Role of β3-adrenergic receptors in the action of a tumour lipid mobilizing factor

ST Russell1, K Hirai2 and MJ Tisdale*,1

1Pharmaceutical Sciences Research Institute, Aston University, Birmingham, B4 7ET, UK; 2Department of Obstetrics and Gynaecology, Osaka City University Medical School, Osaka 545-8585, Japan

Induction of lipolysis in murine white adipocytes, and stimulation of adenylate cyclase in adipocyte plasma membranes, by a tumour-produced lipid mobilizing factor, was attenuated by low concentrations (10^-7 – 10^-3 M) of the specific β3-adrenoceptor antagonist SR59230A. Lipid mobilizing factor (250 nM) produced comparable increases in intracellular cyclic AMP in CHO-K1 cells transfected with the human β3-adrenoceptor to that obtained with isoprenaline (1 nM). In both cases cyclic AMP production was attenuated by SR59230A confirming that the effect is mediated through a β3-adrenoceptor. A non-linear regression analysis of binding of lipid mobilizing factor to the β3-adrenoceptor showed a high affinity binding site with a Kd value 78 ± 45 nM and a Bmax value (282 ± 1 fmoles mg protein^-1) comparable with that of other β3-adrenoceptor agonists. These results suggest that lipid mobilizing factor induces lipolysis through binding to a β3-adrenoceptor.

MATERIALS AND METHODS

Patients

Urine was collected over a 24 h period from patients with unresectable pancreatic cancer and with a weight loss between 0.5 and 3 kg month^-1. No patient had received radiotherapy or chemotherapy. Urine samples were stored at -20°C in the absence of preservatives prior to use.

Chemicals

[^23]P]-ATP (sp. act. 20 Ci mmol^-1) and Na[^125]I (sp. act. > 15 Ci mmol^-1) iodide) were purchased from Amersham Pharmacia Biotech (Bucks, UK). SR59230A was kindly donated by Dr L Manara of the Research Centre Sanofi Midy, Sanofi Winthrop S.p.A., Milan, Italy.

Purification of LMF

LMF was purified from human urine using a combination of batch extraction on DEAE-cellulose and hydrophobic interaction chromatography (Todorov et al., 1998). Urine was centrifuged at 3000 g for 10 min to remove particulate material and was then diluted with 4 vol 10 mM Tris HCl, pH 8.0. DEAE-cellulose, previously activated by washing in 100 mM Tris HCl, pH 8.0 for 5 min was added to the diluted urine (10 g l^-1 of original urine)
and the mixture was stirred for 2 h at 4°C. The DEAE-cellulose was recovered by sedimentation by low speed centrifugation, and the LMF was eluted with 0.5 mM NaCl in 10 mM Tris HCl, pH 8.0. The eluate was equilibrated and concentrated to 1 ml by ultrafiltration, in an Amicon filtration cell (Millipore (UK) Ltd, Watford, Herts, UK) containing a membrane filter with a molecular weight cut-off of 10 kDa, against PBS. Further purification was achieved using a Resource-Iso HPLC column (Pharmacia Biotech, St Albans, Herts, UK), employing a decreasing (NH₄)₂SO₄ concentration from 1.5 M. Active fractions containing LMF eluted at 0.6 M (NH₄)₂SO₄, and were desalted before use by washing five times against PBS using an Amicon filtration cell.

**Lipolytic assay**

A single cell suspension of white adipocytes was prepared from the epididymal adipose tissue of ex-breeder male NMRI mice using collagenase digestion (Beck and Tisdale, 1987). Lipolytic activity was determined by measuring glycerol release (Wieland, 1974) after incubation of LMF with 10⁻⁵–2 × 10⁻⁴ adipocytes for 2 h at 37°C in 1 ml Krebs-Ringer bicarbonate buffer, pH 7.2. Control samples containing adipocytes alone were analyzed to determine the spontaneous glycerol release. Lipid mobilizing activity was expressed as μmole glycerol released 10⁵ adipocytes⁻¹ 2 h⁻¹.

