Metformin inhibits leptin secretion via a mitogen-activated protein kinase signalling pathway in brown adipocytes

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Abstract

Metformin is an anti-diabetic drug with anorexigenic properties. The precise cellular mechanisms of its action are not entirely understood. Adipose tissue has recently been recognized as an important endocrine organ that is pivotal for the regulation of insulin resistance and energy homeostasis. Due to its thermogenic capacity brown adipose tissue contributes to the regulation of energy metabolism and is an attractive target tissue for pharmacological approaches to treating insulin resistance and obesity. Leptin is the prototypic adipocyte-derived hormone inducing a negative energy balance. We investigated effects of metformin on adipocyte metabolism, signalling, and leptin secretion in a brown adipocyte model. Metformin acutely stimulated p44/p42 mitogen-activated protein (MAP) kinase in a dose- (3·2-fold at 1 mmol/l, $P<0·05$) as well as time-dependent (3·8-fold at 5 min, $P<0·05$) manner. This stimulation was highly selective since phosphorylation of intermediates in the stress kinase, janus kinase (JAK)–signal transducer and activator of transcription (STAT), and phosphatidylinositol (PI) 3-kinase signalling pathways such as p38 MAP kinase, STAT3, and Akt was unaltered. Furthermore, chronic metformin treatment for 12 days dose-dependently inhibited leptin secretion by 35% and 75% at 500 μmol/l and 1 mmol/l metformin respectively ($P<0·01$). This reduction was not caused by alterations in adipocyte differentiation. Moreover, the impairment in leptin secretion by metformin was reversible within 48 h after removal of the drug. Pharmacological inhibition of p44/p42 MAP kinase prevented the metformin-induced negative effect on leptin secretion. Taken together, our data demonstrate direct acute effects of metformin on adipocyte signalling and endocrine function with robust inhibition of leptin secretion. They suggest a selective molecular mechanism that may contribute to the anorexigenic effect of this antidiabetic compound.

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Introduction

Metformin is a widely used anti-diabetic agent for the treatment of type 2 diabetes. It enhances insulin sensitivity. Furthermore, this compound displays the unique characteristic of promoting weight loss and reducing appetite (Bailey & Turner 1996, Matthaei *et al.* 2000, Kirpichnikov *et al.* 2002). Although used as a drug since the late 1950s, the mechanisms of action by which metformin lowers glucose and lipid levels remain unclear.

Potential direct effects of metformin on signalling pathways are poorly understood. In muscle, insulin receptor tyrosine kinase activity (Stith *et al.* 1996, 1998) and recruitment of glucose transporter (GLUT) 4 to the plasma membrane (Sarabia *et al.* 1992, Rouru *et al.* 1995) have been shown to be increased by chronic metformin treatment. In hepatocytes metformin inhibits gluconeogenesis and glycogenolysis probably due to a number of mechanisms such as diminished lactate uptake (Radziuk *et al.* 1997), reduction in pyruvate carboxylase–phosphoenolpyruvate carboxykinase activity (Large & Beylot 1999), antagonism to glucagon (Dominguez *et al.* 1996), enhancement of insulin action (Wiernsperger & Bailey 1999), and decreased concentrations of adenosine triphosphate (Argaud *et al.* 1993). In this context, modulation of cellular respiration via unidentified cell-signalling pathways appears to play a role (Dominguez *et al.* 1996, Yki-Jarvinen *et al.* 1999, Kirpichnikov *et al.* 2002). Activation of 5′-AMP-activated protein kinase (AMPK) has been implicated in metformin action in hepatocytes (Zhou *et al.* 2001).

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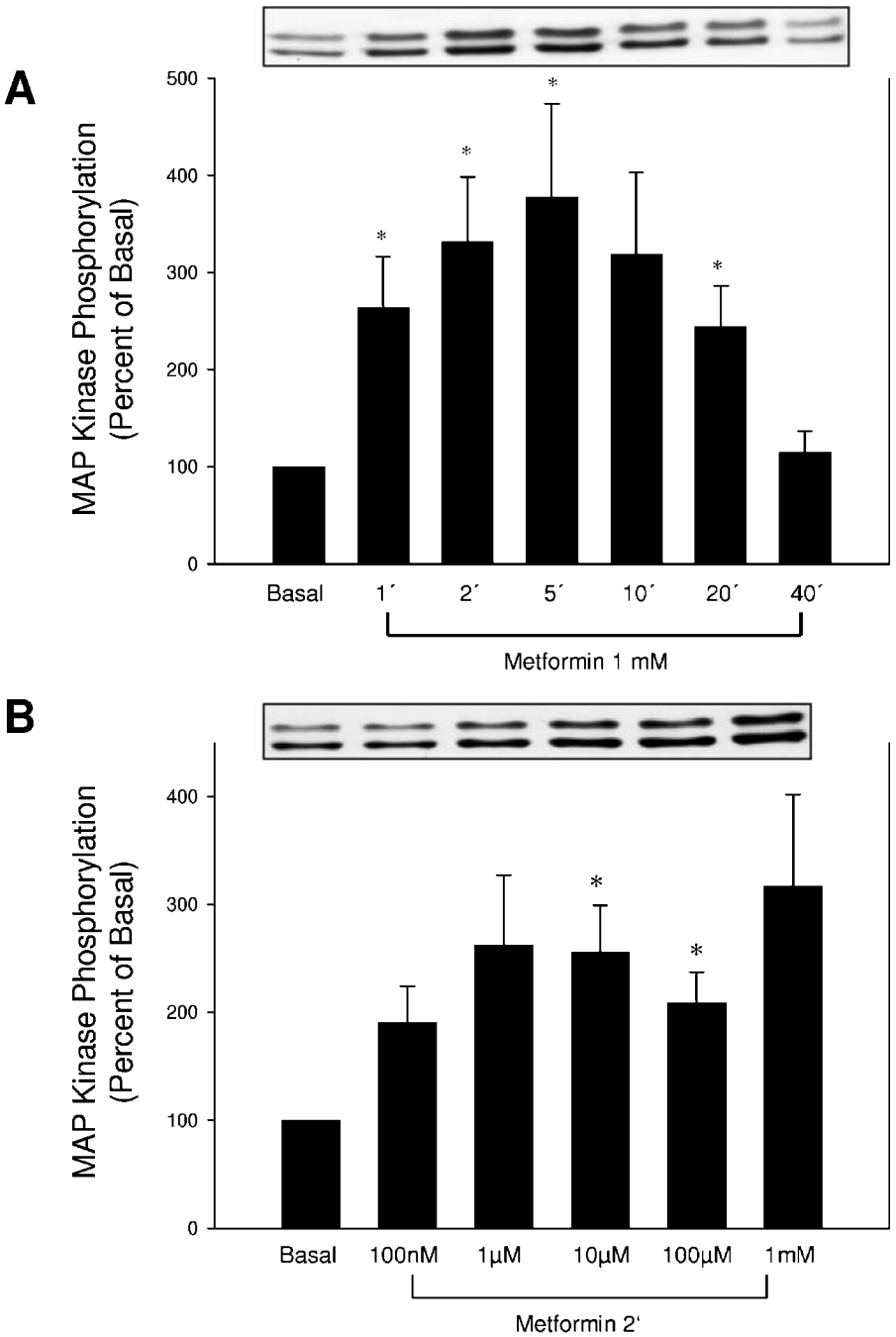

