Modulation of adipocyte G-protein expression in cancer cachexia by a lipid-mobilizing factor (LMF)

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Summary Adipocytes isolated from cachectic mice bearing the MAC 16 tumour showed a 3-fold increase in lipolytic response to both low concentrations of isoprenaline and a tumour-derived lipid mobilizing factor (LMF). This was reflected by an enhanced stimulation of adenylate cyclase in plasma membrane fractions of adipocytes in the presence of both factors. There was no up-regulation of adenylate cyclase in response to forskolin, suggesting that the effect arose from a change in receptor number or G-protein expression. Immunoblotting of adipocyte membranes from mice bearing the MAC16 tumour showed an increased expression of Gαs up to 10% weight loss and a reciprocal decrease in Gx. There was also an increased expression of Gαs and a decrease in Gx in adipose tissue from a patient with cancer-associated weight loss compared with a non-cachectic cancer patient. The changes in G-protein expression were also seen in adipose tissue of normal mice administered pure LMF as well as in 3T3L1 adipocytes in vitro. The changes in G-protein expression induced by LMF were attenuated by the polyunsaturated fatty acid, eicosapentaenoic acid (EPA). This suggests that this tumour-derived lipolytic factor acts to sensitize adipose tissue to lipolytic stimuli, and that this effect is attenuated by EPA, which is known to preserve adipose tissue in cancer cachexia. © 2001 Cancer Research Campaign

Keywords: lipid-mobilizing factor; G-protein expression; lipolysis

Cancer cachexia is associated with a severe and often irreversible loss of total body fat stores, which in mice has been shown to occur before the onset of anorexia (Costa, 1963). This depletion of host lipid stores may occur due to either a reduced rate of fatty acid deposition or an increased rate of mobilization (Thompson et al., 1981). Rates of whole body lipolysis have been shown to be greater in cachectic cancer patients with squamous cell carcinoma of the oesophagus than in healthy controls (Klein and Wolfe, 1990), suggesting production by tumour or host tissues of regulatory molecules involved in fat catabolism.

We have recently isolated a lipid-mobilizing factor (LMF) from the urine of cachectic cancer patients and shown it to be homologous in amino acid sequence with the plasma protein Zn-α2-glycoprotein (Todorov et al., 1998). This material initiates lipolysis through stimulation of adenylate cyclase in a GTP-dependent process in a manner analogous to the lipolytic hormones (Hirai et al., 1998). The elevation of cyclic AMP would lead to an activation of hormone-sensitive lipase (HSL), Thompson et al. (1993) observed a 2-fold elevation in serum triacylglycerol and fatty acid levels in cancer patients compared with normal controls, and a 2-fold increase in the relative level of mRNA for HSL in adipose tissue. There was no change in the relative level of the mRNA for lipoprotein lipase (LPL), suggesting that an increased rate of mobilization, rather than deposition, was most important for the depletion of lipid stores. In addition the results suggest that the increased lipolysis in adipose tissue in cachexia may be due to an up-regulation of the lipolytic response in addition to the production of lipolytic stimuli. The present report examines the sensitivity to lipolytic stimuli of adipose tissue from mice bearing an experimental model of cancer cachexia (MAC16), as well as alterations in adipocyte G-protein expression during the development of cachexia, both in mice and man, and the role of LMF in this process.

MATERIALS AND METHODS

Pure strain male NMRI mice (20–25 g) were obtained from our own inbred colony, and fragments of the MAC16 adenocarcinoma were implanted into the flanks by means of a trocar. Tumours were removed when they weighed between 0.1 and 0.6 g and before weight loss exceeded 20% of the total body weight. All animal experiments meet UKCCCK guidelines and were approved by the Home Office. Polyclonal antisera to Gtxi (AS/7) and Goxs (RM/1) were purchased from NEN Life Science Products, Boston, MA, USA. Affinity purified rabbit anti-rat antibody to HSL was kindly provided by Dr Cecilia Holm, Lund University, Sweden. This antibody cross reacts with mouse HSL. Nitrocellulose membranes were purchased from Hoefer Scientific Instruments, San Francisco, CA, USA. Urine from cachectic cancer patients was kindly provided by Dr A Moses, Department of Surgery, Edinburgh Royal Infirmary, UK.

