

Mechanisms of metformin inhibiting lipolytic response to isoproterenol in primary rat adipocytes

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Abstract

The mobilization of free fatty acids (FFA) from adipose tissue to the bloodstream primarily depends on triacylglycerol lipolysis in adipocytes. Catecholamines are major hormones that govern lipolysis through elevating cellular cAMP production and activating protein kinase, cAMP dependent, catalytic, alpha (PKA) and mitogen-activated protein kinase 1/2 (MAPK1/3). Obesity and type 2 diabetes are associated with elevated levels of systemic FFA, which restricts glucose utilization and induces insulin resistance. The biguanide metformin exerts its antihyperglycemic effect by enhancing insulin sensitivity, which is associated with decreased levels of circulating FFA. In this study, we examined the characteristics and basis of the inhibitory effect of metformin on adrenergic-stimulated lipolysis in primary rat adipocytes. We measured the release of FFA and glycerol as an index of lipolysis and examined the major signalings of the lipolytic cascade in primary rat adipocytes. Metformin at 250–500 μ M efficiently attenuated FFA and glycerol release from the adipocytes stimulated with 1 μ M isoproterenol. To elucidate the basis for this antilipolytic action, we showed that metformin decreased cellular cAMP production, reduced the activities of PKA and MAPK1/3, and attenuated the phosphorylation of perilipin during isoproterenol-stimulated lipolysis. Further, metformin suppressed isoproterenol-promoted lipase activity but did not affect the translocation of lipase, hormone-sensitive from the cytosol to lipid droplets in adipocytes. This study provides evidence that metformin acts on adipocytes to suppress the lipolysis response to catecholamine. This antilipolytic effect could be a cellular basis for metformin decreasing plasma FFA levels and improving insulin sensitivity.

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Introduction

Obesity and type 2 diabetes (diabetes mellitus) are associated with elevated levels of plasma free fatty acids (FFA), which restrict glucose utilization and induce insulin resistance (Groop *et al.* 1989, Bergman & Ader 2000). The concentration of serum FFA primarily depends on the lipolysis of adipose triacylglycerols that releases FFA and glycerol from adipocytes. Catecholamines are the most important hormones that govern lipolysis through elevating cellular cAMP production and activating cAMP-dependent protein kinase, cAMP dependent, catalytic, alpha (PKA; Londos *et al.* 1999). PKA then phosphorylates two proteins, hormone-sensitive lipase (HSL) and perilipin, in adipocytes and causes translocation of HSL from the cytosol to the lipid droplet surface, a crucial step for the lipase access to its triacylglycerol substrates (Sztalryd *et al.* 2003). Perilipin is a structural protein that has a dual role in the control of lipolysis: native perilipins on the lipid droplet surface function as a barrier to prevent

HSL access to the droplet, thus suppressing lipolysis (Sztalryd *et al.* 2003, He *et al.* 2006), whereas phosphorylated perilipins allow the translocation of HSL to the lipid droplet for conferring a full lipolytic reaction (Sztalryd *et al.* 2003). Besides PKA, mitogen-activated protein kinase 1/3 (MAPK1/3; previous aliases: ERK1/2 MAPK) is another important signaling molecule that modulates catecholamine-stimulated lipolysis (Greenberg *et al.* 2001, Robidoux *et al.* 2006).

Biguanide metformin is a popular antihyperglycemic drug used to treat diabetes mellitus (Bailey 1992). Unlike sulfonyleureas, which stimulate insulin secretion, metformin lowers blood sugar levels by improving insulin sensitivity in peripheral tissues. Metformin increases glucose uptake and disposal in muscle, decreases hepatic glucose production, and also helps combat dyslipidemia (Bailey 1992). Clinical studies suggest that promotion of insulin sensitivity by metformin is associated with an overall reduction in plasma FFA levels in obese and diabetic patients (Riccio *et al.* 1991, Perriello *et al.* 1994, Abbasi *et al.* 1998,

Hundal *et al.* 2000). The reduced FFA level is associated with increased glucose disposal. Therefore, the FFA-lowering effect could be essential to mediate the enhanced insulin action of metformin.

