Nonshivering thermogenesis without interscapular brown adipose tissue involvement during conditioned fear in the rat

Andrew Marks,1 Daniel M. L. Vianna,1 and Pascal Carrive

School of Medical Sciences, University of New South Wales, New South Wales, Australia

Submitted 26 August 2008; accepted in final form 8 February 2009

Marks A, Vianna DM, Carrive P. Nonshivering thermogenesis without interscapular brown adipose tissue involvement during conditioned fear in the rat. Am J Physiol Regul Integr Comp Physiol 296: R1239–R1247, 2009. First published February 11, 2009; doi:10.1152/ajpregu.90723.2008.—As with other forms of psychological stress, conditioned fear causes an increase in body temperature. The mechanisms underlying this stress-induced hyperthermia are not well understood, but previous research suggests that nonshivering thermogenesis might contribute, as it does during cold exposure. The major source of nonshivering thermogenesis in the rat is brown adipose tissue (BAT), and the largest BAT deposit in that species is in the interscapular area just below the skin. BAT is also under sympathetic control via β-adrenoceptors. If BAT contributes to fear-induced hyperthermia, then the interscapular skin should warm up faster than other skin areas, and this response should be suppressed by the β-adrenoceptor antagonist, propranolol. We tested this noninvasively by infrared thermography. In conscious rats, 30 min of contextual fear caused hyperthermia (as indicated by a +1.5°C increase in lumbar back skin temperature) and increased the difference in temperature between interscapular and lumbar back skin (TiScap−TBack) by +1°C. Propranolol (10 mg/kg ip) completely abolished this hyperthermia; however, the TiScap−TBack increase was not reduced. In contrast, exposure to cold air (4°C) induced a +2.7°C increase in TiScap−TBack, which was reduced to +1°C after propranolol. The results show that conditioned fear-induced hyperthermia is of nonshivering origin and mediated by β-adrenoceptors, but interscapular BAT does not contribute to it and does not appear to be activated, either.

stress hyperthermia; thermoregulation; tail skin; freezing; sympathetic responses

Many psychological stressors can cause an increase in body temperature (2, 11, 25, 29), an effect also known as stress hyperthermia (2, 18). There are two ways in which body temperature can be elevated, either by reducing heat loss or by increasing heat production. The most effective way of reducing heat loss in the animal is through skin vasoconstriction (13). Increased heat production or thermogenesis can be achieved via two mechanisms: shivering thermogenesis if the heat comes from contracting skeletal muscles (15) or nonshivering thermogenesis if it comes from elsewhere (13, 17). The best studied effector of nonshivering thermogenesis is brown adipose tissue (BAT), which is under sympathetic control (4, 20). Only present in mammals, BAT is classically called into action during cold exposure. It is most often found in newborns, small mammals, and hibernating species, which are the ones who are most prone to suffer from cold stress, but it might also be functional in adult humans (6, 20). Moreover, it is the only site where cold adaptation, that is, the development of increased recruitable thermogenic capacity in response to chronic cold exposure, occurs (4, 9, 12). In rats and mice, the main deposit of BAT is in the interscapular area (iBAT) and lies directly below the skin.

We and others have shown that conditioned fear causes hyperthermia (11, 29), but the origin of this rise in body temperature is not known. One possibility is that it is a reduction in heat loss due to the profound vasoconstriction, which as we have shown occurs in the tail and feet (29). A second possibility is that heat is being generated from the midline and proximal muscles, whose tonic activity during fear causes the characteristic immobile freezing posture. A third possibility is that BAT is implicated, as this tissue has been claimed to be the main source of the hyperthermia observed during immobilization stress (10, 25). In the present study, we sought to determine whether the latter would also be the case for conditioned fear. To make sure the animals would only be exposed to the psychological stress, we used a new approach where no restraint or instrumentation were needed. This approach, which involves measuring skin temperature with an infrared camera has been successfully used before by Alberts and coworkers in rat pups (1, 7). They have shown that during cold exposure, the interscapular skin, which lies directly above iBAT, heats up faster and more than the lumbar back skin (1, 7). This effect also occurs after noradrenaline injection (7) and can be suppressed by injection of the nonspecific β-adrenoceptor antagonist propranolol (7). This is consistent with the notion of interscapular skin warming being due to iBAT thermogenesis, for the latter is triggered by β1 adrenoceptor activation (4).