Lipolytic assay

A single cell suspension of white adipocytes were prepared from finely minced epididymal fat pads of male NMRI mice bearing the MAC16 tumour, using collagenase digestion (Beck and Tisdale, 1987). Samples to be assayed were incubated with 10³–2 × 10⁸ adipocytes (determined by means of a haemocytometer) for 2 h at 37°C in 1 ml of Krebs-Ringer bicarbonate buffer, pH 7.2. The concentration of glycerol released was determined enzymatically...
by the method of Wieland (1974). Control samples containing adipocytes alone were analysed to determine the spontaneous glycerol release. LMF was expressed as µmol glycerol released/10⁶ adipocytes/2 h.

**Purification of LMF**

Material capable of inducing lipolysis in isolated murine epididymal adipocytes was purified either from the solid MAC16 tumour or from the urine of cachectic cancer patients as previously described (Todorov et al, 1998). The steps consisted of ion exchange and exclusion chromatography followed by a Resource™ iso hydrophobic interaction column to give an overall purification of 4200. This yielded a single protein of molecular weight 43 000 on denaturing polyacrylamide gels (Todorov et al, 1998).

**Isolation of adipocyte plasma membranes**

The protocol was based on that previously described by Belsham et al (1980). All procedures were carried out at 4 °C. Adipocytes were homogenized in 10 mM Tris HCl, pH 7.4, containing 0.25 M sucrose and 2 mM EGTA, by aspirating through a Swinny filter at least 10 times. The homogenate was then centrifuged at 300 g for 5 min. The supernatant was removed and centrifuged at 30 000 g for 1 h. Plasma membranes were separated from other organelle membranes in the pellet on a self-forming Percoll gradient. The constituents were 0.25 M sucrose, 2 mM EGTA in 10 mM Tris. HCl, pH 7.4, Percoll and 2 M sucrose, 8 mM EGTA in 80 mM Tris. HCl, pH 7.4 mixed in a ratio of 32:7:1. The mixture was centrifuged at 10 000 g for 30 min and the membrane fractions were resuspended in 10 mM Tris HCl, pH 7.4 containing 0.25 M sucrose, 2 mM EGTA and 4 µM phenylmethylsulfonyl fluoride at 1–2 mg ml⁻¹ snap frozen in liquid nitrogen and stored at −70 °C until use.

**Adenylate cyclase assay**

Adenylate cyclase activity in isolated plasma membranes was determined using the method of Salomon et al (1974). The incubation mixture contained 25 mM Tris. HCl, pH 7.4, 5 mM MgCl₂, 10 µM GTP, 8 mM creatine phosphate, 16 U ml⁻¹ creatine phosphokinase, 1 mM 3-isobutyl-1-methylxanthine, 1 mM cyclic AMP and 1 mM [α-32P] ATP (sp. act. 30 000 Ci mmol⁻¹). The reaction was initiated by the addition of plasma membranes (typically 50 µg protein) to the assay mixture to give a total volume of 100 µl, which was incubated at 30°C for 10 min. Reactions were terminated with 100 µl 2% sodium dodecyl sulfate (SDS) containing 40 mM ATP and 1.4 mM cyclic AMP. One microcurie of [1H] cyclic AMP was added to each assay tube as an internal standard to monitor product recovery. Samples containing labelled nucleotides were diluted to 1 ml with water and loaded onto Dowex 50W×4-400 columns primed with 10 ml of water. After washing twice with 1 ml of water the cyclic AMP was eluted with 3 ml of water into polypropylene tubes containing 200 µl of 1.5 M imidazole, pH 7.2. The samples were then applied to Alumina WN-3 columns (previously washed with 8 ml of 0.1 M imidazole, pH 7.5) and the eluate collected directly into scintillation vials containing Optiphase HiSafe 3 scintillation fluid. A further 1 ml of 0.1 M imidazole was added to the columns and the eluate was combined with the run through. The radioactivity was determined using a Tri-carb 2000A scintillation analyser.