**Adenylate cyclase assay**

Plasma membranes were isolated from epididymal adipocytes, as previously described (Khan and Tisdale, 1999). Briefly isolated adipocytes were homogenized in 250 mM sucrose, 2 mM EGTA and 10 mM Tris HCl pH 7.4, followed by centrifugation at 30,000 g for 1 h at 4°C. The membrane pellet formed was isolated and separated from other organelle membranes on a self forming Percoll gradient, and the mixture was centrifuged at 10,000 g for 30 min at 4°C. The washed plasma membranes were diluted in 10 mM Tris HCl, pH 7.4, containing 250 mM sucrose, 2 mM EGTA and 4 μM phenylmethanesulphonylfluoride at 1–2 mg ml⁻¹, and if not used immediately, snap frozen in liquid nitrogen and stored at −70°C until use. The adenylate cyclase assay was based on that developed by Salomon et al (1973) as previously described (Hirai et al, 1998). Briefly LMF was incubated for 10 min at 30°C together with plasma membrane in 25 mM Tris HCl, pH 7.5, 5 mM MgCl₂, 10 μM GTP, and separated from other organelle membranes on a self forming Percoll gradient, and the mixture was centrifuged at 10,000 g for 30 min at 4°C. The washed plasma membranes were diluted in 10 mM Tris HCl, pH 7.4, containing 250 mM sucrose, 2 mM EGTA and 4 μM phenylmethanesulphonylfluoride at 1–2 mg ml⁻¹, and if not used immediately, snap frozen in liquid nitrogen and stored at −70°C until use. The adenylate cyclase assay was based on that developed by Salomon et al (1973) as previously described (Hirai et al, 1998).

**Cyclic AMP determination**

CHOK1 cells transfected with the human β3-AR, under the control of hygromycin, together with the b-gal reporter construct, selected for resistance to G418, were a gift from Dr Ian Waddell, AstraZeneca, Macclesfield, Cheshire, UK. They were grown in Dulbecco’s modified Eagles medium (DMEM) supplemented with 2 mM glutamine, 50 μg ml⁻¹ hygromycin B and 200 mg ml⁻¹ G418, under an atmosphere of 10% CO₂ in air. For cyclic AMP assays cells were grown in 24 multi-well plates in 1 ml DMEM. Agonists were added to the wells and incubated for 30 min, after which the medium was removed and 0.5 ml 20 mM HEPES, pH 7.5, 5 mM EDTA and 0.1 mM isobutylmethylxanthine was added to each well. The plate was placed in a boiling water bath for 5 min and cooled on ice for 10 min. To 50 μl of the cell extract was added 2 μCi of [8-³²P]-cyclic AMP (Amersham, UK) and 20 μg of cyclic AMP-dependent protein kinase (Sigma Chemical Co. Ltd, Dorset, UK) and incubated for 2 h at 4°C. Unbound cyclic AMP was removed by adsorption onto charcoal and the concentration of cyclic AMP in the sample determined by comparison with standard curves using known concentrations of cyclic AMP.

**Iodination of LMF with [¹²⁵I]**

One iodo-bead (Pierce and Warriner, Chester, UK), washed and dried, was incubated with Na[¹²⁵I] (1 μCi per 100 μg protein) for 5 min in 100 μl PBS. LMF (100 μg protein) was then added and the reaction allowed to proceed for 15 min. The iodo-bead was physically removed and free Na[¹²⁵I] was removed using a Sephadex G25 column eluted with 0.1 M NaI. The [¹²⁵I] LMF was concentrated using a Microcon microconcentrator with a Mₘ 10,000 cut-off against PBS.

**Binding studies**

CHOK1 cells transfected with the human β3-AR were lysed by sonication in 0.5 mM MgCl₂, 2 mM Tris HCl, pH 7.5 and crude membranes were pelleted by centrifugation (45,000 g, 15 min, 4°C). Binding studies were conducted in 400 μl 0.5 mM MgCl₂, 50 mM Tris HCl, pH 7.5, by incubation of membranes (50 μg protein) with various concentrations of [¹²⁵I] LMF for 60 min at 37°C. The samples were then centrifuged at 13,000 g for 20 min, the supernatant was removed and the radioactivity of the pellet was determined using a Packard Cobra Model 5005 Auto-gamma counter. Binding was analyzed using non-linear regression analysis (GraphPad Prism, Version 3.00 for windows, GraphPad Software (San Diego, CA, USA)).