We have previously shown that conditioned fear-related hyperthermia is accompanied by a parallel increase in the temperature of the lumbar back (29). Hence, in the present study, we chose to use lumbar back temperature as an indicator of changes in body temperature, and with the same approach as Alberts and coworkers (1, 7), test the role of iBAT in the hyperthermic response of conditioned fear in the adult rat. Cold exposure, which is known to activate iBAT, was used as a positive control.

MATERIALS AND METHODS

The subjects were 24 experimentally native male Wistar rats (450–550 g) obtained from the colony of specific pathogen-free rats maintained by the University of New South Wales. The animals were housed in individual plastic home boxes (38 cm long × 25 cm wide × 60 cm tall) with food and water provided ad libitum throughout the experiment.

http://www.ajpregu.org 0363-6119/09 $8.00 Copyright © 2009 the American Physiological Society R1239

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 These authors contributed equally to this work.

Address for reprint requests and other correspondence: D. M. L. Vianna, School of Medical Sciences, Univ. of New South Wales, NSW 2052, Australia (e-mail: dmviana@unsw.edu.au).
experiment. The animals were maintained on a 12:12-h light-dark cycle, with all experiments taking place during the light phase. The experimental room was air-conditioned and maintained at a temperature of 22–24°C. All experiments were approved by the Animal Ethics Committee of the University of New South Wales and conformed to the rules and guidelines on animal experimentation in Australia.

Fear conditioning to context was carried out in footshock chambers (23 cm long × 21 cm wide × 60 cm tall, open at the top), as described by Vianna and Carrive (29). One-half of the animals (n = 8) were fear conditioned by subjecting them to four conditioning footshock sessions done on separate days over the course of 5 days. The other half were sham conditioned (n = 8), that is, they underwent the same procedure but never received footshocks. Fear-conditioned and sham-conditioned animals were tested for conditioned fear by re-exposure for 30 min to the same chambers without shock administration.

The environment for cold exposure was a small refrigerator (54 cm long × 50 cm wide × 82 cm tall) set at 4 ± 2°C. A hole (10 cm × 12 cm) was cut at the top to allow infrared imaging of the animal. The rats were lowered through the hole into a Plexiglas box (20 cm long × 23 cm wide × 40 cm tall, open at the top), which was already inside the refrigerator and whose floor was covered with clean bedding. Cold exposure lasted 30 min, after which the animals were removed through opening the refrigerator door. The subjects (n = 8, half fear conditioned, half sham conditioned) were given two pre-exposures in the refrigerator at room temperature (i.e., refrigerator off), each lasting 30 min, over two consecutive days. Half of the cohort (n = 4) was then tested on day 3 with the refrigerator off (room temperature) and on day 4 with the refrigerator on (4°C). The other half of the rats (n = 4) was tested on day 3 with the refrigerator on and on day 4 with the refrigerator off. Therefore, all rats were tested with the refrigerator on and off in a counterbalanced order.

In another series of experiments, propranolol (1-[isopropylamino]-3-[1-naphthylamino]-2-propanol; Sigma, St. Louis, MO) or saline was given intraperitoneally at a dose of 10 mg/kg body wt, immediately before the eight fear-conditioned animals were exposed to the shock box. A new set of eight naïve animals was also habituated to the refrigerator, and then exposed to refrigerator on (4°C) immediately after being treated with propranolol or saline. The order of drug testing was counterbalanced.

Surface temperature of the subject and its immediate surroundings was recorded using an infrared digital thermographic camera (Thermacam P45; FLIR, Danderyd, Sweden) placed 70 cm above the animal (as in ref. 29). The camera has a thermal sensitivity of −0.1°C and a spatial resolution of 640 × 480 pixels. Because Plexiglas and stainless-steel blocks infrared radiation, the lids of home boxes and footshock chambers were replaced by 60-cm high Plexiglas walls.

One day before each experiment started, the rats were lightly anesthetized with Halothane and shaved between the scapulae (5 × 5 cm) and over the lumbar back region (6 × 6 cm), to expose the skin for temperature readings in these areas (TiScap and TBack, respectively). The temperature reading was also obtained from the midsection of the tail (TTail). Infrared images of the whole rat were captured automatically every minute for a total of 150 min: 30 min before test (resting baseline in home box), 30 min during test (in shock box or refrigerator), and 90 min after test (recovery in home box).

