

## 3-HYDROXYBUTYRATE CO-INFUSED WITH NORADRENALINE DECREASES RESULTING PLASMA LEVELS OF NORADRENALINE IN WISTAR RATS

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### Summary

Pentobarbital-anaesthetized male Wistar rats were infused with  $6 \mu\text{g kg}^{-1} \text{min}^{-1}$  of noradrenaline. The infusion was supplemented with  $8.5 \text{ mg kg}^{-1} \text{min}^{-1}$  of D-3-hydroxybutyrate (3-OHB) for 15 min in order to determine its effect on the adrenergic response of the rat. Plasma levels of noradrenaline rose to a plateau of approximately  $50 \text{ nmol l}^{-1}$  with infusion. In the group infused with noradrenaline alone, noradrenaline levels were maintained for 1 h. Supplementation with 3-OHB induced a decrease in plasma noradrenaline level that was inversely correlated with 3-OHB level. Aortic and interscapular brown adipose tissue temperatures increased with noradrenaline infusion, but the rise was arrested by 3-OHB; replacing 3-OHB with glucose had no effect. Infusion of saline, glucose or 3-OHB in the absence of noradrenaline did not induce a rise in

temperature in either tissue. Blood 3-OHB concentration increased to  $1.2 \text{ mmol l}^{-1}$  during 3-OHB infusion, decreasing rapidly at the end of infusion. Blood glucose levels increased with noradrenaline infusion; the presence of high 3-OHB levels decreased glucose concentration. The effects observed were transient and dependent on 3-OHB concentration; these effects may help explain most of the other effects of noradrenaline described here. The role of 3-OHB as a regulator of adrenergic responses seems to be part of a complex fail-safe mechanism which prevents wasting.

Key words: 3-hydroxybutyrate, ketone bodies, thermogenesis, noradrenaline, brown adipose tissue, body temperature, rat.

### Introduction

Adaptive thermogenesis by brown adipose tissue (BAT) is directly related to noradrenergic stimulation (Foster and Frydman, 1978), both under cold-exposure (Foster and Frydman, 1979) or when excess energy is available (Rothwell and Stock, 1979). Continuous infusion of rats with catecholamines results in increased heat production and a rise in blood temperature (Hugie *et al.* 1992; Zylan and Carlisle, 1992) as a consequence of the stimulation of BAT (Trayhurn and Ashwell, 1987). The effects of noradrenaline infusion include increases in circulating glucose levels (Nikami *et al.* 1992), basically a consequence of massive glycogen breakdown (Shiota *et al.* 1992), as well as marked changes in blood flow distribution and cardiac output (Gordon, 1990).

Ketone bodies are synthesized by the liver under conditions of scarce glucose combined with marked lipolysis and the consequent availability of fatty acids (Ubukata *et al.* 1992). Ketone bodies are produced during starvation (Balasse, 1979; Balasse and Féry, 1989), diabetes (Sherwin *et al.* 1976; Féry and Balasse, 1985; Balasse and Féry, 1989) or the consumption of fatty low-carbohydrate (ketogenic) diets (Ide *et al.* 1984).

The large change in blood levels of 3-hydroxybutyrate (3-OHB) and acetoacetate from the basal to the ketotic condition means that these levels may be a signal to determine which substrates are oxidized (Hetenyi and Ferraroto, 1985; Ohmori *et al.* 1990; Metcalfe *et al.* 1992). It has been found that 3-OHB acts as a signal that limits BAT substrate oxidation and thermogenesis (Pokasanieva *et al.* 1993), thus preventing unwanted wastage of substrates under conditions of starvation or altered substrate utilization (De Pergola *et al.* 1990). This effect occurs through modulation of the response of BAT (Pokasanieva *et al.* 1993), but also by a direct interaction between 3-OHB and adrenergic receptors (De Pergola *et al.* 1990). The results presented here show the direct effects of 3-OHB on anaesthetized rats constantly infused with noradrenaline.