The FFA efflux to the bloodstream is governed by the process of adipose lipolysis. To date, information on the direct effect of metformin on adipose lipolysis is scarce and even inconsistent because it was derived from different methodologies. An *in vivo* study suggested that oral administration of metformin does not affect lipolysis in s.c. adipose tissue of diabetic subjects (Jansson *et al.* 1996), whereas another *in vivo* microdialysis study confirmed that direct perfusion of high-concentration (1 mM) metformin into abdominal adipose tissue restricted basal and adrenergic-stimulated glycerol release in obese and diabetic patients (Flechner-Mors *et al.* 1999). *In vitro* studies indicated that metformin at low concentrations (<25 μ M) did not seem to affect the lipolysis response in isolated adipose tissue (Cigolini *et al.* 1984), whereas high-dose metformin (>250 μ M) reduced adrenergic-induced lipolysis in primary rat adipocytes (Ren *et al.* 2006) or in C3H10T1/2 preadipocytes (Lenhard *et al.* 1997). Our previous study suggested that metformin inhibits the lipolysis response to tumor necrosis factor (TNF) or isoproterenol (Ren *et al.* 2006). However, the characteristics and basis of the inhibitory effect of metformin on catecholamine-induced lipolysis has not been clarified.

The aim of this study was to investigate the mechanism by which metformin inhibits catecholamine-stimulated lipolysis in adipocytes. We measured the release of FFA and glycerol as an index of lipolysis and examined the major lipolytic signalings in primary adipocytes. Metformin decreased cellular cAMP production, reduced the activities of PKA and MAPK1, and attenuated the phosphorylation of perilipin during isoproterenol-stimulated lipolysis. Further, metformin suppressed isoproterenol-promoted lipase activity without altering the translocation reaction of HSL. This study provides direct evidence that metformin acts on adipocytes to suppress the lipolysis response to catecholamine. This antilipolytic effect could be a cellular basis for metformin decreasing plasma FFA levels and improving insulin sensitivity.

Materials and methods

Materials

Isoproterenol and phenol red-free DMEM (fDMEM) containing glucose (5 mmol/l) were from Sigma Chemical. Metformin was from Bristol-Myers Squibb Pharmaceuticals (Shanghai, China). Enzyme materials used for enzymatic assays were from Toyobo Co. (Tokyo, Japan). Antibodies against MAPK3 and

phospho MAPK1/3 and HRP (HRP)-conjugated IgG were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against rat perilipin and HSL were generous gifts from Dr Londos at the US National Institutes of Health (Bethesda, MD, USA). Nitrocellulose membrane, a prestained protein molecular weight marker, and ultrasensitive enhanced chemiluminescence (ECL) detection reagents were from Applygen Technologies (Beijing, China). The use of animals was approved by the Animal Care and Use Committee of Peking University Health Science Center.

Isolation and culture of primary rat adipocytes

Adipocytes were isolated from epididymal fat pads of Sprague-Dawley rats (150–180 g) according to our laboratory method (He *et al.* 2006, Jiang *et al.* 2007). The minced fat pads were digested in Krebs-Ringer solution containing 0.75 mg/ml type 1 collagenase, 200 nM adenosine, 25 mM Hepes (pH 7.4), and 1% defatted BSA. After incubation for 40 min at 37 °C in a water bath with shaking at 100 cycles/min, cells were filtered through a nylon mesh and washed three times with warmed DMEM containing 200 nM adenosine. Adipocytes floating on the top of the tube were packed by centrifuging at 200 g for 3 min. The packed cell volume (PCV) of the final suspension was determined (He *et al.* 2006, Jiang *et al.* 2007). Every 25 μ l packed adipocytes were resuspended in 500 μ l serum-free fDMEM containing 2% defatted BSA and preincubated at 37 °C for 1 h prior to treatments (He *et al.* 2006). Adipocytes were incubated in the presence or absence of the tested agents, and then assayed as described below.