**RESULTS**

LMF induced a direct lipolytic response in murine white adipocytes, and this effect was attenuated by low concentrations (10⁻⁵–10⁻⁷ M) of SR59230A (Figure 1A), which has been reported to have a 10-fold selectivity for the β3-AR over the β1-AR (Nisoli et al, 1996). Induction of lipolysis by LMF was associated with a stimulation of adenylate cyclase in isolated adipocyte membranes in the presence of 0.1 μM GTP, and this action was almost completely inhibited by SR59230A at concentrations as low as 10⁻⁷ M (Figure 1B). The difference in sensitivity of intact adipocytes and plasma membranes may be related to access of SR59230A to the β3-AR. SR59230A has been shown to bind strongly to albumin (Nisoli et al, 1996) reducing the effective concentration available in the adipocyte assay. These results suggest that LMF stimulates lipolysis through interaction with a β3-AR.

To investigate this possibility the effect of LMF on cyclic AMP production was determined in CHOK1 cells, which had been transfected with the human β3-AR. The data presented in Figure 2 shows that both isoprenaline and LMF stimulated cyclic AMP production, which reached a comparable maximum level of 25 pmoles per 10⁶ cells with both agents. However maximal cyclic AMP production was achieved with much lower concentrations of isoprenaline (1 nM) than LMF (250 nM), suggesting that LMF had a lower affinity for the β3-AR than isoprenaline. The increase in intracellular cyclic AMP produced by both isoprenaline and LMF in CHOK1/β3 was attenuated by the non-specific β-AR antagonist propranolol (10 μM), while the effect on LMF, although significant, was less than complete. However, cyclic AMP production by both isoprenaline and LMF was almost completely attenuated by SR59230A, confirming that the action of LMF was mediated through a β3-AR.

To determine the affinity of binding of LMF to the β3-AR, LMF was radioiodinated with [¹²⁵I] and the binding to crude plasma membranes from CHOK1/β3 cells was determined. The data is presented in Table 1. Non-linear regression analysis of binding showed a high affinity binding site for LMF with a Kd value about
100-fold lower than that of CGP 12177, a partial agonist of β3-AR (Kubo et al., 1997) and [125I] iodocyanopindolol (Hutchinson et al., 2000), commonly used in binding studies with β3-AR. However, the B_max value for LMF was similar to that for other β3-AR agonists. Binding of [125I] LMF was significantly reduced in the presence of non-labelled LMF, the non-specific β blocker propranolol and the selective β3-AR antagonist SR59230A (Table 1). These results confirm that LMF binds to a β3-AR and stimulates adenylate cyclase.

DISCUSSION

Resting energy expenditure (REE) has been reported to be significantly increased in weight losing patients with lung (Fredrix et al., 1990) and pancreatic cancer (Falceron et al., 1994). Hyltander et al. (1991) found that cancer patients had an elevated REE and increased fat oxidation compared with either weight losing or weight stable controls, and that this was related to an increased heart rate. Such patients were also found to exhibit an increased cardiovascular and metabolic response to adrenaline infusion (Drott et al., 1987), while administration of the non-specific β-blocker propranolol was found to produce a decrease in the basal metabolic rate (BMR) (Gambardella et al., 1999). These results led to the hypothesis of overactivity of the sympathetic nervous system (SNS) in cancer patients.