Figure 1 Metformin acutely activates p44/p42 MAP kinase. Fully differentiated brown adipocytes were stimulated with metformin for the times (1–40 min) (A) and at the concentrations (B) indicated. (A) Cell lysates and immunoblots using phospho-specific antibodies were prepared as described in Materials and Methods. (B) Bar graph analyses with S.E.M. of ≥ 6 independent experiments and representative immunoblots are shown. * Denotes statistically significant ($P<0{\cdot}05$) differences comparing non-treated (Basal) to metformin-treated cells.

By contrast to liver and muscle, relatively little is known about direct metformin actions in adipocytes. In rat adipose tissue glucose uptake has been found to be enhanced (Matthaei *et al.* 1991, 1993) whereas in human adipocytes no change has been described by metformin treatment (Pedersen *et al.* 1989, Ciaraldi *et al.* 2002). Recently, there has been a growing appreciation of adipose tissue as an endocrine organ that is pivotal for the systemic regulation of insulin action and energy homeostasis (Rajala & Scherer 2003). Direct interactions of metformin with adipocyte signalling and endocrine function may thus be instrumental for this compound's effects. Clinical studies with metformin have constantly shown either a decrease in body weight (DeFronzo *et al.* 1991, DeFronzo & Goodman 1995) or at least a significantly smaller increase in body weight compared with other forms of treatment (Yki-Jarvinen *et al.* 1999). The adipocyte-derived hormone, leptin, is an essential player in regulating energy homeostasis (Friedman & Halaas 1998, Spiegelman & Flier 2001, Friedman 2002). Brown adipose tissue importantly contributes to the modulation of energy homeostasis in rodents (Lowell & Flier 1997, Lowell & Bachman 2003), has been implicated in human obesity (Fumeron *et al.* 1996, Oberkofler *et al.* 1997, Fogelholm *et al.* 1998, Valve *et al.* 1998), and is an attractive target tissue for pharmacotherapeutic approaches to obesity (Danforth & Himms-Hagen 1997, Lowell & Flier 1997, Bray & Greenway 1999, Tiraby *et al.* 2003, Klaus 2004). Recent studies suggest the existence of a basal brown adipose phenotype that may be important for the maintenance of normal insulin sensitivity and energy homeostasis (Yang *et al.* 2003). Moreover, transdifferentiation of white to brown adipocytes has been demonstrated and may offer interesting new therapeutic perspectives for treating insulin resistance and energy balance disorders (Tiraby & Langin 2003, Tiraby *et al.* 2003). We have previously demonstrated robust leptin secretion in brown adipocytes (Klein *et al.* 2002, Kraus *et al.* 2002). Investigation of direct metformin interaction with adipose tissue may identify molecular targets and provide insights into mechanisms of insulin resistance and energy homeostasis regulation.

Here, we studied direct metformin effects on adipocyte signalling, differentiation, and leptin secretion (Klein *et al.* 2002, Kraus *et al.* 2002). We demonstrate a selective activation of p44/p42 mitogen-activated protein (MAP) kinase by metformin and a differentiation-independent, robust reduction in leptin secretion that is prevented by pharmacological inhibition of p44/p42 MAP kinase.

Materials and Methods

Materials

Antibodies against the following molecules were employed for immunoblotting: signal transducer and activator of transcription (STAT) 3 (phospho-Tyr705), p44/p42 MAP kinase (phospo-Thr202/Tyr204), Akt (phospho-Ser473) (Cell Signaling Technology, Inc., Beverly, MA, USA), CCAAT enhancer binding protein (C/EBP) α, peroxisome proliferator-activated receptor (PPAR) γ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), uncoupling protein (UCP)-1 (Alpha Diagnostic International, San Antonio, TX, USA). The pharmacological MAP kinase inhibitor, PD98059, was purchased from Cell Signaling Technology, Inc. Unless stated otherwise, all other chemicals were purchased from Sigma-Aldrich Co. (St Louis, MO, USA).