### Materials and methods

Two-month-old male Wistar rats (from Charles River, France) weighing 240–260 g were used. The animals were kept

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under controlled conditions of feeding (B&K, Sant Vicent dels Horts, Spain), illumination (light on from 08:00 to 20:00h), temperature (21–22 °C) and humidity (70%) in individual polypropylene-bottomed cages until the experiments were performed. All experiments took place between 08:00 and 10:00h. The rats were anaesthetized with an intraperitoneal injection of 50 mg kg<sup>-1</sup> sodium pentobarbital (Sigma, St Louis, MO, USA) in 9 g l<sup>-1</sup> NaCl. The rats were then placed in a chamber with the temperature controlled at 28–30 °C. Two K-type thermocouple temperature probes (Kane May, Welwyn Garden City, Herts, UK) were inserted, one on the lower aorta wall and the other between the interscapular BAT masses, as described previously (Closa *et al.* 1993). The probes were connected to a Data logger (type KM-1420, from Kane May) which recorded temperatures at 10 s intervals. These data were converted into 3 min means in order to allow comparisons between series of rats.

The lower vena cava was then cannulated using P10 polyethylene catheter tubing (Clay Adams, Parsippany, NJ, USA) for continuous infusion. A second cannula was also placed on the lower vena cava, more distal with respect to the heart, and filled with heparinized saline to keep it functional for sampling.

The infusion apparatus consisted of a slow infusion syringe pump (Precidor 5003, Infors AG, Bottmingen, Germany) filled with saline; the saline was injected at a rate of 4 ml h<sup>-1</sup> kg<sup>-1</sup>. The syringe efflux was distributed by a Y-piece into two containers filled with 280 µmol l<sup>-1</sup> L-(+)-noradrenaline bitartrate (Sigma) in 9 g l<sup>-1</sup> NaCl; one of these contained, in addition, 1 mol l<sup>-1</sup> D-(-)-3-hydroxybutyric acid sodium salt (Sigma) and 10 mg l<sup>-1</sup> Neutral Red (Sigma, used to colour this solution in order to check its flow) in 50 mmol l<sup>-1</sup> phosphate buffer (pH 7.4). The flow of either noradrenaline or noradrenaline + 3-OHB could be selected by means of a three-way tap, allowing a rapid change of infusion fluid into the lower vena cava without a change in the rate of injection or in the pressure inside the system.

We used an infusion fluid buffered to pH 7.4 to minimize the negative effects of the infusion on the acid–base equilibrium of the rat. The noradrenaline solution was prepared freshly for each rat immediately (5–10 min) before use. Both solutions (noradrenaline alone and supplemented with 3-OHB) were prepared in parallel, i.e. noradrenaline was dissolved in a volume of saline made up with freshly prepared pure water (Milli-Q quality, Millipore, Bedford, MA, USA); a volume of stock 3-OHB (brought to pH 7.4) and Neutral Red solution or plain pure water was then added. Finally, the concentrated buffer (phosphates, final concentration 50 mmol l<sup>-1</sup>) was added and mixed. The parallel containers were then completely filled with the twin solutions. At the end of the experiment, one sample of the infusion solution was taken and frozen after adding glutathione and internal standard as for the blood samples. This method was used to determine the degree of preservation of noradrenaline in the samples. There were no significant differences in noradrenaline content between the standards containing 3-OHB and those not containing it. The

losses of noradrenaline were quite uniform, 5.1 ± 1.7% (mean ± S.E.M., N=10) for freshly taken samples. Storage time affected the noradrenaline content (approximately 8% was lost over 2 weeks).

The closed system used for storage and delivery of the infusion fluid prevented its contact with oxygen, thus limiting noradrenaline oxidation (West, 1952; Hughes and Smith, 1978). Neither the utilization of a thermoneutral chamber (28–30 °C) nor a neutral pH in the buffered infusion fluid affected the noradrenaline concentration in the infused solution for the duration of the experiment and storage.

Rats were anaesthetized and prepared for infusion in 10–13 min; infusion experiments were then carried out in the heated chamber for a further 60 min.

A group of five rats was infused with 100 mmol l<sup>-1</sup> glucose instead of noradrenaline, another group of five rats with 1 mol l<sup>-1</sup> 3-OHB alone, without noradrenaline; a third group of five rats was infused with noradrenaline as indicated above, but the 3-OHB was replaced by 100 mmol l<sup>-1</sup> glucose. Finally, a group of five additional control rats was infused with 9 g l<sup>-1</sup> NaCl only.