Fatty acid assay

The concentration of FFA in the culture medium was determined by colorimetric assay as described (Zu *et al.* 2008). Briefly, 50 μ l culture medium were mixed with 120 μ l isooctane and 80 μ l cupric acetate-pyridine, then vortexed and centrifuged for 10 min at 12 000 g at room temperature. The upper organic phase (80 μ l) was transferred to a clean tube. An amount of 180 μ l of the color development reagent consisting of diphenylcarbazone and diphenylcarbazide in methanol was then added to the tube. The mixture was vortexed, and the reaction was developed immediately for color. The absorbance at 540 nm was spectrophotometrically measured in a 96-well plate.

Glycerol assay

Glycerol content in the culture medium of adipocytes served as an index of lipolysis and was determined at the

absorption of 490 nm (He *et al.* 2006, Zu *et al.* 2008) by use of a colorimetric assay (Trinder reaction) kit from Applygen Technologies. Lipolysis data were expressed as micromoles of glycerol or FFA per milliliter of PCV of adipocytes.

Western blot analysis

Adipocytes were packed and lysed in sample buffer containing 62 mM Tris-HCl (pH 6.8), 5% SDS, 0.1 mM sodium orthovanadate, and 50 mM sodium fluoride. After centrifugation at 12 000 *g* for 10 min at 4 °C, the lysate was collected, and the protein content was determined by use of a bicinchoninic acid protein assay kit (Applygen Technologies). Then, the lysate was included in the sample buffer (62 mM Tris-HCl (pH 6.8), 5% SDS, 1.7% β -mercaptoethanol, 0.04% bromophenol blue, and 10% glycerol, in final; He *et al.* 2006, Jiang *et al.* 2007). After a denaturing at 95 °C for 5 min, equal amounts of proteins were loaded and separated by 10% SDS-PAGE. For immunoblotting of phosphorylated perilipin, a low-bis concentration polyacrylamide gel (10% acrylamide and 0.07% N,N'-methylene-bis-acrylamide) was used, which provides better resolution of proteins in the 60–70 kDa range (He *et al.* 2006). After electrophoresis, the proteins were transferred to nitrocellulose membranes. The membranes were blocked for 1 h in 5% non-fat milk in TBS-T buffer (150 mM NaCl, 20 mM Tris-HCl (pH 7.4), and 0.05% Tween-20), incubated with primary antibodies for 1 h at room temperature or overnight at 4 °C, then incubated for 1 h with HRP-conjugated secondary antibodies (Xu *et al.* 2005, 2006). The blots were developed by use of an ECL detection kit. If required, antibodies bound to membranes were removed by a commercial Stripping Solution from Applygen Technologies, and the blots were then reprobed. Densitometric analysis of protein bands involved use of NIHImage software.

cAMP RIA

As we previously described (Zu *et al.* 2008), adipocytes were lysed in 150 μ l ice-cold buffer containing 50 mM Tris-HCl (pH 7.4), and 1 mM EDTA. After vortexing and centrifugation at 12 000 *g* for 15 min at 4 °C, the cytosolic fraction was collected and the protein content was determined. Then, 90 μ l cytosol fraction were mixed with 30 μ l 40% trichloroacetic acid. The tubes were vortexed and centrifuged at 12 000 *g* for 5 min at 4 °C. The supernatant was collected and used for cAMP assay according to the protocol of a commercial ¹²⁵I RIA kit (Isotope Laboratory of Shanghai University of Chinese Medicine, Shanghai, China). cAMP concentration was normalized and expressed as picomoles per milligram of cytosolic proteins.