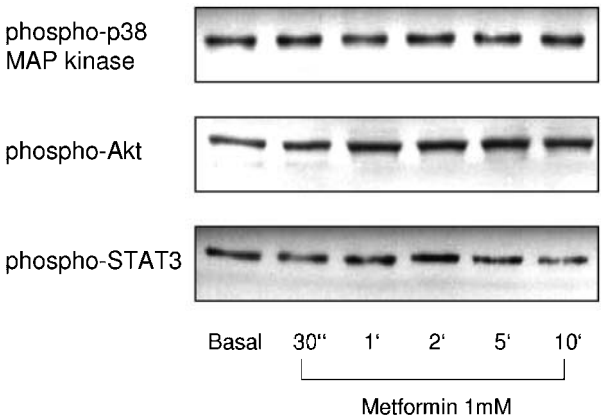

Figure 2 Metformin does not stimulate p38 MAP kinase, Akt or STAT3 phosphorylation. Adipocytes were stimulated with metformin (1 mM) for the indicated times (30 s and 1, 2, 5 and 10 min). Cell lysates and immunoblots using phospho-specific antibodies were prepared as described in Materials and Methods. Representative blots of phospho-p38 MAP kinase (upper panel), phospho-Akt (middle panel), and phospho-STAT3 (lower panel) of ≥ 5 independent experiments are shown.

Cell culture

SV40T-immortalized brown adipocytes from the FVB strain of mice – generated as described elsewhere (Klein *et al.* 1999) – were used for all experiments. Preadipocytes were seeded on tissue culture plates (Sarstedt, Nümbrecht, Germany) and grown to confluence in culture medium with Dulbecco's modified Eagle's medium (Life Technologies, Paisley, Strathclyde, UK), supplemented with 20% fetal bovine serum, 4·5 g/l glucose, 20 nM insulin, 1 nM triiodothyronine ('differentiation medium'), and penicillin/streptomycin. Adipocyte differentiation was induced by complementing the medium further with 250 μM indomethacin, 500 μM isobutylmethylxanthine and 2 μg/ml dexamethasone for 24 h when confluence was reached. After this induction period, cells were changed back to differentiation medium. Cell culture was continued for 5 more days before cells were starved for 24 h with serum-free medium prior to carrying out the

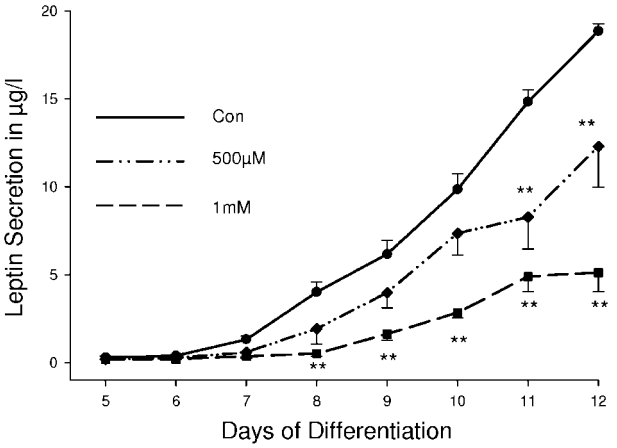

Figure 3 Chronic metformin treatment dose-dependently inhibits leptin secretion. Cells were chronically exposed to the indicated concentrations of metformin over the entire differentiation course. Medium was collected every 24 h. Secretion of leptin was analysed in the culture medium using a mouse leptin RIA. A line graph with S.E.M. of ≥ 3 independent experiments is shown comparing untreated cells (Con, ●) with 500 µM (♦) and 1 mM (■) metformin treatment. ** Denotes high statistical significance ($P<0{\cdot}01$).

immunoblotting experiments. For leptin secretion experiments, cell culture was continued for up to 9 days after induction of differentiation.

Determination of leptin secretion

Cells were chronically treated with or without metformin and medium was collected every 24 h from day 4 to day 12 of the differentiation course. Treatment with the pharmacological MAP kinase inhibitor, PD98059, was begun 30 min prior to adding metformin. The amount of leptin released into the medium was determined using a mouse leptin RIA (Linco Research, Inc., St Louis, MO, USA).

Oil Red O staining

Tissue culture plates were washed twice with PBS and fixed with 10% formalin for at least 1 h at room temperature. Cells were then stained for 1 h at room temperature with a filtered Oil Red O solution (0·5 g Oil Red O in 100 ml isopropyl alcohol). The staining solution was washed off the cells with distilled water twice.

Western blotting

SV40T-immortalized mouse brown adipocytes were used between passages 10 and 25. For p44/p42 MAP kinase, Akt, p38 MAP kinase, and STAT3 analysis fully differentiated cells were starved for 24 h in serum-free medium prior to carrying out the experiments. Following treatment with metformin as indicated, proteins were isolated using whole cell lysis buffer containing 2 mM vanadate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 2 mM PMSF. Protein content of lysates was determined by the Bradford method using the dye from Bio-Rad (Hercules, CA, USA). Lysates were submitted to SDS-PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell Inc., Keane, NH, USA). Membranes were blocked with rinsing buffer (10 mM Tris, 150 mM NaCl, 0·05% Tween, pH 7·2) containing 3% bovine serum albumin ('blocking solution') overnight. Membranes were then incubated in blocking solution for 1–2 h with the antibodies indicated. Protein bands were visualized using the chemiluminescence kit from Roche Molecular Biochemicals (Mannheim, Germany) and enhanced chemiluminescence films (Amersham Pharmacia Biotech, Freiburg, Germany).

Statistical analysis

Data are presented as means ± S.E.M. Sigma Plot software (SPSS Science; Chicago, IL, USA) was employed for statistical analysis of all data. Statistical significance was determined using the unpaired Student's *t*-test. *P* values $<0{\cdot}05$ are considered significant, $<0{\cdot}01$ highly significant.