**Western blot analysis**

Samples of adipocyte plasma membrane (5 µg for Gs and 50 µg for Gi) were resolved on 10% sodium dodecylsulfate polyacrylamide gels and transferred to 0.45 µm nitrocellulose membranes, which had been blocked with 5% Marvel in Tris buffered saline, pH 7.5, at 4°C overnight. The primary antibodies were used at a 1:2000 dilution, while the secondary antibody peroxidase conjugated monoclonal anti-rabbit immunoglobulin (Sigma Chemical Co, Dorset, UK) was used at a dilution of 1:1000. Incubation was for 2 h at room temperature and development was by enhanced chemiluminescence (ECL) (Amersham, UK). Blots were scanned by a densitometer to quantitate differences, and a parallel gel was silver stained to ensure equal loading. All blots were repeated at least 3 times on different incubations.

**3T3L1 maintenance and differentiation**

3T3 preadipocytes were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum under an atmosphere of 10% CO₂ in air. Cells were differentiated using a mixture of methylisobutylxanthine (0.5 mM), dexamethasone (0.25 µM) and insulin (1 µg ml⁻¹) according to the method of Frost and Lane (1985). The extent of differentiation to adipocytes was determined by staining for fat globules using Oil Red O. Cells were used for experimentation between 8 and 10 days after initiation of differentiation.

**RESULTS**

Adipocytes isolated from the epididymal fat pads of male NMRI mice bearing the MAC 16 tumour and with 15% weight loss...
showed over a 3-fold increase in response to lipolytic stimulation by both isoprenaline and LMF (Figure 1). The effect was more marked with low concentrations of isoprenaline. Plasma membranes prepared from adipocytes from mice bearing the MAC 16 tumour showed an enhanced stimulation of adenylate cyclase in the presence of isoprenaline (Figure 2A) or LMF (Figure 2B) when compared with adipocyte membranes from non-tumour bearing mice. The effect on isoprenaline stimulation was marked at submaximal concentrations (0.001 µM), where little or no response was observed in normal membranes, while there was no difference in response between adipocyte membranes prepared from cachectic and non-cachectic mice at optimal stimulatory concentrations (Figure 2A). The difference between adipocyte plasma membranes and intact cells may reflect differing GTP requirements for the adipocytes from cachectic mice. The activity of the LMF was potentiated in adipocyte membranes from cachectic mice in a dose-dependent manner (Figure 2B). Up-regulation of the response may be associated with the number or activity of receptors, G proteins or the cyclase catalytic element.

The effect on the latter was determined by the use of forskolin, which acts directly to stimulate adenylate cyclase. There was no up-regulation of response to forskolin in adipocyte membranes from cachectic mice (Figure 3), and there appeared to be greater cyclic AMP production using plasma membranes from non-cachectic animals.

Immunoblotting of plasma membranes with the specific Gαs antibody showed a marked increase in both 46 and 54 kDa Gαs splice variants with increasing weight loss up to 10% (Figure 4A). Thereafter, the levels of both variants decreased to that found in mice with 5% weight loss, but were still significantly increased above that found in mice with no weight loss. In contrast there was a decrease in the intensity of the 40 kDa band detected with anti-Gαi with increasing weight loss reaching a minimum at 10% weight loss (Figure 4B) and thereafter increased with additional host wasting. The ratio of Gαs to Gαi detected in adipocyte plasma membranes increased markedly with increasing weight loss (Table 1) reaching a maximum at 10% weight loss (Beck et al, 1991) returned the Gαs/Gαi ratio back to control levels (Table 1).