Before the beginning of the infusion procedure, the aortic temperature of the rats was checked. Even in the heated chamber, there was a slow tendency to hypothermia caused by anaesthesia (Sessler, 1991); infusion began when the rat maintained a temperature of 36 °C for 30 s, thus ensuring an added degree of uniformity in the responses observed.

Blood samples in this series of rats were limited to approximately 0.120 ml (every 6 min) in each extraction by drawing the blood through short pieces of capillary tube used as blood containers. Blood samples were blown out of the tubes into small Eppendorf plastic vials; they were centrifuged at 10 000 g for 7 min to obtain plasma. Samples of 75 µl of plasma were mixed with 675 µl of water, 50 µl of 15 mmol l<sup>-1</sup> reduced glutathione (Sigma), 50 µl of 90 mmol l<sup>-1</sup> EDTA (Sigma) and 60 µl of 130 nmol l<sup>-1</sup> dihydroxybenzylamine (Sigma, internal standard). The samples were then shaken (with an orbital mixer for 30 min at 4 °C) with 20 mg of dry alumina (Merck, Darmstadt, Germany) and 400 µl of 500 mmol l<sup>-1</sup> Tris–HCl buffer, pH 8.6. The samples were centrifuged for 10 min at 2000 g at 4 °C. The pellet was resuspended in 1 ml of 3 mmol l<sup>-1</sup> EDTA buffered at pH 7.0 and centrifuged again for 7 min; this washing was repeated three times. The last clean pellet was resuspended in 150 µl of 200 mmol l<sup>-1</sup> perchloric acid, shaken for 1 min and then centrifuged for 10 min at 2000 g at 4 °C. The supernatant was then stored at –80 °C until used for noradrenaline estimation.

Blood was withdrawn from a second series of animals in extractions of 0.050 ml (at 3 min intervals) and then deproteinized with perchloric acid (Merck); the acid was then removed with potassium bicarbonate and by centrifugation (10 000 g for 7 min). The supernatants were stored at –80 °C until processed for glucose (Trinder, 1969) and 3-OHB (Williamson *et al.* 1962) estimation.

Noradrenaline was determined by using a high-performance liquid chromatography (HPLC) apparatus (model 1050,

Hewlett-Packard, Palo Alto, CA, USA) fitted with an electrochemical detector (model 5100A, Coulochem, Bedford, MA, USA). Samples of 50  $\mu\text{l}$  of purified extracts and standards were injected into a 15 cm  $\times$  0.46 cm column of Spherisorb ODS2 5  $\mu\text{m}$  (Tecnokroma, Sant Cugat del Vallès, Spain), and a mobile phase of 100 mmol l<sup>-1</sup> sodium acetate, 40 mmol l<sup>-1</sup> citric acid, 60 mmol l<sup>-1</sup> sodium hydroxide, 5 mmol l<sup>-1</sup> 1-octane sulphonic acid and 100 ml l<sup>-1</sup> methanol, pH 5.2, was run through at a pressure of 12.4 MPa and a rate of 0.80 ml min<sup>-1</sup> (Mefford *et al.* 1981). Noradrenaline was eluted as a distinct peak at 4.5–5 min, adrenaline at 5.5–6 min and dihydroxybenzylamine at 8.5–9 min. The assay was completed in 20 min.

Estimation of differences between groups was performed using standard one- or two-way analysis of variance (ANOVA), and the Student's *t*-test was used for direct comparison between pairs of groups. The limit of significance was set at  $P < 0.05$ .

## Results

Plasma noradrenaline levels rose sharply at the beginning of the infusion and stabilized at approximately 50 nmol l<sup>-1</sup> (Fig. 1), a level that was maintained for up to 60 min. The presence of 3-OHB in the infusion lowered the levels of noradrenaline significantly (to approximately 30 nmol l<sup>-1</sup>). Removal of the 3-OHB infusion restored the noradrenaline levels.

