Regulation of Heat Production of Brown Adipocytes via Typical and Atypical \( \beta \)-Adrenoceptors in the Rat

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Abstract Previous studies in our laboratory demonstrated that microcalorimetry is an appropriate method for estimating the physiological function of isolated rat brown adipocytes. In the present study, to elucidate the mode of action of typical and atypical \( \beta \)-adrenoceptors on heat production of this cell, the effect of novel adrenergic \( \beta_1 \)-agonists was compared with that of other typical adrenergic reagents by direct microcalorimetry. Isoproterenol and \( \beta_2 \)-agonists, BRL37344, ICI215001, and CGP12177, increased heat production in a dose-dependent manner, however, phenylephrine had no effect. Propranolol and pindolol did not increase the heat production but attenuated the effect of isoproterenol and BRL37344 in a dose-dependent manner. Molar IC\(_{50}\) values of propranolol and pindolol for BRL37344 were about \( 10^{-5} \) and \( 3 \times 10^{-6} \) M, respectively, whereas those of the two antagonists for isoproterenol were about \( 3 \times 10^{-7} \) M. The \( pA_2 \) values by Schild analysis of propranolol vs. isoproterenol and BRL37344 were 7.91 and 6.13, respectively. These results suggest that heat production may be regulated via both \( \beta_1 \)- and typical \( \beta \)-adrenoceptors in brown adipocytes.

Key words: \( \beta \)-adrenoceptor, brown fat, calorimetry, sympathetic nervous system, BRL 37344.

Brown adipose tissue is the principal site of nonshivering thermogenesis in a large number of mammalian species including humans. Heat production by brown adipose tissue is very important, for example, during early postnatal life, on arousal from hibernation, during cold exposure, and in the overfed state (see reviews [1–4]). Owing to the difficulty in measuring heat production of brown adipocytes

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directly, the activity of the adipocytes in vitro has mainly been estimated indirectly by β-adrenergic-mediated metabolic responses, e.g., lipolysis, oxygen consumption, cAMP accumulation, or noradrenaline turnover (see the above reviews). However, since thermogenesis is the most important physiological function of this tissue, a direct measure of heat production would be the best way to estimate the cell’s physiological function. Various calorimetric methods have been used [5–9] and we have also succeeded in measuring heat production of isolated brown adipocytes with a new microcalorimeter ESCO-3000 in vitro [10]. Our method has the great advantage of sensitivity and it only takes a short time to measure the sample because it is a flow type calorimeter [11].

Besides the β1- and β3-adrenoceptors, an atypical β-adrenoceptor called β3-adrenoceptor has recently been identified in adipocytes and selective β3-agonists which predominantly stimulate lipolysis, have been discovered [12–16]. These reports indicate that the β3-adrenoceptor mediates the sympathetic control of the various metabolic processes mentioned above, involving adenylyl cyclase activity [17, 18] and uncoupling protein gene expression [19]. However, the β3-adrenoceptor may not be involved in some adrenergic-mediated processes; for example, DNA synthesis by noradrenaline was stimulated via the β1-adrenoceptor, while it was inhibited by the α2-adrenoceptor [20], and synthesis of uncoupling protein was partially regulated via the α1-adrenoceptor [21].

The gene encoding the β3-adrenoceptor has been isolated and expressed in Chinese hamster ovary cells [13, 15, 17, 18]. These cells and the selective β3-agonists would conveniently enable the analysis of the properties and physiological function of the β3-adrenoceptor. However, discrepancies in the pharmacological responses have been found between isolated adipocytes and transfected cells. For example, the nonselective β-antagonist alprenolol inhibited the effect of isoproterenol on adenylyl cyclase activity in isolated brown adipocytes but not at all in the β3-adrenoceptor-transfected cells [18]. Thus, although the pharmacological properties of β3-adrenoceptors were revealed using the transfected cells and/or selective β3-agonists, it is still unknown whether or to what extent the β3-adrenoceptor contributes to heat production in brown adipocytes in the physiological state.