Each infrared digital image simultaneously captured TiScap, TBack, and TTail. In addition, during conditioned fear, an experimenter recorded freezing behavior, sampled every 2 s for the 30 min of conditioned-fear exposure. Freezing was defined as a complete absence of movement, while the animal assumed a characteristic tense posture. Infrared images were analyzed using the Thermacam Reporter 7.0 Professional SR-4 software (FLIR). The hottest pixel from each area of interest was extracted and recorded. This gave a temperature value for each of the interscapular, back and tail regions every minute of every test. Occasionally, depending on the posture of the animal, an area would be momentarily concealed and therefore not imaged. Emissivity was set to 0.98, which is the emissivity of the skin (29).

As mentioned in the introduction, we used TiScap and TBack as indicators of the changes in temperature occurring in iBAT and in the body, respectively. To verify the equivalence of these measures, we compared them to the temperature readings of radio-telemetric thermocouples implanted underneath iBAT or in the peritoneal cavity (Fig. 1). Thus, TiScap was compared with the temperature readings of a thermocouple placed between iBAT and the muscles surrounding the scapulae using a TA-F40 probe (DSI, St. Paul, MN, USA). Fig. 1A shows one example of this dual recording during a test for conditioned fear (Fig. 1A). As can be seen, the temperature recorded by the thermocouple was on average 2.4°C higher than the temperature of the skin directly over the iBAT, but the relative changes were similar and well correlated throughout the recording (r = 0.97; P < 0.0001). TBack was compared with the temperature reading of a C50-PXT probe (DSI, St. Paul, MN, USA) implanted in the peritoneal cavity. One example of such a dual recording during a cold exposure test is shown in Fig. 1B. As can be seen, sudden changes in ambient temperature (from warm to cold and vice versa) cause quick shifts in surface temperature (a physical effect, which is observed with inanimate objects as well), but when the ambient temperature is constant (warm or cold), the relative changes in TBack are parallel to those recorded in the peritoneal cavity. In this particular example, both temperatures dropped by 1°C during cold exposure and were well correlated during this period (r = 0.75; P < 0.0005). Finally, as explained in the introduction, potential iBAT thermogenesis was estimated by computing the difference between the two surface temperature TiScap and TBack (TiScap − TBack).

Data were analyzed by repeated-measures ANOVA. The significance threshold was set at the 0.05 level. The surface temperatures at rest were relatively constant throughout the study. On average, these baselines were 34.9°C for TiScap, 34.4°C for TBack, and 28.6°C for TTail.

RESULTS

Conditioned fear. The temperature and behavioral changes evoked by re-exposure of fear-conditioned and sham-conditioned animals are shown in Fig. 2 and Fig. 3. Fear-conditioned animals displayed freezing immobility during most of the re-exposure (85% of the time), indicating a strong and sustained fear response to context. As previously reported (29), this was associated with a marked drop in TTail (−3.95 ± 0.28°C, down to room temperature) and a steady rise in TBack (+1.58 ± 0.16°C at the end of re-exposure, Figs. 2 and 3). TiScap also increased steadily but peaked higher (+2.40 ± 0.19°C at the end of the re-exposure; Figs. 2 and 3). This difference is clearly visible from TiScap-TBack, which demonstrates that the interscapular area got gradually warmer throughout the re-exposure. It reached a maximum difference of 1.31 ± 0.13°C toward the end of the re-exposure, which corresponds to an increase of +1.00°C from baseline (Fig. 3).

Sham-conditioned animals re-exposed to the same box did not freeze since they had never received footshocks in that context (Fig. 3). As previously reported (29), these animals were active walking and rearing for the first 15 min, then went to rest. A clear difference in the temperature response was observed between the first and second 15-min periods of the re-exposure. In the first half of that session, TTail dropped, while TBack and TiScap increased. The changes were almost identical to that of the fear-conditioned animals except that TiScap increased faster to reach a maximum of +1.20 ± 0.14°C, 14 min earlier than the fear-conditioned animals. As
TiScap-TBack shows, this difference can be attributed to a faster warming of the interscapular area (0.95°C in 7 min). As the animal went to rest in the second half of the re-exposure, TTail stopped dropping and stabilized half-way between room temperature and baseline (1.99 ± 0.66°C), TBack also stopped increasing and stabilized (+0.87 ± 0.16°C), TiScap slowly decreased down to +0.84 ± 0.14°C above baseline by the end of re-exposure, and TiScap-TBack gradually returned to baseline levels.