Noradrenaline induced a gradual increase in aortic (core) temperature to 40–41 °C. The addition of 3-OHB reduced the rate of temperature increase significantly (Fig. 2A). When 3-OHB infusion was stopped, the rate of temperature increase was restored. Infusion of saline resulted in a slow decrease in core temperature; a similar slow decrease was obtained with glucose infusion. Infusion of 3-OHB alone (data not shown) also caused a similar pattern of temperature change. The replacement of 3-OHB by glucose in noradrenaline-infused rats had no effect on the rate of increase induced by noradrenaline alone (data not shown).

The temperature changes observed in the aorta reflected changes in the temperature of the interscapular BAT. Infusions of saline, glucose (Fig. 2B) or 3-OHB alone (data not shown) induced a slow decrease in temperature identical to that of aortic core temperature. Noradrenaline infusion resulted in an increase in interscapular BAT temperature to 41–42 °C. Infusion of 3-OHB and noradrenaline resulted in a marked slowing of the rate of increase in temperature, an effect that was maintained after the cessation of 3-OHB infusion. Substitution of 3-OHB by glucose (data not shown) resulted in the same pattern as described for noradrenaline alone. The differences between interscapular BAT and aortic temperature (i.e. the differential heating induced by BAT) in the rats infused with noradrenaline and 3-OHB are shown in Fig. 2C; a temperature difference of up to 2 °C was maintained in noradrenaline-infused rats up to 45 min of infusion, after which this difference began to decrease at a comparable rate.

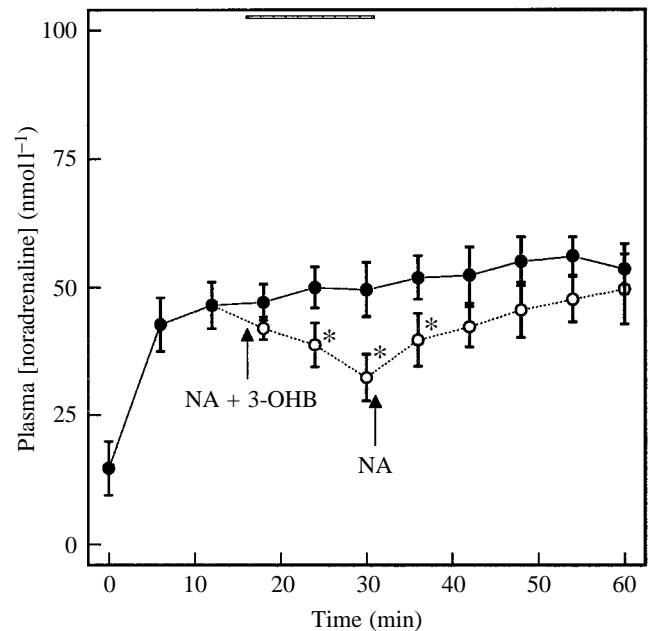


Fig. 1. Plasma noradrenaline levels in rats infused with noradrenaline (NA) (filled circles) or noradrenaline + 3-hydroxybutyrate (3-OHB) (open circles). The data represent the mean  $\pm$  S.E.M. for 7–8 different animals. The arrows indicate the period of infusion of 3-OHB (also marked as a bar at the top of the figure). Significance of the differences between groups (Student's *t*-test): the differences between the noradrenaline and noradrenaline + 3-OHB groups were significant only at 24, 30 and 36 min (asterisks).

Moreover, 3-OHB infusion halted the rise in temperature differential, an effect that continued after infusion ceased.

3-Hydroxybutyrate levels were not affected by noradrenaline treatment. Infusion of 3-OHB increased noradrenaline concentration to above 1 mmol l<sup>-1</sup> (Fig. 3). After 3-OHB infusion ceased, levels fell again to the basal concentrations.

The curve for the decrease in blood 3-OHB levels after its infusion ceased was fitted to a decay equation:

$$H = Ae^{-Kt},$$

where  $t$  is time,  $H$  is the concentration of 3-OHB,  $A$  is a constant, and  $K$  is the decay constant. The half-time for this process was 4.8 min.