| Weight loss or treatment | Gαs/Gαi |
|--------------------------|---------|
| 0                        | 0.83    |
| 5                        | 1.72    |
| 10                       | 3.14    |
| 15                       | 2.18    |
| 20                       | 0.73    |
| LMF                      | 2.23    |
| MAC 16 + EPA             | 0.66    |

* Determined from densitometric analysis of the blot shown in Figure 4.
A similar change in G-protein expression was found in adipose tissue of a patient with cancer cachexia, when compared with a non-cachectic cancer patient, both with colonic cancer. Thus there was an enhanced expression of Gαs in both omental and subcutaneous adipose tissue in the cachectic subject (Figure 5A), while expression of Gαi was markedly reduced (Figure 5B). Thus the Gαs/Gαi ratios were increased to a comparable extent as that found in mice bearing the MAC16 tumour (Table 2).

To determine if changes in G-protein expression in adipose tissue were due to LMF ex-breeder male mice were injected i.v. with purified human LMF (8 μg b.d.) for 48 h which produced 7% weight loss and animals were terminated 12 h after the last injection and adipocyte membranes were blotted for Gαs and Gαi (lane 6), while mice bearing the MAC16 tumour were treated with EPA (0.5 g kg⁻¹) (lane 7). Band volume was measured as an indication of the quantity of G-protein present.

Table 2  Gαs/Gαi ratio in subcutaneous (s.c.) and omental (om) adipocyte

| Subject           | Gαs/Gαi |
|-------------------|---------|
| Non-cachectic (s.c.) | 0.32    |
| Cachectic (s.c.)    | 1.17    |
| Non-cachectic (om)  | 0.53    |
| Cachectic (om)      | 1.29    |

*aDetermined from densitometric analysis of the blot shown in Figure 5.

Table 3  Gαs/Gαi ratio in adipocyte membranes from 3T3L1 cells in the absence (A) or presence (B) of 50 μM EPA

| Concentration of LMF (nM) | A       | B       |
|--------------------------|---------|---------|
| 0                        | 1.00    | 1.00    |
| 12                       | 1.58    | 0.94    |
| 58                       | 1.46    | 0.94    |
| 174                      | 2.25    | 1.08    |
| 406                      | 2.85    | 1.23    |
| 580                      | 2.62    | 1.26    |

*Normalized to control value of 1.00.

Figure 5  Immunodetection of Gαs (A) and Gαi (B) in subcutaneous (lanes 1 and 3) and omental (lanes 2 and 4) adipose tissue from a cancer patient with no weight loss (lanes 1 and 2) and from a cachectic subject with 15% weight loss (lanes 3 and 4).

Figure 6  Immunodetection of Gαs (A and B) and Gαi (C and D) in 3T3L1 adipocytes incubated either alone (lane 1) or with 12 nM (lane 2); 58 nM (lane 3); 174 nM (lane 4); 406 nM (lane 5) and 580 nM (lane 6) LMF for 24 h in either the absence (A or C) or with a 2 h pretreatment with 50 μM EPA (B and D).
ratio of cells treated with LMF with and without EPA is shown in Table 3. Over 2-fold stimulation of Gs:G\(_i\) ratio was seen at concentrations of LMF greater than 174 nM, while in cells pretreated with EPA the Gs:G\(_i\) ratio remained near unity at all concentrations of LMF. These results confirm the ability of LMF to directly alter levels of expression of Gs and G\(_i\) and provide an explanation for the inhibitory effect of EPA on this process.

Low concentrations of LMF (12–58 nM) also caused an increased expression of HSL in 3T3L1 adipocytes (Figure 7). Thus LMF not only stimulates adenylate cyclase in adipocytes (Price and Tisdale, 1998), but also modifies G-protein expression and HSL to maximally activate lipolysis.

DISCUSSION

Both the induction of lipolysis and formation of cyclic AMP by both isoprenaline and LMF have been shown to be up-regulated in epididymal fat pads from cachectic mice bearing the MAC16 tumour compared with non-tumour-bearing mice. This suggests that a feature common to the pathways of both agents has been up-regulated. This appears not to be adenylate cyclase since forskolin, which bypasses receptor binding and activates adenylate cyclase directly, did not produce an enhanced response using adipocyte membranes from cachectic animals. This suggests that the up-regulated response in adipose tissue from cachectic animals is due to modification of receptor number of guanine nucleotide-binding protein (G-protein) expression.