Classical β1 and β2-AR mediate response to noradrenaline released from the SNS. In addition a third β-AR subtype has been identified (reviewed in Howe, 1993), which shares only 40–50% amino acid sequence identity with β1 and β2-AR, and is referred to as a β3-AR. These receptors mediate lipolysis in white adipose tissue in mice and rats (Arch et al., 1984; Arch and Wilson,
1996), and thermogenesis in BAT (Arch, 1989), and are also responsible for the unexpected negative inotropic effects of catecholamines in the heart (Gauthier et al, 1996). However, the evidence that \( \beta_3 \)-AR can mediate lipolysis in human adipocytes is controversial, since \( \beta_3 \)-AR mRNA is expressed at a much lower level than in rat or mouse (Langin et al, 1991), although lipolysis has been induced in human omental fat cells by the selective \( \beta_3 \)-AR agonist CGP 12177 (Hoffstedt et al, 1995), and LMF (Hirai et al, 1998).

We have previously shown that cachexia in both mice and humans is associated with LMF production by the tumour and excretion in the urine (Todorov et al, 1998), and that LMF stimulated lipolysis like a classical lipolytic hormone through increases in intracellular cyclic AMP as a result of the stimulation of adenylate cyclase (Hirai et al, 1997). This study shows that LMF exerts this effect through a \( \beta_3 \)-AR, although the affinity for this receptor appears to be less than seen with classical \( \beta_3 \)-AR agonists. In white adipocytes both the induction of lipolysis and the stimulation of adenylate cyclase were attenuated by the \( \beta_3 \)-AR antagonist SR59230A (Nisoli et al, 1996), while in CHO cells transfected with the human \( \beta_3 \)-AR LMF stimulated cyclic AMP production in a similar manner to isoprenaline, although the concentration required to produce maximal stimulation was 250-fold greater. In addition SR59230A attenuated the increase in cyclic AMP confirming the effect was mediated through a \( \beta_3 \)-AR. The effect of propranolol was less complete than with isoprenaline, suggesting that the mechanism of stimulation by LMF may be different.

Previous studies (Khan and Tisdale, 1999) have shown propranolol to act as a non-competitive inhibitor of the induction of lipolysis in murine white adipocytes by LMF. This suggests that it may act at a site distal to the \( \beta_3 \)-AR and may attenuate the action of two \( \beta_3 \) agonists to different extents. In this study we have used intact cells, since the coupling efficiency of \( \beta_3 \)-AR to adenylate cyclase is highly dependent upon the integrity of the cells (Granneman, 1995). However, it is known that the coupling efficiency of \( \beta_3 \)-AR is greater than that for \( \beta_1 \)-AR, thus offsetting the low binding affinity. Also unlike \( \beta_1 \) and \( \beta_2 \)-AR the \( \beta_3 \)-AR has fewer potential phosphorylation sites and is resistant to agonist-induced desensitization (Granneman, 1995). The \( \beta_3 \)-AR mediated coupling of LMF to lipolysis would explain the lowered maximal response of human omental adipocytes to lipolysis when compared with murine white adipocytes (Hirai et al, 1998). However, the increased coupling efficiency together with the induction of UCP1 in brown adipose tissue (BAT) (Russell et al, 2000) would ensure maximum fat mobilization and utilization together with a net increase in energy expenditure. These results suggest that selective \( \beta_3 \)-AR antagonists may be useful in controlling energy expenditure and fat mobilization in cancer cachexia.

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Table 1 Kd and B\(_{\text{max}}\) values for LMF and other agonists to \( \beta_3 \)-AR

| Agent | Kd (nm) ± s.e.m. | \( B_{\text{max}} \) (fmol mg protein \(^{-1}\)) ± s.e.m. | Reference |
|-------|-----------------|---------------------------------|-----------|
| CGP 12177 | 0.42 ± 0.06 | 312 ± 22 | Kabo et al, 1997 |
| \[^{[125}I\]CVP | 0.78 ± 0.28 | 63 ± 10 | Hutchinson et al, 2000 |
| \[^{[125}I\]LMF | 78 ± 4.5 | 282 ± 1 | This study |
| \[^{[125}I\]LMF + propranolol (10 μM) | 123 ± 6.1 | 3.9 ± 0.2 | This study |
| \[^{[125}I\]LMF + SR59230A (10 μM) | 182 ± 3.98 | 2.7 ± 0.75 | This study |
| \[^{[125}I\]LMF + cold LMF | 109 ± 13 | 1.7 ± 0.6 | This study |

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