Noradrenaline infusion increased blood glucose concentration gradually by almost 5 mmol l<sup>-1</sup> in 1 h (Fig. 4). However, infusion of 3-OHB reversed this effect, first causing a slow decrease in glucose concentration that then stabilized. The difference in blood glucose concentration compared with infusion of noradrenaline alone was almost 4 mmol l<sup>-1</sup>.

## Discussion

Anaesthesia affects thermoregulation in the rat; its effects are often associated with a fall in core temperature due to lower rate of heat production (Ohlson *et al.* 1992) and a diminished ability to retain heat, consistent with destabilized temperature

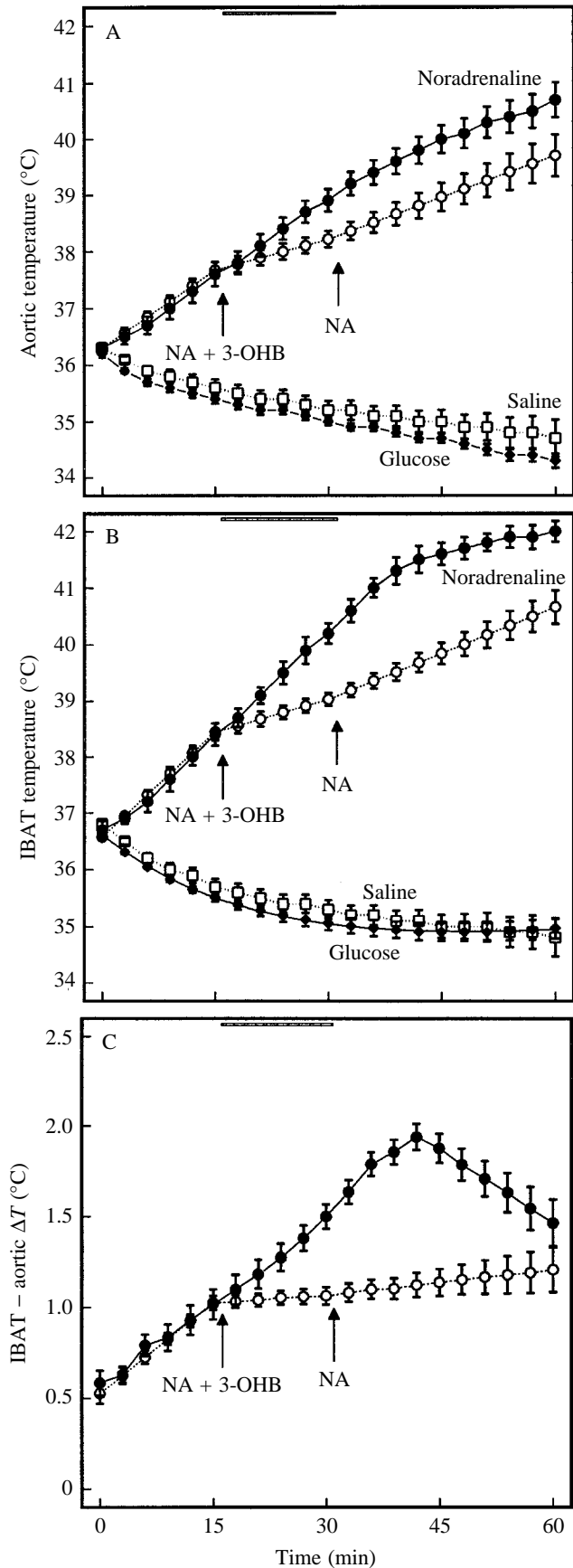


Fig. 2. Temperature changes in rats infused with noradrenaline (NA) (filled circles), noradrenaline + 3-OHB (open circles), saline (open squares) or glucose (filled diamonds). (A) Aortic temperature; (B) interscapular brown adipose tissue (IBAT) temperature; (C) difference between IBAT and aortic temperatures ( $\Delta T$ ). Noradrenaline and noradrenaline + 3-OHB data represent the mean  $\pm$  S.E.M. for 9–10 different animals. Saline and glucose data are the mean  $\pm$  S.E.M. for 5–6 different animals. The arrows indicate the period of infusion of 3-OHB (also marked as a bar at the top of the figure). Significance of the differences between groups (ANOVA): (A) there were no differences between the saline- and glucose-infused groups; the differences between the noradrenaline and noradrenaline + 3-OHB groups were significant, as were those between these two groups and the saline and glucose groups; (B) there were no significant differences between the saline- and glucose-infused groups; the differences between the noradrenaline and noradrenaline + 3-OHB groups were significant, as were those between them and the saline and glucose groups; (C) the differences between both groups were significant.