In this paper, the role of adrenoceptors including that of β3-adrenoceptors on heat production in brown adipocytes was estimated by means of direct microcalorimetry using selective β3-agonists, BRL37344, ICI215001, and CGP12177.

MATERIALS AND METHODS

Isolation of brown adipocytes. All procedures were conducted in accordance with the Guiding Principles for the Care and Use of Animals approved by the Council of the Physiological Society of Japan. Male Wistar rats (200–250 g) fed laboratory chow ad libitum were kept at 24 ± 1°C of ambient temperature with 30–40% humidity. The room was light from 19:00–07:00, and dark from 07:00–19:00. The rats were killed by decapitation at around 10:00. Adipocytes were
isolated from interscapular brown adipose tissue by the method of Bukowiecki et al. [22] with some modifications. Instead of the bicarbonate buffer, we used HEPES buffer of the following composition (mM): HEPES 5, NaCl 136.9, KCl 2.7, CaCl₂ 1.8, MgCl₂ 0.5, glucose 5.6; 4% bovine serum albumin was added. The final pH was adjusted to 7.4 by 1 M NaOH. The isolated cells were counted and adjusted to 0.5–1.5 × 10⁶ cells/ml in the medium.

**Heat production.** Heat production was measured by the stopped-flow method using a microcalorimeter (Thermoactive Cell Analyzer ESCO-3000, ESCO Ltd., Tokyo, Japan) [10, 11]. When all the cells, which were suspended in a total volume of 0.5 ml, were allowed to enter the heat detector part, the flow of the cell suspension was stopped and heat was measured in microwatts (μW) being micro-joules per second (μJ/s). The detection limit was 0.2 μW. In preliminary experiments, we confirmed that the heat production of the cell suspension was directly proportional to the number of cells (data not shown).

Saline (5.5 μl) with or without reagents was added to the cell suspension (540 μl) 6 min before beginning the heat production measurements. The preparations obtained from one animal were used for one series of experiments that was performed sequentially with one kind of reagent; each experiment was made with a new cell suspension.

**Reagents.** L-Phenylephrine hydrochloride, DL-propranolol hydrochloride, and pindolol were purchased from Waco Pure Chemical Industries, Osaka, Japan; L-isoproterenol d-bitartrate from Nacalai Tesque, Kyoto, Japan; and fatty acid-free (less than 0.02%) bovine serum albumin from Sigma Chemical, St. Louis, USA. The three β-agonists were generously donated: BRL37344 (4-[2-[(2-hydroxy-2-(3-chlorophenyl)ethyl)amino]propyl]-phenoxyacetic acid) by SmithKline-Beecham, Epsom, UK; ICI215001 ((S)-4-[2-(2-hydroxy-3-phenoxypropylamino)ethoxy]phenoxyacetic acid) (the active metabolite of ICI D7114) by Zeneca, Cheshire, UK; and CGP12177 (4-(3-tert-butylamino-2-hydroxypropoxy) benzimidazol-2-one) by Ciba-Geigy, Basel, Switzerland. Other chemicals were of analytical grade.

**Statistical analysis.** All data obtained in this study are expressed as means ± SE. We applied paired t-test to determine the significant differences between pairs of groups. Differences were considered significant at p < 0.05. In order to estimate the effect of antagonists, the pA₂ value was calculated by Schild analysis.

**RESULTS**

**Effect of adrenergic agonists on heat production**

To identify adrenoceptor subtypes regulating heat production in isolated brown adipocytes, we examined phenylephrine, isoproterenol, and selective adrenergic β₂-agonists BRL37344, ICI215001, and CGP12177. The dose-response curves of these agonists are shown in Fig. 1. Isoproterenol and the three β₂-agonists increased the heat production in a concentration-dependent manner, showing that the maximal effect was 364 ± 75 pW/cell (isoproterenol at a concentration of 10⁻⁶
Fig. 1. Effect of adrenergic agonists on heat production of brown adipocytes.
Values are means ± SE for phenylephrine (▲, n = 4), isoproterenol (●, n = 6),
BRL37344 (■, n = 6), ICI215001 (◆, n = 3), and CGP12177 (▼, n = 3).

