Metformin reduces lipolysis in primary rat adipocytes stimulated by tumor necrosis factor- α or isoproterenol

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Abstract

In patients with type 2 non-insulin-dependent diabetes mellitus (NIDDM), the biguanide, metformin, exerts its antihyperglycemic effect by improving insulin sensitivity, which is associated with decreased level of circulating free fatty acids (FFA). The flux of FFA and glycerol from adipose tissue to the blood stream primarily depends on the lipolysis of triacylglycerols in the adipocytes. Adipocyte lipolysis is physiologically stimulated by catecholamine hormones. Tumor necrosis factor- α (TNF- α), a cytokine largely expressed in adipose tissue, stimulates chronic lipolysis, which may be associated with increased systemic FFA and insulin resistance in obesity and NIDDM. In this study, we examined the role of metformin in inhibiting lipolytic action upon various lipolytic stimulations in primary rat adipocytes. Treatment with metformin attenuated TNF- α -mediated lipolysis by suppressing phosphorylation of extracellular signal-related kinase 1/2 and reversing the downregulation of perilipin protein in TNF- α -stimulated adipocytes. The acute lipolytic response to adrenergic stimulation of isoproterenol was also restricted by metformin. A high concentration of glucose in the adipocyte culture promoted the basal rate of glycerol release and significantly enhanced the lipolytic action stimulated by either TNF- α or isoproterenol. Metformin not only inhibits the basal lipolysis simulated by high glucose, but also suppresses the high glucose-enhanced lipolysis response to TNF- α or isoproterenol. The antilipolytic action in adipocytes could be the mechanism by which cellular action by metformin reduces systemic FFA concentration and thus improves insulin sensitivity in obese patients and the hyperglycemic conditions of NIDDM.

Journal of Molecular Endocrinology (2006) **37**, 175–183

Introduction

Obesity and type 2 non-insulin-dependent diabetes mellitus (NIDDM) are associated with increased levels of circulating free fatty acids (FFA), which are thought to induce insulin resistance (Groop *et al.* 1989). The flux of FFA and glycerol from adipose tissue to the blood stream primarily depends on the lipolysis of triacylglycerols in adipocytes. Under physiological conditions, adipocyte lipolysis is stimulated by catecholamine hormones by elevating cellular cAMP content and activating cAMP-dependent protein kinase A (Londos *et al.* 1999). Tumor necrosis factor- α (TNF- α) is a cytokine largely expressed in adipose tissue, which may be associated with increased plasma FFA level and impaired insulin sensitivity in obesity and NIDDM (Hotamisligil *et al.* 1993, 1995). TNF- α stimulates chronic lipolysis in primary (Green *et al.* 1994) or differentiated adipocytes (Souza *et al.* 1998a,b, Ryden *et al.* 2002, Zhang *et al.* 2002, Green *et al.* 2004) by activating extracellular signal-related kinase (ERK) 1 and ERK2 (Ryden *et al.* 2002, Zhang *et al.* 2002, Souza

et al. 2003). Perilipin protein coats the surface of intracellular lipid droplets in adipocytes and is involved in lipolytic regulation by functioning as a substrate-associating factor (Londos *et al.* 1999, Sztalryd *et al.* 2003, He *et al.* 2006). Downregulated perilipin has been proposed to account for increased lipolytic action in TNF- α -stimulated differentiated adipocytes (Souza *et al.* 1998a,b, Ryden *et al.* 2004). In addition, a high concentration of glucose increases basal lipolytic activity and enhances lipolysis in adipocytes stimulated by adrenergic agonist (Moussalli *et al.* 1986, Szkudelski & Szkudelska 2000) or TNF- α (Green *et al.* 2004). A high level of plasma glucose is associated with a high concentration of systemic FFA in NIDDM (Abbasi *et al.* 1998). Therefore, excess glucose may act to boost lipolytic stimulation in adipose cells under hyperglycemic conditions, thereby further increasing the level of circulating FFA and insulin resistance.