Repeated-measures ANOVAs comparing the fear- and sham-conditioned groups were done for the entire 30-min period of testing, as well as for the second 15-min periods since differences were apparent during that period for some variables. Only TTail was significantly different between fear and sham (F1,14 = 6.06, P = 0.027) when the entire 30 min of the exposure was considered (F1,14 < 3.57, P > 0.08 for the other variables). In contrast, apart from TBack (F1,14 = 0.85, P = 0.37), there were significant group effects in the second half of the testing period for TiScap (F1,14 = 16.65, P = 0.001), TiScap-TBack (F1,14 = 9.35, P = 0.009), and TTail (F1,14 = 8.51, P = 0.01). Hence, conditioned fear to context and exposure to a known but nonaversive context (sham-conditioned animals) produced comparable hyperthermic responses (in the range of +1.2°C to +1.5°C as shown by the sustained increase in TBack), but only fear produced a sustained increase in TiScap-TBack.

Cold exposure. Cold exposure is known to cause robust activation of iBAT. To find out how this strong activation would affect skin surface temperature, we compared two groups of rats placed in a small refrigerator set, either at 4°C or room temperature (Fig. 4). Exposure to the cold refrigerator immediately cooled down the skin: within the first minute of exposure, TTail had dropped by −4.43°C, TBack dropped by −3.30°C, and TiScap dropped by −2.38°C. TTail kept falling at a fast rate (−16.16°C within the first 10 min) and then slowed down but kept cooling until the end of the session, when it reached 8.69 ± 0.49°C. TBack remained the same for
the rest of the exposure (30.96 ± 0.31°C), indicating that the animals were maintaining their core temperature, but TiScap gradually increased during the first half of the exposure, to peak at a maximum of 34.14 ± 0.17°C, 1.94°C higher than at the beginning of the cold exposure. The difference TiScap-TBack reveals the heat that is locally generated under the interscapular skin (i.e., potentially of iBAT origin). The % of time spent freezing is also indicated for each minute during re-exposure. Values are expressed as means ± SE.

As expected, exposure to the small refrigerator at room temperature produced a similar type of temperature response (although slightly more intense) to that described above for sham-conditioned animals re-exposed to the shock box, indicating that the increased in TiScap-TBack to cold was not due to the refrigerator environment itself but to the lower temperature. The effect of cold on TiScap-TBack was highly significant ($F_{1,14} = 29.51, P < 0.001$).

Conditioned fear after propranolol. The role of β-adrenoceptors in the temperature responses of conditioned fear was then tested by comparing the effects of intraperitoneal injections of propranolol and saline (Fig. 5). The fear response evoked after saline was the same as described previously (see Fig. 3). After propranolol, small reductions in the freezing and TTail responses were observed, but neither were significantly different to the saline-injected animals ($F_{1,12} = 1.40, P = 0.259$ and $F_{1,13} = 0.33; P = 0.574$, respectively). In contrast, propranolol caused a marked reduction of the TiScap and TBack responses ($F_{1,14} = 6.99, P = 0.019$ and $F_{1,13} = 4.87,$...
In fact, the hyperthermic response as indicated by the rise in TBack was abolished since TBack remained unchanged throughout the re-exposure. However, the TiScap response was not abolished; a small increase remained (+0.7°C by the end of re-exposure). This corresponds to an increase of TiScap-TBack, which was practically the same as in the saline-injected animals ($F_{1,13} = 1.69; P = 0.217$, Fig. 5). Thus, propranolol had no effect on the fear-evoked increase in TiScap-TBack but completely abolished the hyperthermic response (as indicated by the rise in TBack).