adjustment mechanisms (Takemoto, 1992). The preparation used here cushions the fall in body temperature by maintaining a high environmental temperature. Infusion of saline or saline supplemented with 3-OHB or glucose elicited neither a rise in temperature nor an alteration in the basal hypothermic condition induced by pentobarbital anaesthesia (Sessler, 1991; Takemoto, 1992).

Most of the alterations to the effects of noradrenaline described here may be traced to the decrease in circulating

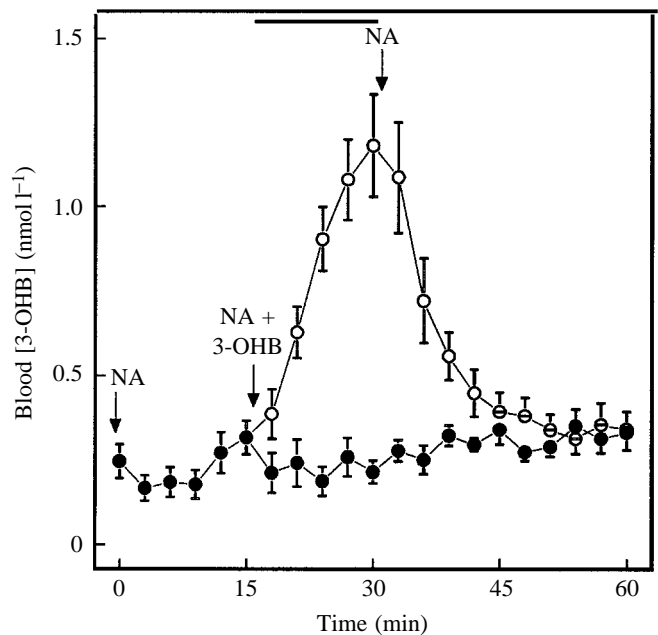


Fig. 3. Blood 3-OHB levels of rats infused with noradrenaline (NA) (filled circles) or noradrenaline + 3-OHB (open circles). The data represent the mean  $\pm$  S.E.M. for 7–8 different animals. Arrows indicate the period of infusion of 3-OHB (also marked as a bar at the top of the figure). Significance of the differences between groups (Student's *t*-test): the differences between the noradrenaline and noradrenaline + 3-OHB groups were significant only between 22 and 40 min.

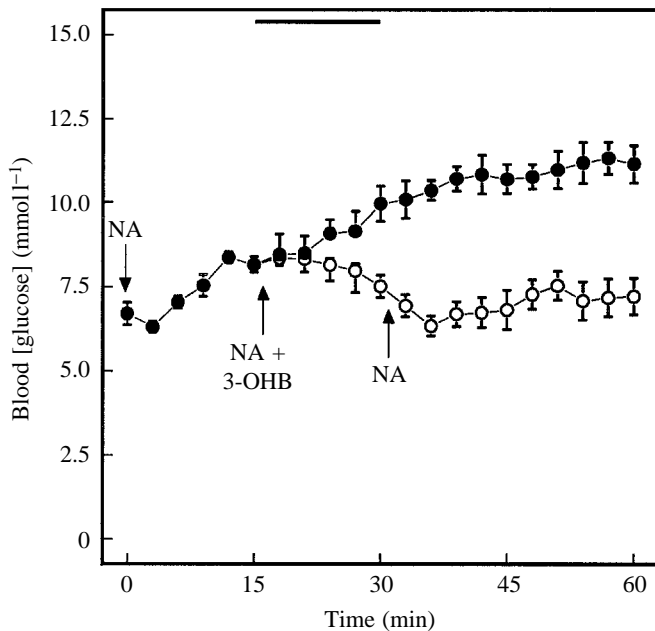


Fig. 4. Blood glucose levels of rats infused with noradrenaline (NA) (filled circles) or noradrenaline + 3-OHB (open circles). The data represent the mean  $\pm$  S.E.M. for 7–8 different animals. Arrows indicate the period of infusion of 3-OHB (also marked as a bar at the top of the figure). Significance of the differences between groups (Student's *t*-test): the differences between the noradrenaline and noradrenaline + 3-OHB groups were significant from 28 min onwards.