The biguanide, metformin, is widely used for the treatment of NIDDM. Unlike the sulfonylureas that stimulate insulin secretion, metformin can reduce blood glucose levels in a reduced concentration of

plasma insulin (DeFronzo *et al.* 1991). Metformin exerts its antihyperglycemic effect by increasing insulin sensitivity in peripheral tissues, where it suppresses hepatic glucose output and increases glucose transport and utilization (Bailey 1992). Clinical studies suggest that the improvement of insulin sensitivity by metformin is associated with an overall reduction in circulating FFA levels in NIDDM patients (Groop *et al.* 1989, Riccio *et al.* 1991, Perriello *et al.* 1994, Abbasi *et al.* 1998). However, the cellular basis by which metformin decreases plasma FFA level has not yet been elucidated.

In this study, we examined the role of metformin in inhibiting lipolytic action upon various lipolytic stimulations in primary rat adipocytes. Treatment of adipocytes with metformin attenuated lipolytic action, stimulated by TNF- α , isoproterenol, or a high concentration of glucose. The inhibitory effect of metformin on TNF- α -stimulated lipolysis is accompanied by suppressed phosphorylation of ERK1/2 and reversed the downregulation of perilipin. Moreover, metformin not only inhibits the basal lipolysis simulated by high glucose alone but also suppresses the excess glucose-enhanced lipolytic response to TNF- α or isoproterenol. This study provides the first direct evidence that the antilipolytic action of metformin in adipocytes may allow this agent to decrease the circulating FFA levels and thus increase insulin sensitivity.

Materials and methods

Materials

TNF- α was purchased from PeproTech EC (London, UK). Metformin was from Bristol-Myers Squibb Pharmaceuticals (Shanghai, China). Phenol red-free Dulbecco's modified Eagle's medium (fDMEM) containing 5 mM glucose was from Sigma. Antibodies against ERK1 or phospho-ERK1/2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phospho-p38 or phospho-JNK were purchased from the Cell Signaling Technology Inc. (Danvers, MA, USA). Rabbit antibody raised against rat perilipin (Brasaemle *et al.* 1997) was a generous gift from Dr Londos at the US National Institutes of Health (Baltimore, MD, USA). Nitrocellulose membrane, pre-stained protein molecular weight marker, and Super Plus chemiluminescence detection kit were purchased from Applygen Technologies (Beijing, China).

Isolation, culture, and treatment of primary rat adipocytes

Adipocytes were isolated from the epididymal fat pads of Sprague–Dawley rats (160–180 g). A total of 200 nM

adenosine (Sigma) and 1% defatted BSA were included in all subsequent incubations (Honnor *et al.* 1985). The fat pads were minced and digested by 1 mg/ml type I collagenase in Krebs–Ringer solution (120 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 15 mM NaHCO₃) buffered with 25 mM Hepes, pH 7.4. After incubation for 40 min at 37 °C in a water bath shaken at 100 cycles/min, the cells were filtered through a nylon mesh and washed three times in a pre-warmed phenol red- and serum-free DMEM (fDMEM) (He *et al.* 2006). Adipocytes floating in the tube were packed by centrifuging at 200 g for 3 min, resuspended in fDMEM, and incubated at 37 °C for 1 h prior to treatments (He *et al.* 2006). Next, adipocytes were incubated in the absence or presence of TNF- α and/or metformin at the concentration indicated in the text or figure captions, under a glucose concentration of 5 or 25 mM.

Lipolysis assay

Packed adipocytes (20 μ l) were suspended in 500 μ l fDMEM and incubated in an atmosphere of 5% CO₂ at 37 °C. After the treatments, the media were collected and heated at 70 °C for 10 min to inactivate residual lipase activity in the culture, according to our prior method (He *et al.* 2006). Glycerol released in the media was determined by the use of a colorimetric assay (Trinder reaction) from the absorption at 490 nm and served as an index of lipolysis (McGowan *et al.* 1983). Lipolysis data were expressed as micromolecules of glycerol released per milliliter packed cell volume (PCV) of adipocytes (He *et al.* 2006).