Results

Metformin acutely induces p44/p42 MAP kinase but not p38 MAP kinase, Akt and STAT3 phosphorylation

P44/42 MAP kinase is an important signalling intermediate of growth factor signalling pathways and a major regulator of gene transcription. Treatment of fully differentiated brown adipocytes with metformin resulted in a time- and dose-dependent stimulation of p44/p42 MAP kinase as assessed using phospho-specific antibodies (Fig. 1A and B). Metformin-induced activation was most prominent after 5 min (Fig. 1A) with a maximal 3·5-fold phosphorylation increase at a concentration of 1 mM (Fig. 1B). There was no change in protein amounts of MAP kinase as assessed by immunoblots using p44/p42 MAP kinase antibodies (data not shown). Furthermore, metformin treatment did not induce significant changes in phosphorylation of p38 MAP kinase, Akt and STAT3 – key signalling molecules of the stress kinase, phosphatidylinositol 3-kinase (PI 3-kinase), and janus kinase (JAK)/STAT signalling pathways respectively (Fig. 2).

Metformin treatment inhibits leptin secretion in a dose-dependent manner

When cells were chronically exposed to metformin, there was a dose-dependent impairment in leptin secretion. Non-treated control cells displayed a differentiation-dependent increase in leptin secretion over two orders of

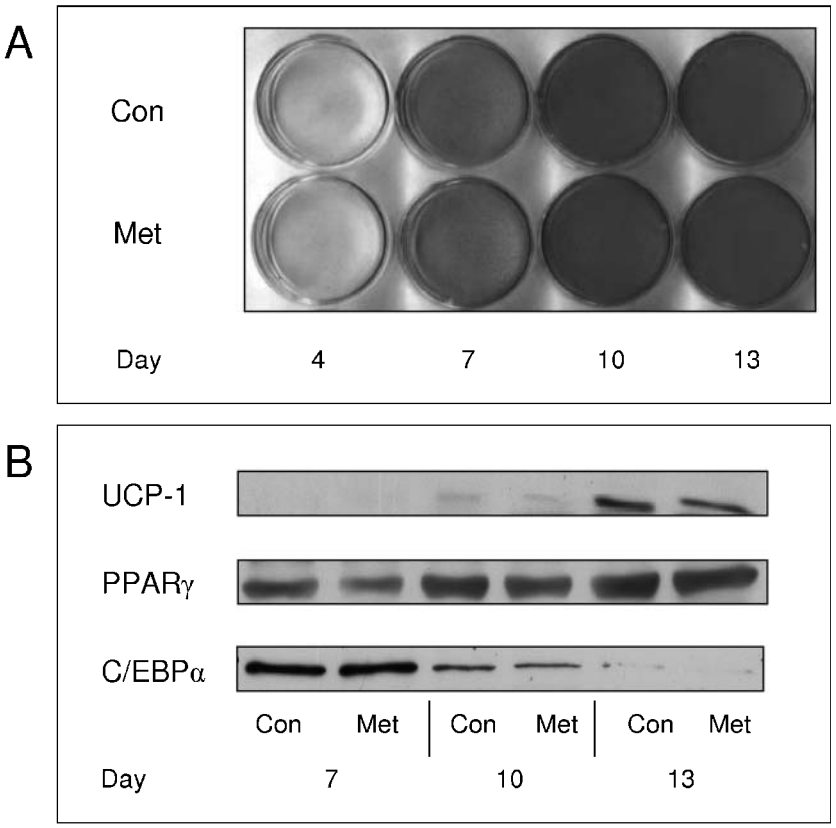

Figure 4 The inhibitory effect of metformin on leptin secretion is not caused by alterations in adipocyte differentiation. (A) Differentiation was assessed in cell lines either non-treated (Con) or chronically exposed to metformin (Met, 1 mM) using the fat-specific Oil Red O staining. (B) Using specific antibodies as applicable, protein expression of the differentiation markers uncoupling protein-1 (UCP-1, upper panel), peroxisome proliferator-activated receptor gamma (PPARγ, middle panel) and CCAAT enhancer-binding protein alpha (C/EBPα, lower panel) was analysed in immunoblots. Representative blots and staining results of ≥ 2 independent experiments are shown.

magnitude with the lowest detectable leptin levels at a concentration of 0·2 μg/l rising to the maximum detectable level of 20 μg/l during a 12-day-differentiation course (Fig. 3). Chronic metformin treatment dose-dependently inhibited this increase in leptin secretion with a maximum reduction of 35% and 75% at the end of the differentation course at concentrations of 500 μM and 1 mM metformin respectively. These changes were highly significant (Fig. 3). A significant inhibition of leptin secretion was also seen at 100 μM metformin (data not shown). Furthermore, metformin did not influence glucose utilization and lactate production (data not shown).

The inhibitory effect of metformin on leptin secretion is not caused by alterations in differentiation

To separate the impairment in leptin secretion from a differentiation-dependent effect, we next investigated adipocyte differentiation under chronic metformin treatment. When differentiating adipocytes were stained with the fat-specific Oil Red O at days 4, 7, 10 and 13 of the differentiation course there was no difference between metformin-treated and non-treated control cells (Fig. 4A). Furthermore, protein expression of early and late adipocyte differentiation markers such as C/EBPα, PPARγ, and UCP-1 did not differ between metformin-treated and non-treated control cells throughout the differentiation course (Fig. 4B).