levels of noradrenaline induced by 3-OHB infusion. This transient effect cannot be reproduced by either saline or glucose infusion. However, it can explain, at least in part, the arrest by 3-OHB of temperature increases and the noradrenaline-induced rise in blood glucose level because, during 3-OHB infusion, there was less circulating noradrenaline left to act on BAT and the liver glycogenolytic pathway.

The observed effects of 3-OHB infusion on the adrenergic response may be directly related to its concentration in the plasma, since the maximal effect on BAT temperature coincides with the highest plasma 3-OHB level, a value close to the values reached during short-term starvation (Palou *et al.* 1981) but far below the upper physiological limit attainable during starvation (Balasse, 1979). The ability of the rat to remove 3-OHB from the blood was also concentration-dependent, suggesting that its utilization by peripheral tissues was enhanced by a fast turnover.

Noradrenaline elicits a significant thermogenic response in BAT, whose temperature differential from that of the aortic core was increased. Noradrenaline increases heat production in BAT (Foster and Frydman, 1978) and other tissues (Ma *et al.* 1987), thus raising core temperature (Foster and Frydman, 1978), as observed here, by promoting heat storage. The infusion of 3-OHB strongly influences the effect of noradrenaline on BAT temperature, i.e. on BAT heat production. This agrees with its role as a modulator of

adrenergically induced thermogenesis when energy substrates are scarce, such as during starvation. The effect of 3-OHB on the stimulation of brown adipocytes by noradrenaline (Pokasanieva *et al.* 1993) may be related to its direct effects on  $\beta$ -adrenergic receptors (De Pergola *et al.* 1990; Gómez *et al.* 1996), but the overall effect is a 3-OHB-induced decrease in BAT thermogenesis.

Glucose levels increased during noradrenaline infusion, a direct consequence of generalized glycogenolysis (Shiota *et al.* 1992). The increase in temperature observed may result in higher demands for substrates, e.g. glucose, to fuel the increased thermogenesis, since this is the main substrate for the BAT thermogenesis that is elicited by noradrenaline or exposure to cold (Foster and Frydman, 1978, 1979). The slow rise in blood glucose levels reflects the balance between noradrenaline-elicited release of glucose from glycogen and its consumption by BAT and other tissues, which is also in part dependent on noradrenaline stimulation. The rapid utilization of infused 3-OHB as an alternative source of energy would spare glucose and tend to increase glucose levels (Senior and Loridan, 1968; Amiel *et al.* 1991). In spite of this sparing of glucose stores, plasma glucose levels actually decreased in response to 3-OHB infusion, which suggests that its effects on glycogenolysis are even more marked than those on glucose consumption. Thus, it may be assumed that 3-OHB inhibits noradrenaline-induced glycogenolysis by decreasing the levels of circulating noradrenaline but also by altering the response of adrenergic receptors to this hormone (De Pergola *et al.* 1990; Gómez *et al.* 1996).

However, the decrease in circulating noradrenaline levels elicited by 3-OHB is difficult to explain, since the rate of infusion of noradrenaline did not change throughout the experiment: a decrease in noradrenaline levels could only be explained by an increase in its rate of removal/destruction. This may be due to enhanced disposal of the hormone, which in this case would be dependent on the concentration of 3-OHB. The decreased affinity of  $\beta$ -adrenergic receptors for noradrenaline (De Pergola *et al.* 1990; Gómez *et al.* 1996) and the modulation of central sympathetic brain activity induced by 3-OHB (Maekubo *et al.* 1977; Fisler *et al.* 1995) may contribute to the role of 3-OHB as an important regulating agent that shows multiple interactions with noradrenaline within the physiological concentration range. This interaction may be part of an elaborate and complex fail-safe mechanism to prevent wastage of stored substrates.

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