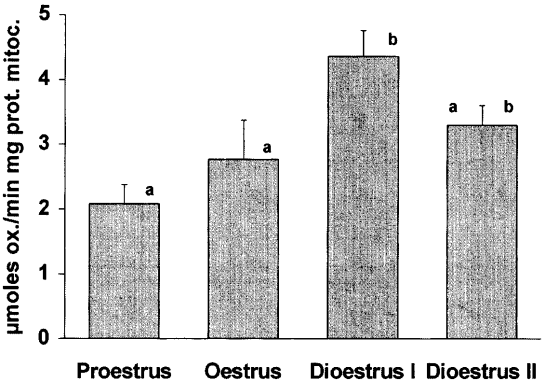


**Figure 1** Mitochondrial COX activity of cold-acclimated rats during the oestrous cycle. Bars represent means ± S.E. of the number of animals of Table 2. Different superscript letters mean $P < 0.05$ when comparing values from different phases by one-way ANOVA. (ox, oxidized).

At the time of killing, the rats in the first experiment had spent about 50 days in the cold (Table 1) and were therefore fully cold acclimated. BAT mass remained at around 970 mg, irrespective of the phase of the cycle. Since the protein content of the tissue was also unaltered during the cycle (Table 1) these result showed that the oestrous cycle sex hormone fluctuations do not bring about major changes in tissue mass or gross composition. However, as the machinery for thermogenesis is in the mitochondria, we measured the total mitochondrial content and the COX-specific activity of the mitochondria of rats in different phases of the oestrous cycle. As can be seen in Table 3, the mitochondrial content of BAT was unchanged during the oestrous cycle. However, the COX activity per mg mitochondrial protein was not constant but changed during the cycle, with a minimum at proestrus (Fig. 1). Since neither IBAT mass nor gross composition was changed, this change in specific activity meant that total oxidative capacity of mitochondria, either per 100 mg tissue, or per IBAT depot was not constant but was related to the phase of the oestrous cycle (Table 4).

**Table 4.** Total oxidative capacity (means ± S.E.) of cold-acclimated rats during the oestrous cycle. The number of animals is in parentheses.

| | COX activity | |
|---|---|---|
| | µmol ox/min per 100 mg IBAT | µmol ox/min per IBAT |
| Proestrus (8) | 9.0 ± 0.9$^{a}$ | 88.2 ± 12.8$^{a}$ |
| Oestrus (7) | 10.3 ± 2.0$^{a}$ | 98.8 ± 16.0$^{a}$ |
| Dioestrus I (5) | 16.3 ± 1.4$^{b}$ | 151.6 ± 4.6$^{b}$ |
| Dioestrus II (6) | 12.9 ± 1.9$^{a,b}$ | 124.7 ± 18.9$^{a,b}$ |

Different superscript letters mean $P < 0.05$ when comparing values from different phases by one-way ANOVA.
(ox, oxidized).

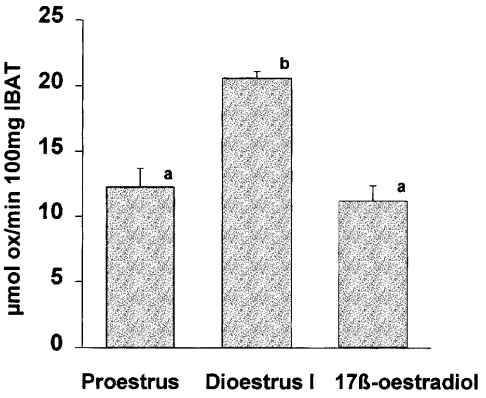


**Figure 2** COX activity in cold-acclimated rats during the oestrous cycle and after oestradiol treatment. Bars represent means ± S.E. of four animals. Different superscript letters mean $P < 0.05$ when comparing values from different phases by one-way ANOVA. (ox, oxidized).