Immunoblotting

After being packed and washed, the adipocytes were lysed and the protein content was determined by the Bradford protein assay. The extracts were adjusted equivalently by mixing with a concentrated SDS-sample buffer (50 mM Tris–Cl, pH 6.8, 5% SDS, 1% β -mercaptoethanol, 0.01% bromophenol blue, and 10% glycerol, in final), then separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked for 1 h with 5% non-fat milk in TBS-T buffer (150 mM NaCl, 20 mM Tris–Cl, pH 7.4, 0.05% Tween-20) and incubated for 1 h with selected primary antibodies, according to our prior method (Xu *et al.* 2001, 2005, 2006). The blots were washed and probed for 1 h with secondary peroxidase-conjugated antibodies, then developed with the use of an enhanced chemiluminescence detection kit (Applygen Technologies). Next, the antibodies bound to membranes were removed in a commercial Stripping Solution (Applygen Technologies). Blots were then

reprobed with the other indicated antibodies and developed as described above.

Statistical analysis

Data are expressed as means $\pm$ S.E.M. One-way ANOVA Tukey's test involved the use of GraphPad Prism version 4.0 (San Diego, CA, USA). *P* values <0.05 were considered statistically significant.

Results

Metformin blocks lipolysis stimulated by TNF- α

Triacylglycerol hydrolysis proportionally releases glycerol and FFA from adipocytes. We assayed glycerol release in the culture medium as an index of lipolysis. Primary rat adipocytes were pre-incubated with metformin for 1 h, then treated with TNF- α , metformin, or both for 23 h. The addition of TNF- α at 25 or 50 ng/ml elevated glycerol release by 2.1- or 2.8-fold (Fig. 1A) respectively, beginning as early as 8 h after the addition and significantly increasing at 16 or 24 h incubation (Fig. 1B). Treatment with metformin alone at 250 or 500 μ M did not affect basal lipolytic rate but significantly blocked TNF- α -stimulated glycerol release. The antilipolytic action of metformin at 500 μ M was more effective than that at 250 μ M (Fig. 1A).

Metformin attenuates the phosphorylation of ERK1/2 in TNF- α -treated adipocytes

Activation of ERK signaling participates in TNF- α -induced lipolysis in differentiated primary rat

adipocytes (Ryden *et al.* 2002, Zhang *et al.* 2002, Souza *et al.* 2003). To investigate the mechanism by which metformin inhibits TNF- α -stimulated lipolysis, we performed immunoblot analysis to examine the phosphorylation of three mitogen-activated protein kinases (MAPKs), including ERK1/2, p38 (also termed stress-cytokine-activated kinase) and c-Jun-NH₂-terminal kinase (JNK, also termed stress-activated protein kinase, SPAK). Primary rat adipocytes were pre-incubated for 1 h with or without metformin and then treated for 30 min with 50 ng/ml TNF- α or 500 μ M metformin, or both. ERK1/2 and p38 were constitutively phosphorylated (Fig. 2A and C). Treatment with TNF- α further promoted the ERK1/2 phosphorylation by 1.5-fold without affecting the level of total ERK1; metformin did not alter the constitutive phosphorylation of ERK1/2 but largely reduced the TNF- α -mediated phosphorylation of ERK1/2 (Fig. 2A and B). Figure 2C shows that the phosphorylation of p38 in the same adipocytes was not activated by TNF- α , but unusually and modestly promoted by metformin. Under the same conditions, JNK phosphorylation was undetectable with or without TNF- α treatment (data not shown). These results suggest that metformin exerts its antilipolytic action mainly through blocking TNF- α -activated phosphorylation of ERK.

Metformin reverses the downregulation of perilipin caused by TNF- α

Perilipin is a substrate-associating protein and an active participant in lipolytic modulation (He *et al.* 2006). Downregulated perilipin was thought to

Figure 1 Metformin inhibits TNF- α -stimulated lipolysis in adipose cells. Isolated adipocytes were preincubated for 1 h (A) with metformin and then treated for 23 h or (B) the indicated time after the addition of TNF- α , metformin (Met), or both. Glycerol release in the culture media was determined as an index of lipolysis and expressed as micromoles glycerol per milliliter packed cell volume (PCV) of adipocytes. Data are the means $\pm$ S.E.M. of three separate experiments performed in triplicate. **P* <0.01 vs control; †*P* <0.05 vs 25 ng/ml TNF- α alone; ‡*P* <0.01 vs 50 ng/ml TNF- α alone.