Subacute metformin treatment induces a reversible impairment in leptin secretion

To further define the kinetics of the inhibitory metformin effect on leptin secretion, we pretreated adipocytes for various periods of time with 1 mM metformin on day 8 of the differentiation course, collected the medium every 24 h, and continued cell culture for two more days without metformin exposure. Interestingly, metformin

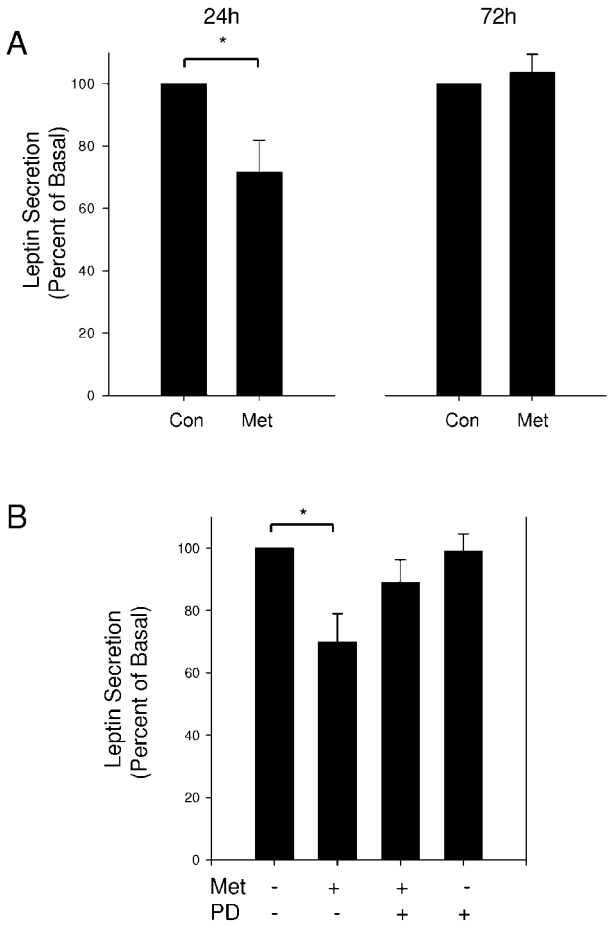

Figure 5 Subacute metformin treatment induces an impairment in leptin secretion that can be prevented by inhibition of p44/p42 MAP kinase. On day 8 of the differentiation course cells were either left untreated (Con) or treated with metformin (Met, 1 mM) for 24 h. Medium was collected 24 h (A, left panel) or 72 h (A, right panel) after removal of metformin. (B) The MAP kinase inhibitor, PD98059 (PD, 50 μM), was added 1 h prior to metformin treatment for 24 h, and the medium was analysed for leptin concentrations 24 h later. A bar graph analysis with S.E.M. of ≥ 5 independent experiments is shown. * Denotes statistical significance ($P<0{\cdot}05$).

treatment for 24 h resulted in a significant 30% reduction of leptin secretion within the next 24 h (Fig. 5A, left panel). This effect was completely reversible 72 h after metformin removal from the medium (Fig. 5A, right panel). Furthermore, there was a time-dependent trend towards impaired leptin secretion after 8 and 16 h of metformin treatment whereas shorter periods of time did not show significant alterations in leptin secretion as compared with control cells (data not shown).

Inhibition of p44/p42 MAP kinase prevents the inhibitory metformin effect

The impairment of leptin secretion by subacute metformin treatment in concert with the acute induction of p44/p42 MAP kinase phosphorylation suggested an involvement of this signalling intermediate in the mediation of this effect. Metformin treatment for 24 h again significantly diminished leptin secretion by 30% on the following day as compared with non-treated control cells (Fig. 5B). However, when cells were pretreated with the p44/p2 MAP kinase inhibitor, PD98059, exposure to metformin failed to significantly inhibit leptin secretion (Fig. 5B). Treatment with the pharmacological inhibitor alone did not change basal leptin secretion (Fig. 5B).

Discussion

In this study, we show direct effects of the anorexigenic anti-diabetic drug, metformin, on adipocyte signalling and endocrine function with robust inhibition of leptin secretion.

Metformin directly induced p44/p42 MAP kinase activation. To our knowledge, this is the first report demonstrating stimulation of this important growth factor signalling intermediate by metformin. Apart from p44/p42 MAP kinase, only AMPK and p38 MAP kinase have been shown to be implicated in intracellular metformin action so far. Zhou *et al.* (2001) and Hawley *et al.* (2002) described activation of AMPK by chronic treatment with metformin in rat hepatocytes and skeletal muscle. In skeletal muscle, Kumar & Dey (2002) also found an increase in p38 MAP kinase activity by metformin. Interestingly, however, p38 stress kinase-, PI 3-kinase-, and JAK/STAT-signalling pathways remained unaffected by metformin treatment in our study using adipocytes. These discrepancies may indicate tissue- and cell-specific effects of metformin.

Of note, stimulation of p44/p42 MAP kinase occurred acutely and was time- and dose-dependent. In concert with the demonstrated selectivity of action, these findings suggest a receptor-mediated signalling mechanism employed by metformin in adipocytes. However, no specific receptor mediating the effects of metformin has been identified so far. Rather, this lipophilic compound may exert its effects by alterations of the cellular membrane structure (Meuillet *et al.* 1999).