m, n = 6), 370 ± 81 (BRL37344 at 10^{-7} M, n = 6), 236 ± 10 (ICI215001 at 10^{-6} M,
n = 3), and 315 ± 105 pW/cell (CGP12177 at 10^{-4} M, n = 3). However, phenylephrine
had no effect on heat production below a concentration of 10^{-6} M (n = 4).
Basal heat production in these five series was 42 ± 5 pW/cell (n = 22).

Effect of β-antagonists on heat production

The effect on heat production of β-antagonists, propranolol, and pindolol, were
examined. At a concentration of 10^{-5} M, propranolol showed significant reduction
compared with the basal heat production (28 ± 3 vs. 38 ± 6 pW/cell, n = 6, p < 0.05,
paired t-test), while pindolol had no significant effect (32 ± 7 vs. 37 ± 5 pW/cell, n =
7, paired t-test). Any lower concentrations of the antagonists had no effect.

The inhibition curves of propranolol on β-agonist-induced heat production are
depicted in Figs. 2 and 3. The effect of propranolol on various concentration of
isoproterenol and BRL37344 was evaluated (Fig. 2A and B, respectively). Increasing
concentrations of propranolol produced a rightward shift of the concentration-
response curves of two agonists. Antagonist potencies were evaluated by Schild
plots calculating their pA2 values (Fig. 2C and D). The pA2 values of propranolol
versus isoproterenol and BRL37344 were 7.91 and 6.13, respectively.

Figure 3A illustrates the dose-dependent effects of propranolol and pindolol in
the presence of the submaximum concentration (10^{-7} M, see Fig. 1) of isoproter-
enol. Propranolol and pindolol attenuated the effect of isoproterenol in a dose-
dependent manner. The concentrations at which these two antagonists inhibited
50% of the maximal response (IC50) induced by isoproterenol were about 3 × 10^{-7}
M, which was only 3-fold higher than the concentration of the agonist. At a
concentration of 10^{-4} M, these two antagonists blocked isoproterenol completely.

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Fig. 2. Dose-response curves for the antagonistic effect of increasing concentrations of propranolol on isoproterenol- (A) and BRL37344-stimulated heat production (B), and determination of the $pA_2$ values for propranolol. The corresponding Schild plots used for the calculation of the $pA_2$ values for propranolol versus isoproterenol (C) and BRL37344 (D) are shown; $pA_2 = -\log(\text{agonist}/\text{DR} - 1)$, where DR is the dose-ratio between the EC$_{50}$ value for an agonist in the presence of a certain antagonist concentration. The concentration of propranolol were $0$ (●), $10^{-7}$ (▲), $10^{-5}$ (▼), $10^{-5}$ (■), and $10^{-4}$ M (◆). Data are from 1–6 separate experiments.

In Fig. 3B the dose-dependent effects of these two antagonists in the presence of the submaximum concentration ($10^{-4}$ M, see Fig. 1) of BRL37344 is shown. Above the concentrations of $10^{-6}$ M, both antagonists attenuated the effect of BRL 37344 in a dose-dependent way. Molar IC$_{50}$ values of propranolol and pindolol were about $10^{-5}$ and $3 \times 10^{-6}$ M, respectively, which were 300- and 1,000-fold higher than the concentration of the agonist. At a concentration of $10^{-4}$ M, propranolol blocked the effect of BRL37344, however the blocking effect of pindolol was not complete.