Figure 2 Metformin inhibits TNF- α -stimulated phosphorylation of ERK1/2. Adipocytes were preincubated for 1 h with 500 μ M metformin (Met), then treated for 30 min with 50 ng/ml TNF- α , 500 μ M metformin, or both. (A) Cells were lysed and the equivalent amounts of proteins underwent immunoblotting with the use of primary antibodies against phosphorylated-ERK1/2 (p-ERK). To detect total ERK-1, the membranes were stripped and reprobed with anti-ERK-1 antibodies (ERK). The blots shown are representative of three separate experiments. (B) The densitometric measurement of phosphorylated-ERK1/2 bands. The data (means $\pm$ S.E.M.) of three separate experiments are presented as percentage of the control; * $P < 0.05$ vs TNF- α alone. (C) Immunoblotting of phosphorylated p38 (p-p38). Note that the phosphorylated JNK was undetectable (data not shown).

account for the increased lipolytic action of TNF- α in differentiated adipocytes (Souza *et al.* 1998a,b, Ryden *et al.* 2004). We investigated the effect of metformin on the regulation of perilipin during TNF- α -stimulated lipolysis in primary adipocytes. Immunoblotting revealed that the protein level of perilipin was decreased in adipocytes treated with either 25 or 50 ng/ml TNF- α , but metformin prevented the decrease in perilipin level in TNF- α -treated adipocytes (Fig. 3A and B).

Metformin restricts the chronic lipolysis stimulated by TNF- α in normal- and high-glucose environments

Metformin has therapeutic effects in hyperglycemic conditions. Glucose at high concentrations increases basal lipolysis (Moussalli *et al.* 1986, Szkudelski & Szkudelska 2000) and enhances lipolysis response to an adrenergic agonist (Moussalli *et al.* 1986, Szkudelski & Szkudelska 2000) or TNF- α (Green *et al.* 2004), an action that may contribute to the elevation in circulating FFA level in NIDDM (Abbasi *et al.* 1998).

We examined the antilipolytic actions of metformin under a normal- or high-glucose environment. Adipocytes were pre-treated for 1 h with metformin and then incubated for 23 h in the media supplemented with 5 (normal) or 25 mM (high) glucose, in the presence of 50 ng/ml TNF- α or 500 μ M metformin, or both. After the treatments, we immediately determined the 24 h glycerol accumulation in the culture media, and then measured a 1 h glycerol release after washing and incubating the cells for another 1 h with normal glucose. Glycerol accumulation after a 24 h incubation under high-glucose conditions (Fig. 4A) or a subsequent 1 h release (Fig. 4B) was elevated by 1.5- or 1.6-fold respectively, as compared with that under normal-glucose conditions. Thus, treatment with high glucose at 25 mM not only increased basal lipolysis but also significantly promoted TNF- α -stimulated lipolysis. However, pre-treatment with metformin largely suppressed the lipolytic reaction stimulated by 50 ng/ml TNF- α or 25 mM glucose alone and attenuated the TNF- α -mediated lipolysis enhanced by 25 mM glucose (Fig. 4A and B).

Figure 3 Metformin reduces TNF- α -mediated downregulation of perilipin. Adipocytes were preincubated for 1 h with 500 μ M metformin (Met), then treated for 23 h in the presence of TNF- α , metformin, or both. (A) The cells were lysed and the equivalent amounts of proteins separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were immunoblotted with anti-perilipin antibodies and peroxidase-conjugated secondary antibodies. The blot shown is a representative of three separate experiments. (B) Densitometric measurement of perilipin bands. The data (means $\pm$ S.E.M.) of three separate experiments are presented as percentage of the control; * $P < 0.05$ vs TNF- α alone.