Activation of p44/p42 MAP kinase plays an important role in regulating gene expression, insulin signalling and – specifically in brown adipocytes – thermogenesis (Porras *et al.* 1998, Klein *et al.* 2000). Therefore, it appears plausible to propose important functional consequences of metformin-induced acute changes in p44/p42 MAP kinase signalling in adipocytes. Indeed, we found that metformin directly affected endocrine function and inhibited leptin secretion. We used a previously well characterised adipocyte model (Klein *et al.* 2002) that displays strong leptin secretion (Kraus *et al.* 2002). A decrease in leptin levels in metformin-treated individuals has been found in several studies (Freemark & Bursey 2001,

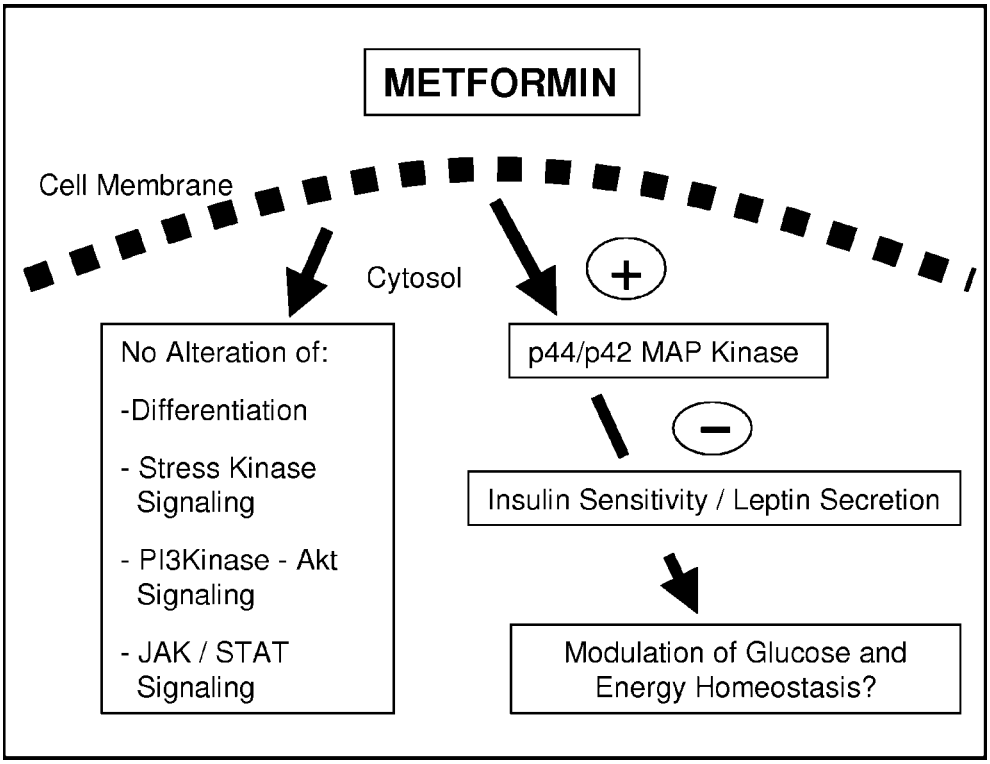

Figure 6 Metformin directly modulates adipocyte signalling and endocrine function. Metformin activates p44/p42 MAP kinase and impairs leptin secretion unless p44/p42 MAP kinase is inhibited. This effect is reversible and is not caused by alterations in adipocyte differentiation. Furthermore, it is selective since there is no activation of stress kinase, PI 3-kinase, and JAK/STAT signalling pathways. Modulation of endocrine adipocyte function by metformin may be important in the regulation of energy homeostasis.

Glueck et al. 2001, Fruehwald-Schultes *et al.* 2002); however, in other studies, no effect on serum leptin was found (Guler *et al.* 2000, Mannucci *et al.* 2001, Uehara *et al.* 2001, Ciaraldi *et al.* 2002, Sivitz *et al.* 2003). Possible explanations for these discrepancies may be the length of treatment and the study population, with obese people showing a decrease in leptin levels after long-term treatment. A negative correlation of the length of metformin therapy with circulating leptin levels in this setting could possibly be accounted for by a direct subacute effect of this anti-diabetic drug on adipose tissue as described in this study.

In a previous study in rat white adipocytes, a negative influence of chronic metformin exposure on leptin secretion has also been reported (Mueller *et al.* 2000). As we show here, the direct metformin-induced impairment in leptin secretion is independent of changes in adipocyte morphology and differentiation. Furthermore, it is already evident after 24 h of treatment, and it is reversible. As was the case with activation of p44/p42 MAP kinase, these observations point towards a selective signalling mechanism mediating these effects. In favour of this assumption, we found that inhibition of p44/p42 MAP kinase signalling prevented the metformin-induced reduction in leptin secretion, thus suggesting an involvement of this important growth factor signalling intermediate in the modulation of endocrine adipocyte function.

In summary, our data show a direct selective interaction of metformin with adipocyte p44/p42 MAP kinase signalling and leptin secretion. They describe a potential molecular mechanism mediating this anorexigenic compound's effects on adipose tissue. Selective modulation of adipose tissue function could have important implications for therapeutic strategies of the insulin resistance syndrome.

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References

Argaud D, Roth H, Wiernsperger N & Leverve XM 1993 Metformin decreases gluconeogenesis by enhancing the pyruvate kinase flux in isolated rat hepatocytes. *European Journal of Biochemistry* **213** 1341–1348.

Bailey CJ & Turner RC 1996 Metformin. *New England Journal of Medicine* **334** 574–579.

Bray GA & Greenway FL 1999 Current and potential drugs for treatment of obesity. *Endocrine Reviews* **20** 805–875.

Ciaraldi TP, Kong AP, Chu NV, Kim DD, Baxi S, Loviscach M, Plodkowski R, Reitz R, Caulfield M, Mudaliar S & Henry RR 2002 Regulation of glucose transport and insulin signalling by troglitazone or metformin in adipose tissue of type 2 diabetic subjects. *Diabetes* **51** 30–36.

Danforth E Jr & Himms-Hagen JH 1997 Obesity and diabetes and the beta-3 adrenergic receptor. *European Journal of Endocrinology* **136** 362–365.