DISCUSSION

Studies with several kinds of adrenergic agents including selective $\beta_2$-agonists have been useful in elucidating the regulatory mechanisms of heat production in
brown adipocytes. In the present study, we confirmed that the three β3-adrenergic agents, BRL37344, ICI215001, and CGP12177, increased heat production of this cell in a dose-dependent manner. The results that α-agonist phenylephrine had no effect on heat production suggest that heat production of brown adipocytes is unlikely to be stimulated via α-adrenoceptors. With membranes of Chinese hamster ovary cells that express human or rat β3-adrenoceptor, the order of adenyl cyclase activity of β-agonists has been found to be as follows: isoproterenol, noradrenaline, and adrenaline [23]. This report and our previous findings that the order of these three typical agonists on heat production was the same as that above [10] support the hypothesis that heat production of brown adipocytes is regulated, at least in a part, via β3-adrenoceptors. The results that the selective β3-agonist BRL37344 increased heat production, as did isoproterenol, suggest that isoproterenol and BRL37344 bind to the β3-adrenoceptor probably with similar affinity or that isoproterenol binds to other adrenoceptor(s) which can also regulate heat production. The other β3-agonists ICI215001 and CGP12177 also increased heat production; however, they seemed to be less effective than BRL37344 on heat production. The reason for the weakness of CGP12177 may be explained by its antagonistic effect [24], but that of ICI215001 was unclear. The selectivity for the β3-adrenoceptor of these two agents was not examined in this study.

The present study showed that neither the nonselective β-agonist propranolol nor pindolol stimulated heat production in brown adipocytes in the absence and presence of β-agonists, whereas Emorine et al. [13] reported that
pindolol stimulated cAMP accumulation in the presence of isoproterenol in the β3-adrenoceptor-transfected cell. The discrepancy between these studies may come from differences in the methods and/or materials used. For example, 10^-7 M isoproterenol increased heat production in this cell, while the same concentration of this agent did not increase lipolysis at all [10]. We performed the present experiments on cells with heterogeneous receptors, measuring heat production as the final step of metabolic cascades, while Emorine et al. [13] observed only the changes in cAMP on transfected cell membrane with homogeneous receptors.

As well as β-adrenergic agents, studies on transfected cells have made a contribution to our understanding of receptors and cell function. Judging from a comparison between the IC₅₀ values and the concentration of the agonist, nonselective β-antagonists did not inhibit isoproterenol in the β3-adrenoceptor-transfected cell with only pure β3-adrenoceptor [13]; and similar results were observed in the membrane of this cell [18]. Here, the IC₅₀ values divided by the concentration of the agonist were always more than 100. On the other hand, in native adipocytes and their membrane with heterogeneous β-adrenoceptors, nonselective β-antagonists seemed to inhibit nonselective β-agonists (here the IC₅₀ values divided by the concentration of the agonist, were always less than 10; Fig. 3A [18, 20, 25]), but not BRL37344 (Fig. 3B [21]). However, in the cardiac cell with no β3-adrenoceptors, the action of BRL37344 on the sinus rate was easily inhibited by propranolol [26]; in the β1-adrenoceptor-transfected cell membrane the effect of isoproterenol was inhibited by alprenolol [18]. These results indicate that: 1) non-β3-adrenoceptors are easily blocked by nonselective β-antagonists even when stimulated by a selective β3-agonist, 2) β3-adrenoceptors are partially blocked by nonselective β-antagonists even when stimulated by nonselective β-agonists, and 3) in native adipocytes with heterogeneous β-adrenoceptors including β3, the metabolic responses mediated by non-β3-adrenoceptors are easily blocked by nonselective β-antagonists, whereas those by β3-adrenoceptors are partially blocked. From these three observations, we may deduce that heat production in brown adipocytes is regulated via both typical β-adrenoceptors (non-β3, presumably β1) and β3-receptors; isoproterenol stimulates heat production predominantly via typical β-adrenoceptors which are inhibited by nonselective β-antagonists, while BRL37344 does so mainly via the β3-adrenoceptors which are not inhibited by nonselective β-antagonists. This hypothesis was also supported by the result of the Schild plots analysis, where the discrepancy between the pA₂ values of propranolol versus isoproterenol and BRL37344 was more than one unit. However, the dominance of β3 over typical β-adrenoceptors referring to heat production in brown adipocytes is still unclear.

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