**Cold exposure after propranolol.** The effect of propranolol and saline on the temperature response to cold exposure is...
shown on Fig. 6. The response after saline was the same as described in Fig. 4, except for the TiScap-TBack increase, which was not as marked (+2.14°C vs. +2.69°C, from baseline). Notwithstanding, propranolol significantly reduced this increase to a +1.03°C increase from baseline at its peak ($F_{1,12} = 19.42; P = 0.001$). There was also a gradual drop in TiScap and TBack throughout the entire session (-3.94°C and -2.58°C, respectively), suggesting that the ability to maintain body temperature was impaired. TiScap was significantly different between saline- and propranolol-treated animals throughout the session ($F_{1,12} = 10.78; P = 0.007$), while TBack was different during the second half only ($F_{1,12} = 5.15; P = 0.042$). Finally, propranolol treatment also slightly reduced the speed of the TTail fall ($F_{1,12} = 6.12; P = 0.029$), even though at the end of the session, both groups reached identical values. Thus, propranolol markedly reduced the cold-evoked increase in TiScap-TBack and the ability to generate heat in response to cold.

**DISCUSSION**

In this study, we used infrared thermography to indirectly assess iBAT thermogenesis and changes in body temperature evoked by conditioned fear and cold exposure. Using this approach, we could show a marked, β-adrenoceptor-mediated activation of iBAT during cold exposure. However, no β-ad-
renoceptor-mediated activation of iBAT could be observed during conditioned fear, even though the associated hyperthermic response was entirely β-adrenoceptor mediated. Thus, the hyperthermic response of conditioned fear is of nonshivering origin, but iBAT does not contribute to it. In fact, iBAT does not appear to be activated.

Infrared thermography is easy to use, noninvasive, and because recording is done at a distance from the target, ideal
for studies in conscious freely moving animals. Here, we recorded the changes in skin temperature in three different parts of the body (tail, lumbar back, interscapular back), and from each of these regions, we extracted a different component of the physiological response to conditioned fear and cold exposure (tail skin vasoconstriction, changes in body temperature, and potential changes in iBAT thermogenic activity, respectively). This approach has previously been used by Alberts and colleagues (1, 7) to record changes in body temperature and iBAT thermogenesis in rat pups. We show here that it also works well on the shaved skin of adult rats.

We assessed changes in body temperature from the temperature of the shaved skin of the lumbar back. As shown in Fig. 1B and in our previous work (29), this measure can be used as an indicator of the changes in body temperature, at least in the conditions of this study, and as long as ambient temperature remains constant. The rise in lumbar back temperature seen during conditioned fear directly reflects the hyperthermia associated with this response: it starts within the first minutes, is of the same magnitude as the temperature change recorded from the peritoneal cavity or rectum (11, 29), and has a time course consistent with that of a temperature response.

Potential thermogenesis from iBAT was assessed from the shaved skin that lies directly over it in the interscapular back area. This was done by computing the difference in temperature between the interscapular and lumbar back skin (TiScap-TBack), since both are exposed to the same changes in ambient and body temperature. However, iBAT is also surrounded by important postural muscles of the neck and shoulders that can potentially generate heat when activated by efferent somatic nerve. Thus, an increase in TiScap-TBack does not necessarily mean an increase in iBAT activity. This can be verified, however, with the nonspecific β-adrenoceptor antagonist, propranolol. When administered in a sufficiently high dose (i.e., 10 mg/kg ip) propranolol blocks the β3-adrenoceptors that mediate iBAT activation (4, 30).

The first experiment showed that the rise in TBack (i.e., the hyperthermia) evoked by conditioned fear was associated with an increase of TiScap-TBack, which gradually built up to a maximum of +1°C at the end of the fear response. At first, we thought that this increase in local temperature was due to iBAT thermogenesis, but propranolol did not reduce it. In contrast, cold exposure produced a marked +2.69°C increase, which was clearly reduced by propranolol to +1°C, an increase similar to that seen with conditioned fear. This positive control confirmed that increases in iBAT activity can be detected by infrared thermography and that this activation can be blocked by propranolol at the dose that we used. Thus, the +1°C increase, which was detected in fear-conditioned rats and that remained in cold-exposed animals after propranolol, does not appear to be of iBAT origin. We suspect that this locally generated heat originates from somatic activation of neck and shoulder muscles. These muscles would be activated during fear because their tonic contraction contributes to the tense immobile freezing posture, characteristic of conditioned fear. They would also be activated during cold exposure because the animals were behaviorally active during the test (personal observation, 19). In fact, it may also be the origin of the quick but short increase in differential temperature (also in the +1°C range) displayed by sham-conditioned rats and rats exposed to the refrigerator at room temperature. These animals were also active, and those changes in temperature correlate well with the 10- to 15-min burst of behavioral activity that characterizes such responses (29).