The G-proteins involved in the adenylate cyclase pathway consist of Gs and Gi families that stimulate and inhibit adenylate cyclase, respectively (Levitzki, 1987). Adipocyte plasma membranes contain 2 Gs sub-units and 2 Gi sub-units, but not Go (Hirsch et al, 1988). A number of stimuli control G-protein expression in adipose tissue. Thus depletion of testosterone by castration has been shown to induce a down-regulation of both Gs and Gi (1 + 2, with testosterone replacement restoring Gs expression (Dieudonne et al, 1993). A concurrent up-regulation of the expression of G\(_i\) 1, G\(_i\) 2 and G\(_i\) 3, but not Gs, has been noted in adipocytes isolated from hypothyroid rats (Milligan and Saggerson, 1990), indicating an increased efficiency of antilipolytic agents such as adenosine, and correlating with the gain in body weight often observed in individuals exhibiting hypothyroidism. Patients with Gs deficiency are obese and show a decreased lipolytic response to adrenaline (Carel et al, 1999). In addition heterologous desensitization of lipolysis appears at least partly due to down-regulation of Gi (Green et al, 1992). Stimulation of lipolysis by tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) appears to be by Gi protein down-regulation blunting endogenous inhibition of lipolysis by adenosine (Gasic et al, 1999). Thus in addition to agonist-mediated receptor expression, adipocyte response to lipolytic stimuli also depends upon the expression and conformation of G-proteins.

To date there have been no measurements of G-protein expression in adipose tissue in cancer cachexia. This study reports alterations in adipocyte G-proteins with the development of the cachectic state in both mice bearing the cachexia-inducing MAC16 tumour and in a patient with cancer cachexia. The changes consisted principally of a reduction in membrane Gs expression allied with increased expression of G\(_i\)s, changes which would favour mobilization of lipid stores from adipocytes and hence facilitate host tissue catabolism. Such alterations were observed in mice manifesting up to 10–15% loss of their original body weight and correlates with maximal production of LMF by the tumour (Groundwater et al, 1990).

Similar changes in G-protein expression were also seen in mice administered LMF, as well as in 3T3L1 adipocytes in vitro, showing that the observed changes in G-protein expression were a direct effect of LMF. LMF was also found to stimulate expression of HSL in 3T3 adipocytes, which may explain the increased levels observed in adipose tissue of cancer patients (Thompson et al, 1993).

The polyunsaturated fatty acid, eicosapentaenoic acid (EPA) has been shown to attenuate the development of cachexia in both the MAC16 model (Beck et al, 1991) and in patients with unresectable pancreatic cancer (Wigmore et al, 1996), with preservation of adipose stores. Using isolated white adipocytes we have previously shown EPA inhibition of both lipolysis and adenylate cyclase stimulation by LMF to be a pertussis toxin-sensitive process, suggesting the involvement of G\(_i\) (Price and Tisdale, 1998). The effect appears to arise from a direct attenuation of the action of LMF on G-protein expression in adipose tissue, resulting in an increased G\(_i\)/G\(_s\) expression.

Thus tumours secreting LMF will maximize catabolism of adipose tissue by the continuous production of this lipolytic stimulus, together with increases in G\(_i\)/G\(_s\) and HSL, which will sensitize the adipocytes to a range of lipolytic stimuli. Such changes would explain the 85% fall in total body fat reported for lung cancer patients who had lost 30% of their pre-illness stable weight (Fearon, 1992) if energy utilization was also increased. In mice administered LMF a 3-fold increase in oxygen consumption by interscapular brown adipose tissue was observed (Hirai et al, 1998) suggesting an increase in thermogenesis. The mechanism by which this occurs will be the subject of further investigation.

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