Metformin restricts the acute lipolysis stimulated by isoproterenol under normal- and high-glucose environments

Finally, we examined the effect of metformin on acute lipolytic stimulation of adrenergic isoproterenol. Adipocytes were pre-incubated for 24 h with 500 μ M metformin in the presence of 5 or 25 mM glucose in the culture media. The cells were then washed and resuspended in the freshly changed media and stimulated for 30 min with 1 μ M isoproterenol. The glycerol released in the culture medium was determined as an index of lipolysis. As shown in Fig. 5, treatment with isoproterenol greatly stimulated lipolysis. The incubation of adipocytes with high glucose, 25 mM, promoted the basal level of glycerol release and further enhanced the lipolysis response upon adrenergic stimulation by isoproterenol, as compared with the culture with 5 mM glucose. Pre-treatment with metformin

significantly attenuated glycerol release from isoproterenol-stimulated adipocytes in the presence of 5 or 25 mM glucose (Fig. 5). Thus, metformin not only inhibits the lipolysis stimulated by isoproterenol or high glucose alone but also restricts the adrenergic lipolytic stimulation enhanced by excess glucose.

Discussion

Various biological activities of the biguanide, metformin, may explain in part its antihyperglycemic effect. Metformin does not directly affect insulin secretion in the pancreas. Instead, it acts on the peripheral tissues to increase insulin sensitivity (Bailey 1992). Many clinical studies have suggested that the improved insulin sensitivity with metformin is associated with decreased circulating FFA levels in patients with NIDDM (Groop *et al.* 1989, Riccio *et al.* 1991, Perriello *et al.* 1994, Abbasi *et al.* 1998). However, the cellular mechanism by which metformin restricts plasma FFA is completely unknown. In this study, we demonstrate that metformin directly inhibits the lipolysis of triacylglycerols in primary rat adipocytes stimulated with TNF- α , isoproterenol, and/or high concentrations of glucose. These data suggest a novel mechanism by which metformin reduces serum FFA and thus increases insulin sensitivity.

Triacylglycerol lipolysis is a key process that proportionally releases FFA and glycerol from adipose tissue to the blood stream. The catecholeamines are the most important hormones that physiologically stimulate lipolysis in mammalian fat cells. In contrast, TNF- α , an adipocytokine, is known to stimulate chronic lipolysis in primary (Green *et al.* 1994) or differentiated adipocytes (Souza *et al.* 1998a,b, Ryden *et al.* 2002, Zhang *et al.* 2002, Green *et al.* 2004). The production of TNF- α in adipose tissue is greatly increased in obesity. This increase may be associated with increased plasma FFA levels and impaired insulin sensitivity in obese and NIDDM patients (Hotamisligil *et al.* 1993, 1995). We show that TNF- α at 25 or 50 ng/ml stimulated chronic and extensive lipolysis in primary adipocytes. This result is consistent with previous observations in isolated fat cells (Green *et al.* 1994). Treatment of adipocytes with metformin did not affect basal lipolysis but rather blocked the lipolytic action mediated by TNF- α . This observation provides the first evidence that metformin directly inhibits lipolysis in TNF- α -stimulated adipose cells.

In 3T3-L1 adipocytes (Souza *et al.* 2003) or differentiated human adipocytes (Ryden *et al.* 2002, Zhang *et al.* 2002), the activation of ERK1/2 appears to be a major pathway for the regulation of TNF- α -stimulated lipolysis (Ryden *et al.* 2002, Zhang *et al.* 2002, Souza *et al.* 2003), whereas the phosphorylation of JNK but not p38 may also participate in this process (Ryden *et al.* 2002).

Figure 4 Metformin restricts lipolytic stimulation of TNF- α enhanced by high-concentration glucose. Adipocytes were pretreated for 1 h with metformin and cultured for 23 h in the media supplemented with 5 or 25 mM glucose, in the presence of 50 ng/ml TNF- α or 500 μ M metformin (Met). (A) Glycerol accumulation in the culture media was determined after 24 h treatment. (B) The cells were then washed and incubated for 1 h in freshly changed media containing 5 mM or 25 mM glucose and glycerol release was assayed. Data are the means $\pm$ S.E.M. of three separate experiments performed in triplicate. * $P < 0.01$, vs the incubations with 5 mM glucose; $^{\dagger}P < 0.01$ vs TNF- α plus 25 mM glucose.