Propranolol had a clear effect on TBack in both cold-exposed and fear-conditioned rats; in both cases, reductions in TBack were observed, which are consistent with decreases in body temperature. The drop in TBack in cold-exposed animals is consistent with a drop in body temperature due to the sympathetic blockade of iBAT and other BAT deposits throughout the body, although this may not be the only source of β-adrenoceptor-mediated thermogenesis (see below). Thus, the animals were not able to generate enough heat to compensate for the heat lost to the cold environment, and therefore their body temperature (and Tback) dropped. With conditioned fear, propranolol abolished the increase in TBack, an effect, which can only be interpreted as a complete blockade of the hyperthermic response of fear. This was clearly not due to a reduction in fear because the animals still froze (see also ref. 5), and their tail temperature still dropped (an α-adrenoceptor-mediated response). There was a small reduction of these responses, suggesting a possible central anxiolytic effect of propranolol, but the effect was not significant and too small. This important result shows that the hyperthermic response of fear is not due to skin vasoconstriction (reduced heat loss) or somatic activation of skeletal muscles but to a sympathetically, β-adrenoceptor-mediated thermogenesis. Where is this heat coming from? Is it apparently not from iBAT since it was not activated. One possibility is that it comes from other BAT deposits around the body, since iBAT cannot account for more than 40% of all BAT deposits (24). However, there is no evidence to suggest that BAT deposits could be activated separately (8, 22, 26, 27). The other possibility is that it comes from non-BAT tissues. Blood flow measurements performed by Foster and Frydman (9) show that BAT contributes to less than half of the calorigenic response to noradrenaline in warm acclimated animals. The same is observed in mice lacking uncoupling protein-1 (4, 12, 21). Various tissues in the body may contribute to this thermogenesis, but according to blood flow measurement, the main contributor is the heart muscle itself (9). This would be consistent with our observations since cardiac acceleration, which is observed during fear, cold exposure, and arousal, is under β-adrenergic control (5, 19, 20, 28, 29).

To our knowledge, this is the first time that iBAT thermogenesis has been assessed during conditioned fear. Our finding that iBAT is not activated during this pure form of psychological stress is somewhat in contrast to previous reports showing that it is activated during immobilization or restraint, another form of psychological stress which, unlike fear, has a strong physical component. Gao et al. (10) showed an increase in uncoupling protein 1 activity (the protein responsible for thermogenesis in BAT) after a complete immobilization that lasted 3 h. This test is very strong and known to produce ulcers (3). The other two studies used thermistor probes implanted below iBAT. Shibata and Nagasaka (25) used a subtraction method similar to ours to estimate iBAT thermogenesis and demonstrated that the increase they observed after a 2-min immobilization was abolished by sympathectomy. Ootsuka et al. (23) used a more common restraint paradigm that lasted 30 min; however, body temperature was not subtracted, and no propranolol injection or sympathectomy was made to verify the
Thermogenesis without iBAT activation during fear

iBAT origin of the local temperature increase. Clearly, further work is needed to clarify the role of BAT in the hyperthermic response of different forms of psychological stress. The present findings suggest that recruitment of BAT is less likely to occur in the more psychological forms of stress.

Perspectives and Significance

The present results suggest that thermogenesis during fear is the result of sympathetic activation of organs whose primary role is not heat production, e.g., the heart. It may come as a surprise that the sympathetic nervous system can increase body heat via β-adrenoceptors without necessarily activating iBAT. Yet we know that BAT thermogenesis cannot account for the whole metabolic increase caused by nonshivering thermogenesis (14, 16) and that animals lacking BAT can also display noradrenaline-mediated nonshivering thermogenesis (4). To our knowledge, this study is the first demonstration in the rodent of a naturally evoked, noradrenergic, nonshivering thermogenic response, in which the participation of the main BAT deposit has been ruled out.

ACKNOWLEDGMENTS

This work was supported by a University of New South Wales Goldstar grant and a grant from the National Heart Foundation of Australia.

REFERENCES