DeFronzo RA & Goodman AM 1995 Efficacy of metformin in patients with non-insulin-dependent diabetes mellitus. The Multicenter Metformin Study Group. *New England Journal of Medicine* **333** 541–549.

DeFronzo RA, Barzilai N & Simonson DC 1991 Mechanism of metformin action in obese and lean noninsulin-dependent diabetic subjects. *Journal of Clinical Endocrinology and Metabolism* **73** 1294–1301.

Dominguez LJ, Davidoff AJ, Srinivas PR, Standley PR, Walsh MF & Sowers JR 1996 Effects of metformin on tyrosine kinase activity, glucose transport, and intracellular calcium in rat vascular smooth muscle. *Endocrinology* **137** 113–121.

Fogelholm M, Valve R, Kukkonen-Harjula K, Nenonen A, Hakkarainen V, Laakso M & Uusitupa M 1998 Additive effects of the mutations in the beta3-adrenergic receptor and uncoupling protein-1 genes on weight loss and weight maintenance in Finnish women. *Journal of Clinical Endocrinology and Metabolism* **83** 4246–4250.

Freemark M & Bursey D 2001 The effects of metformin on body mass index and glucose tolerance in obese adolescents with fasting hyperinsulinemia and a family history of type 2 diabetes. *Pediatrics* **107** E55.

Friedman JM 2002 The function of leptin in nutrition, weight, and physiology. *Nutrition Reviews* **60** S1–S14

Friedman JM & Halaas JL 1998 Leptin and the regulation of body weight in mammals. *Nature* **395** 763–770.

Fruehwald-Schultes B, Oltmanns KM, Toschek B, Sopke S, Kern W, Born J, Fehm HL & Peters A 2002 Short-term treatment with metformin decreases serum leptin concentration without affecting body weight and body fat content in normal-weight healthy men. *Metabolism* **51** 531–536.

Fumeron F, Durack-Bown I, Betoulle D, Cassard-Doulcier AM, Tuzet S, Bouillaud F, Melchior JC, Ricquier D & Apfelbaum M 1996 Polymorphisms of uncoupling protein (UCP) and beta 3 adrenoreceptor genes in obese people submitted to a low calorie diet. *International Journal of Obesity and Related Metabolic Disorders* **20** 1051–1054.

Glueck CJ, Fontaine RN, Wang P, Subbiah MT, Weber K, Illig E, Streicher P, Sieve-Smith L, Tracy TM, Lang JE & McCullough P 2001 Metformin reduces weight, centripetal obesity, insulin, leptin, and low-density lipoprotein cholesterol in nondiabetic, morbidly obese subjects with body mass index greater than 30. *Metabolism* **50** 856–861.

Guler S, Cakir B, Demirbas B, Gursoy G, Serter R & Aral Y 2000 Leptin concentrations are related to glycaemic control, but do not change with short-term oral antidiabetic therapy in female patients with type 2 diabetes mellitus. *Diabetes, Obesity and Metabolism* **2** 313–316.

Hawley SA, Gadalla AE, Olsen GS & Hardie DG 2002 The antidiabetic drug metformin activates the AMP-activated protein kinase cascade via an adenine nucleotide-independent mechanism. *Diabetes* **51** 2420–2425.

Kirpichnikov D, McFarlane SI & Sowers JR 2002 Metformin: an update. *Annals of Internal Medicine* **137** 25–33.

Klaus S 2004 Adipose tissue as a regulator of energy balance. *Current Drug Targets* **5** 241–250.

Klein J, Fasshauer M, Ito M, Lowell BB, Benito M & Kahn CR 1999 Beta(3)-adrenergic stimulation differentially inhibits insulin signalling and decreases insulin-induced glucose uptake in brown adipocytes. *Journal of Biological Chemistry* **274** 34795–34802.

Klein J, Fasshauer M, Benito M & Kahn CR 2000 Insulin and the beta3-adrenoceptor differentially regulate uncoupling protein-1 expression. *Molecular Endocrinology* **14** 764–773.

Klein J, Fasshauer M, Klein HH, Benito M & Kahn CR 2002 Novel adipocyte lines from brown fat: a model system for the study of differentiation, energy metabolism, and insulin action. *Bioessays* **24** 382–388.

Kraus D, Fasshauer M, Ott V, Meier B, Jost M, Klein HH & Klein J 2002 Leptin secretion and negative autocrine crosstalk with insulin in brown adipocytes. *Journal of Endocrinology* **175** 185–191.

Kumar N & Dey CS 2002 Metformin enhances insulin signalling in insulin-dependent and -independent pathways in insulin resistant muscle cells. *British Journal of Pharmacology* **137** 329–336.

Large V & Beylot M 1999 Modifications of citric acid cycle activity and gluconeogenesis in streptozotocin-induced diabetes and effects of metformin. *Diabetes* **48** 1251–1257.

Lowell BB & Flier JS 1997 Brown adipose tissue, beta 3-adrenergic receptors, and obesity. *Annual Review of Medicine* **48** 307–316.

Lowell BB & Bachman ES 2003 Beta-adrenergic receptors, diet-induced thermogenesis, and obesity. *Journal of Biological Chemistry* **278** 29385–29388.

Mannucci E, Ognibene A, Cremasco F, Bardini G, Mencucci A, Pierazzuoli E, Ciani S, Messeri G & Rotella CM 2001 Effect of metformin on glucagon-like peptide 1 (GLP-1) and leptin levels in obese nondiabetic subjects. *Diabetes Care* **24** 489–494.