In primary rat adipocytes, we observed that not only did TNF- α -stimulated lipolysis, it further stimulated the phosphorylation of ERK1/2 but not p38 and JNK; metformin did not suppress basal ERK phosphorylation but greatly inhibited the TNF- α -stimulated activation of ERK1/2. Therefore, the inhibitory effect of metformin in TNF- α -induced ERK1/2 activation may account for its antilipolytic action.

MAPK signaling data obtained from primary rat adipocytes in the present study are apparently different from data obtained from differentiated human or 3T3-L1 adipocytes (Jain *et al.* 1999, Ryden *et al.* 2002). In primary rat adipocytes, ERK1/2 and p38 but not JNK were constitutively activated, and only ERK1/2 was sufficiently phosphorylated by TNF- α . By contrast, in 3T3-L1 adipocytes, all three MAPKs were constitutively phosphorylated, but no further induction could be observed in response to TNF- α stimulation (Jain *et al.* 1999, Ryden *et al.* 2002). Moreover, different degrees of the constitutive and TNF- α -induced phosphorylation of ERK1/2 were observed by two groups of investigators, both using the differentiated human adipocytes (Ryden *et al.* 2002, Zhang *et al.* 2002). These discrepancies are unclear. However, as discussed earlier by Ryden *et al.* (2002), the differences in cellular contexts and/or experimental conditions such as the preadipocyte differentiation cocktail (insulin, dexamethasone, isobutylmethylxanthine, rosiglitazone, and triiodothyronine), may partly explain the phenomenon.

Perilipin coats the surface of intracellular lipid droplets in adipocytes. Perilipin has a firmly established function to modulate lipolysis (He *et al.* 2006) by

limiting the lipase access to the triacylglycerol core stored within the lipid droplets (Londos *et al.* 1999, Sztalryd *et al.* 2003). The lipolytic action of TNF- α is mediated by downregulation of the perilipin proteins in differentiated 3T3-L1 adipocytes, an action that can be prevented by adenoviral overexpression of perilipin

Figure 5 Inhibitory effect of metformin on isoproterenol-stimulated lipolysis. Adipocytes were preincubated for 24 h with 500 μ M metformin (Met) in the presence of 5 or 25 mM glucose. The cells were washed, suspended in freshly changed media containing 5 mM or 25 mM glucose and stimulated for 30 min with 1 μ M isoproterenol (Iso). Glycerol release in the culture media was assayed. Data are the means $\pm$ S.E.M. of three separate experiments performed in triplicate.

(Souza *et al.* 1998b) or by BRL 49653 (Souza *et al.* 1998a). BRL 49653 is a thiazolidinedione, an antidiabetic agent and also a peroxisome proliferator-activated receptor (PPAR) γ agonist which can transcriptionally upregulate perilipin (Arimura *et al.* 2004). The present study confirms that TNF- α has a similar effect in downregulating perilipin in primary rat adipocytes. Decreased perilipin which is downregulated by TNF- α may impair the barrier function of the protein and thus lead to increased lipolysis (Londos *et al.* 1999). Our data suggest that metformin protects the loss of perilipins in TNF- α -stimulated adipocytes. Thus, metformin not only attenuates the ERK1/2 activation but also preserves the barrier function of the perilipins. These two effects could predominantly account for the molecular basis of the antilipolytic action of metformin in TNF- α -stimulated adipocytes.

In contrast to TNF- α , which causes chronic lipolysis, catecholamines are a class of physiological hormones that stimulate acute lipolysis. An *in situ* microdialysis study indicated that pre-perfusion of metformin inhibited the release of glycerol *in vivo* in abdominal adipose tissue of obese and hyperinsulinemic subjects after administration of catecholamine (Flechtner-Mors *et al.* 1999). However, this study did not investigate the cellular basis by which metformin lowered glycerol release from adrenaline-stimulated adipose tissue. The present study reveals that metformin directly reduces the lipolysis in adipocytes upon isoproterenol stimulation. Although the detailed pathway for this effect remains to be identified, this antilipolytic action might serve as a primary mechanism for metformin to reduce the flux of FFA from adipose tissue into plasma in response to adrenergic stimulations.