Assay of PKA activity ratios

An amount of 50 μ l packed adipocytes was added in 150 μ l ice-cold homogenization buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 1 mM 3-isobutyl-1-methylxanthine. The cells were homogenized by ten strokes with a ground glass pestle in a 1 ml ground glass homogenizing tube. The homogenate was transferred immediately to a precooled 1.5 ml tube and centrifuged at 10 000 *g* for 15 min at 4 °C to prepare the cytosolic fraction. Corrected PKA activity ratio, which more faithfully reflects the true activity of cAMP-dependent PKA in response to lipolytic or antilipolytic stimulators in adipocytes (Honnor *et al.* 1985a,b), was determined and calculated as described (Honnor *et al.* 1985a) with minor modifications. Briefly, 20 μ l of the cytosolic extract were added to a tube at 4 °C, and the assay medium was added for a final volume of 50 μ l. PKA activity in each cytosol extract was assayed six times under three separate conditions: a) in a quiescent reaction consisting of 50 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 1 mM 3-isobutyl-1-methylxanthine, 20 μ Ci/ml γ -³²P-ATP, 0.3 mM ATP, and 100 μ M Kempptide (a specific PKA substrate); b) a stimulatory condition consisting of reaction (a) in the presence of 2 μ M cAMP to activate PKA; and c) an inhibitory condition consisting of reaction (a) in the presence of 10 μ M PKI inhibitor to suppress PKA. The reactions were incubated for 10 min at 30 °C, and then terminated by cooling the tubes in a 0–4 °C ice bath. Then, 40 μ l of reaction mixture were collected and spread on a P81 phosphocellulose filter (diameter 2.5 cm). The filter was washed for 4 \times 5 min with ice-cold 75 mM phosphoric acid, then underwent 2 \times 5 min washes with 100% ethanol. The filter paper was air dried and placed into 3 ml liquid scintillation fluid for counting the ³²P radioactivity. Results are expressed as corrected PKA activity ratio (a-c)/(b-c; Honnor *et al.* 1985a).

Assay of adipose lipase activity

An amount of 50 μ l packed adipocytes was lysed in 120 μ l buffer containing 50 mM Tris-HCl (pH 7.4), and 1 mM EDTA. The lysate was vortexed and centrifuged at 12 000 *g* for 15 min at 4 °C. The infranant phase below the fat cake fraction was transferred to a new tube and then centrifuged at 12 000 *g* for 5 min at 4 °C. The supernatant was used for the determination of cellular lipase activity against emulsified triolein substrate. The mixture was incubated for 30 min at 37 °C, when the lipases hydrolyze emulsified triolein to produce glycerol. The release of glycerol from triolein hydrolysis represented the activity of adipose lipase and was assayed as described previously (Zu *et al.* 2008).

Differentiation of rat preadipocytes and immunostaining of HSL

Adipose precursor cells (vascular stromal cell) were isolated from epididymal fat pads and differentiated into adipocytes for 3 days in serum-free DMEM:F12 (1:1) medium supplemented with 5 µg/ml insulin, 33 µM biotin, and 200 pM triiodothyronine, as we described previously (He *et al.* 2006, Jiang *et al.* 2007). The differentiated adipocytes (day 5) were treated with metformin, isoproterenol, or both. Immunostaining was performed according to prior methods. The cells were fixed for 20 min with 4% paraformaldehyde and 0.01% Triton X-100 in PBS buffer at room temperature, then rinsed three times (Xu *et al.* 2005, He *et al.* 2006) for 5 min each with PBS. Nonspecific binding sites in the cells were blocked with 2% donkey serum for 60 min and washed three times for 5 min each. The cells were incubated with rabbit polyclonal serum against HSL at 1:500 overnight at 4 °C and then with FITC-conjugated donkey anti-rabbit IgG at 1:300 for 2 h in the darkness at room temperature. Immunofluorescent signaling of HSL was observed with use of a Nikon Eclipse TE2000-U microscope.

Statistical analysis

Data are expressed as means ± s.e.m. One-way ANOVA Tukey's test was used for statistical analysis. A $P < 0.05$ was considered statistically significant.