Matthaei S, Hamann A, Klein HH, Benecke H, Kreymann G, Flier JS & Greten H 1991 Association of metformin's effect to increase insulin-stimulated glucose transport with potentiation of insulin-induced translocation of glucose transporters from intracellular pool to plasma membrane in rat adipocytes. *Diabetes* **40** 850–857.

Matthaei S, Reibold JP, Hamann A, Benecke H, Haring HU, Greten H & Klein HH 1993 *In vivo* metformin treatment ameliorates insulin resistance: evidence for potentiation of insulin-induced translocation and increased functional activity of glucose transporters in obese (fa/fa) Zucker rat adipocytes. *Endocrinology* **133** 304–311.

Matthaei S, Stumvoll M, Kellerer M & Haring HU 2000 Pathophysiology and pharmacological treatment of insulin resistance. *Endocrine Reviews* **21** 585–618.

Meuillet EJ, Wiernsperger N, Mania-Farnell B, Hubert P & Cremel G 1999 Metformin modulates insulin receptor signalling in normal and cholesterol-treated human hepatoma cells (HepG2). *European Journal of Pharmacology* **377** 241–252.

Mueller WM, Stanhope KL, Gregoire F, Evans JL & Havel PJ 2000 Effects of metformin and vanadium on leptin secretion from cultured rat adipocytes. *Obesity Research* **8** 530–539.

Oberkofler H, Dallinger G, Liu YM, Hell E, Krempler F & Patsch W 1997 Uncoupling protein gene: quantification of expression levels in adipose tissues of obese and non-obese humans. *Journal of Lipid Research* **38** 2125–2133.

Pedersen O, Nielsen O, Bak J, Richelsen B, Beck-Nielsen H & Sorensen N 1989 The effects of metformin on adipocyte insulin action and metabolic control in obese subjects with type 2 diabetes. *Diabetic Medicine* **6** 249–256.

Porras A, Alvarez AM, Valladares A & Benito M 1998 p42/p44 mitogen-activated protein kinases activation is required for the

insulin-like growth factor-I/insulin induced proliferation, but inhibits differentiation, in rat fetal brown adipocytes. *Molecular Endocrinology* **12** 825–834.

Radziuk J, Zhang Z, Wiernsperger N & Pye S 1997 Effects of metformin on lactate uptake and gluconeogenesis in the perfused rat liver. *Diabetes* **46** 1406–1413.

Rajala MW & Scherer PE 2003 Minireview: The adipocyte - at the crossroads of energy homeostasis, inflammation, and atherosclerosis. *Endocrinology* **144** 3765–3773.

Rouru J, Koulu M, Peltonen J, Santti E, Hanninen V, Pesonen U & Huupponen R 1995 Effects of metformin treatment on glucose transporter proteins in subcellular fractions of skeletal muscle in (fa/fa) Zucker rats. *British Journal of Pharmacology* **115** 1182–1187.

Sarabia V, Lam L, Burdett E, Leiter LA & Klip A 1992 Glucose transport in human skeletal muscle cells in culture. Stimulation by insulin and metformin. *Journal of Clinical Investigation* **90** 1386–1395.

Sivitz WI, Wayson SM, Bayless ML, Larson LF, Sinkey C, Bar RS & Haynes WG 2003 Leptin and body fat in type 2 diabetes and monodrug therapy. *Journal of Clinical Endocrinology and Metabolism* **88** 1543–1553.

Spiegelman BM & Flier JS 2001 Obesity and the regulation of energy balance. *Cell* **104** 531–543.

Stith BJ, Goalstone ML, Espinoza R, Mossel C, Roberts D & Wiernsperger N 1996 The antidiabetic drug metformin elevates receptor tyrosine kinase activity and inositol 1,4,5-trisphosphate mass in *Xenopus* oocytes. *Endocrinology* **137** 2990–2999.

Stith BJ, Woronoff K & Wiernsperger N 1998 Stimulation of the intracellular portion of the human insulin receptor by the antidiabetic drug metformin. *Biochemical Pharmacology* **55** 533–536.

Tiraby C & Langin D 2003 Conversion from white to brown adipocytes: a strategy for the control of fat mass? *Trends in Endocrinology and Metabolism* **14** 439–441.

Tiraby C, Tavernier G, Lefort C, Larrouy D, Bouillaud F, Ricquier D & Langin D 2003 Acquirement of brown fat cell features by human white adipocytes. *Journal of Biological Chemistry* **278** 33370–33376.

Uehara MH, Kohlmann NE, Zanella MT & Ferreira SR 2001 Metabolic and haemodynamic effects of metformin in patients with type 2 diabetes mellitus and hypertension. *Diabetes, Obesity and Metabolism* **3** 319–325.

Valve R, Heikkinen S, Rissanen A, Laakso M & Uusitupa M 1998 Synergistic effect of polymorphisms in uncoupling protein 1 and beta3-adrenergic receptor genes on basal metabolic rate in obese Finns [see comments]. *Diabetologia* **41** 357–361.

Wiernsperger NF & Bailey CJ 1999 The antihyperglycaemic effect of metformin: therapeutic and cellular mechanisms. *Drugs* **58 (**Suppl 1) 31–39; discussion 75–82.

Yang X, Enerback S & Smith U 2003 Reduced expression of FOXC2 and brown adipogenic genes in human subjects with insulin resistance. *Obesity Research* **11** 1182–1191.

Yki-Jarvinen H, Nikkila K & Makimattila S 1999 Metformin prevents weight gain by reducing dietary intake during insulin therapy in patients with type 2 diabetes mellitus. *Drugs* **58 (**Suppl 1) 53–54; discussion 75–82.

Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ & Moller DE 2001 Role of AMP-activated protein kinase in mechanism of metformin action. *Journal of Clinical Investigation* **108** 1167–1174.

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