The present and prior studies have demonstrated that a high concentration of glucose supplied in the adipocyte culture elevates basal lipolysis and enhances lipolysis stimulated by TNF- α (Green *et al.* 2004) or adrenergic agonists (Moussalli *et al.* 1986, Szkudelski & Szkudelska 2000). Therefore, excess glucose could further promote the FFA flux from adipocytes to the blood stream, thus increasing the circulating FFA and impairing insulin sensitivity. We further show that metformin inhibits the basal lipolysis simulated by high glucose alone, and also strongly restricts the high glucose-enhanced lipolytic response to either TNF- α or isoproterenol. The therapeutic concentrations of metformin fall between ~ 10 and ~ 2000 $\mu\text{mol/l}$ or even higher in plasma or tissues in human or diabetic animals (Pentikainen 1986, Wilcock & Bailey 1994, Wang *et al.* 2003). The concentration of metformin we used for adipocytes is between 250 and 500 $\mu\text{mol/l}$. At such concentrations, metformin directly inhibits adipocyte lipolysis response to various lipolytic stimulations such as TNF- α , isoproterenol, and/or a high

concentration of glucose. By contrast, a low concentration (2–4 mg/l, equivalent to 12–24 $\mu\text{mol/l}$) of metformin can enhance insulin-stimulated glucose conversion into both triglycerides and CO_2 , but neither inhibits norepinephrine-stimulated lipolysis nor enhances the antilipolytic effect of insulin in isolated human abdominal s.c. adipose tissue (Cigolini *et al.* 1984). Thus, if metformin is administered at relatively high concentrations, its antilipolytic effects could be particularly beneficial in reducing systemic FFA concentrations via restricting FFA efflux from adipose tissue and hence improving insulin sensitivity in obese and NIDDM patients. Metformin increases the activity of AMP-activated protein kinase (AMPK) in rat hepatocytes (Zhou *et al.* 2001) and skeletal muscles of NIDDM patients (Zhou *et al.* 2001), and is associated with low hepatic glucose production and high muscle glucose uptake. This effect is proposed as one of the major mechanisms of metformin action (Zhou *et al.* 2001). The present results suggest that the clinical effects of metformin may be attributed not only to the effects on the muscle and liver but also to the reduced lipolysis in adipose tissue.

However, our study is unable to elucidate the detailed molecular basis by which metformin inhibits lipolysis. This weakness is, in part, due to the fact that the common mechanism of metformin, in spite of its antihyperglycemic effect, remains a mystery. However, lipolytic cascades in response to TNF- α , catecholamine, and/or excess glucose are not well understood. For example, the lipolytic action of TNF- α may be mediated in part by G_i protein downregulation (Gasic *et al.* 1999) or cellular cAMP elevation in differentiated 3T3-L1 adipocytes (Zhang *et al.* 2002). Nevertheless, our preliminary experiments suggest that TNF- α neither downregulates G_i protein nor elevates cellular cAMP level in primary rat adipocytes (T Ren and G Xu, unpublished data). Thus, the effects of metformin on these lipolytic factors were inaccessible. Apparently, the molecular pathway by which metformin inhibits adipocyte lipolysis remains to be further clarified.

The antilipolytic effect is not limited to the biguanide, metformin but is also seen with other types of antidiabetic drugs. For example, insulin is a major antilipolytic hormone (Londos *et al.* 1999); thiazolidinediones block TNF- α -mediated lipolysis (Souza *et al.* 1998a, Mayerson *et al.* 2002) and sulfonylureas inhibit catecholamine-induced lipolysis (Muller *et al.* 1994, Shi *et al.* 1999). Thus, an antilipolytic action that decreases FFA flux from adipose cells seems to be a common mechanism for those antidiabetic drugs by which reduce systemic FFA content and exert their antihyperglycemic effects. Further studies are necessary to clarify this relationship.

Acknowledgements

This work was supported by grants 30270506, 30370535, and 30540035 from the National Natural Science Foundation of China (to G X), grant 5042015 from the Natural Science Foundation of Beijing Province of China (to G X), and grant NECT-04-0023 from the Program for New Century Excellent Talents in the University of the Education Ministry of China. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 1 May 2006

Accepted 30 May 2006

Made available online as an Accepted Preprint 7 June 2006