The maximum capacity for substrate oxidation in proestrus was only 58% of that in dioestrus I. Therefore, our results show that the oestrous cycle influences oxidative capacity of BAT. However, the actual level of heat production occurring in BAT via uncoupled thermogenesis is not indicated by oxidative capacity, rather by the level of GDP-binding to UCP-1, since this measures at any moment the amount of UCP-1 that is active as a proton channel. When we measured GDP-binding we observed it to be unaltered during the oestrous cycle (Table 3). In other words, BAT-uncoupled thermogenesis remained unchanged during the oestrous cycle. This conclusion was supported by measuring the level of multilocularity of brown adipocytes (a sensitive, though inferential index, of the level of thermogenesis), which also remained unchanged during the cycle (Table 2). Taken together our results showed that BAT undergoes a functional rhythm mirroring the oestrous cycle. However, such a rhythm does not affect BAT-specific uncoupled thermogenic machinery as indicated by the constant GDP-binding, but rather the total oxidative capacity as revealed by the fluctuating COX activity. Up till now it was thought that the lower the acclimation temperature, the greater the BAT mitochondrial content, UCP-1-specific activity and GDP-binding and COX-specific activity. At a given constant environmental temperature, it was thought that the mitochondrial-specific activity of both COX and GDP-binding remained constant. This work has shown that cold-acclimated female rats do have a constant level of uncoupled thermogenesis, but a changing oxidative capacity due to a changing level of specific mitochondrial COX activity, in phase with the reproductive cycle.

Two plausible reasons can be suggested to explain the changes in COX activity. Either the total amount of the enzyme is changed during the cycle, as a consequence of reduced synthesis or enhanced breakdown, or both; or its activity is modulated by some factor with the total amount of the enzyme remaining constant. Considering the first alternative, it is appropriate to point out that for detection of a diminution in synthesis it would be necessary for the half-life of COX to be shorter than the duration of the proestrus phase of 12–18 h. The reported half-live of COX varies with the species; as brief as 1.3 days in the shrimp *Artemia franciscana* (17) and as long as 5 days in rat liver (18–20). As far as we are aware, BAT COX half-life has not been determined, but from the above reported values of half-lives it seems improbable that the changes in COX activity we detected during the proestrus of the rat (lasting 12–18 h) were due to altered synthesis rate. Therefore, it seems more plausible to suggest that COX activity undergoes modulation, perhaps allosteric in nature, by some unknown factor associated with the oestrous cycle. Further studies are required to investigate this.

What may be the agent(s) responsible for the rhythm in COX activity? Plasma levels of several hormones are increased during proestrus making them candidates for the cause of the inhibition found (11). Since we have previously demonstrated an inhibitory effect of oestradiol on BAT thermogenesis (5), we repeated this study to see whether indeed oestradiol would reduce COX activity to the low levels observed in proestrus. The result is presented in Fig. 2, which shows that oestradiol does indeed reduce COX-specific activity to levels similar to those found during proestrus in spontaneously cycling female rats. Therefore, a suppressive effect of oestradiol on BAT COX-specific activity is demonstrated. We conclude that oestradiol may be the agent bringing about the modulation of COX-specific activity during proestrus in the cycling female rat. This possibility is supported by the observation that oestradiol does indeed modulate the activity of other enzymes, for example lipoprotein lipase (21). Nevertheless, we cannot preclude the possibility of other hormones causing or contributing to the inhibition of COX activity which we have observed.

What might be the physiological function of the COX activity rhythm in BAT? The answer may well be none during the short time scale of the oestrous cycle since: (i) the laboratory determination of COX activity is done at saturation levels of substrates, levels that are unlikely to be reached within brown adipocytes *in vivo,* and (ii) proestrus lasts only a short period of time (12–18 h of a 4–5 day cycle). Possibly we are observing an effect that in other circumstances with a different, more extended time scale, would become physiologically significant. Such circumstances could be late pregnancy or chronic oestradiol treatment where COX activity is reduced (5, 7).

To sum up, BAT oxidative capacity fluctuates during the oestrous cycle with a minimum in proestrus. BAT-uncoupled thermogenesis is, however, unchanged. As the plasma levels of oestradiol also fluctuate in the

oestrous cycle, peaking at proestrus, oestradiol is a candidate as an agent involved in this inhibitory effect.

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