Results

Metformin inhibited isoproterenol-stimulated lipolysis in primary adipocytes

Hydrolysis of triacylglycerol by cellular lipases results in the release of FFA and glycerol from fat cells. To determine the antilipolytic effect of metformin on isoproterenol-stimulated lipolysis, we measured the levels of FFA and glycerol, an index of lipolysis, released in the incubation medium. Primary rat adipocytes were incubated for the indicated periods in the presence or absence of 250 or 500 µM metformin, then the media were replaced with warm fDMEM before 1 µM isoproterenol was added for stimulation for 30 min for glycerol assay (Fig. 1B and D) or 60 min for FFA measurement (Fig. 1A and C). Preincubation with metformin at 500 but not 250 µM readily inhibited the basal FFA efflux (Fig. 1A and C) but not the basal glycerol release from unstimulated adipocytes (Fig. 1B). However, metformin at both 250 and 500 µM suppressed isoproterenol-stimulated release of FFA (Fig. 1A) and glycerol (Fig. 1B) from adipocytes. Metformin at 100 µM or less had no significant effect on the basal or isoproterenol-stimulated lipolysis (data not shown). Next, preincubation with 500 µM metformin for 4 h readily attenuated isoproterenol-mediated release of FFA (Fig. 1C) and glycerol (Fig. 1D). At 8 or

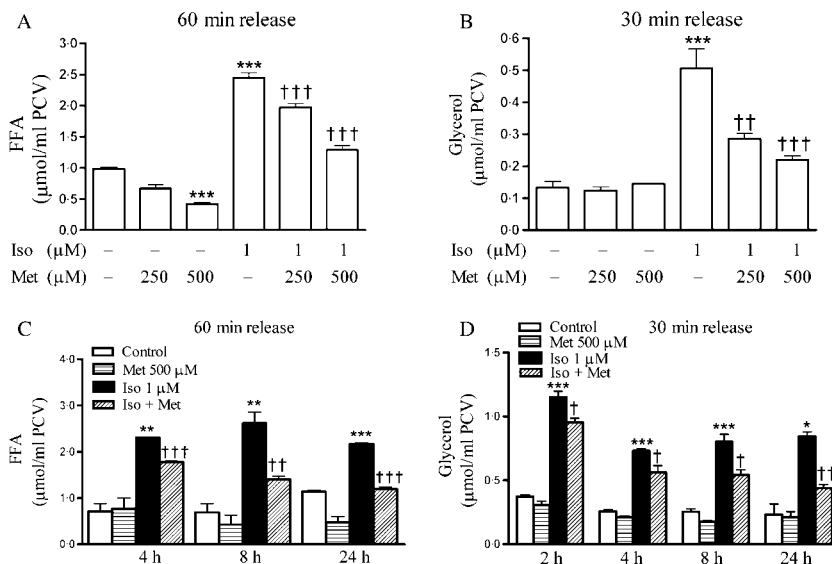


Figure 1 Metformin inhibits the release of FFA and glycerol from primary adipocytes stimulated by isoproterenol. Primary rat adipocytes were preincubated for 6 h (A and B) or for the indicated times (C and D) in the presence or absence of 250 or 500 µM metformin (Met), then washed and stimulated with 1 µM isoproterenol (Iso) under fresh medium. The 60 min release of FFA (A and C) and 30 min release of glycerol (B and D) was measured. The data are expressed as micromoles of FFA or glycerol per milliliter packed cell volume (PCV) of adipocytes. Results are means ± s.e.m. of three separate experiments performed in triplicate. *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$ versus control; †, $P < 0.05$, ††, $P < 0.01$ and †††, $P < 0.001$ versus Iso alone.

24 h of pretreatment, metformin blunted basal or isoproterenol-induced lipolysis (Fig. 1C and D).

Metformin suppressed elevated cAMP level mediated by isoproterenol

Isoproterenol is well known to stimulate lipolysis primarily through postreceptor activation of adenylyl cyclase and elevation of intracellular cAMP level in adipocytes (Londos *et al.* 1999). To elucidate the mechanism whereby metformin inhibits isoproterenol-stimulated lipolysis, we performed a cAMP immunoradioassay to measure the cAMP levels in primary rat adipocytes. Adipocytes were preincubated for 16 h with or without 500 μ M metformin, then washed and stimulated for 15 min with 100 nM isoproterenol in the freshly changed medium. Pretreatment with metformin alone slightly decreased the cAMP content by 27% in adipocytes (Fig. 2A); a short stimulation (15 min) with 100 nM isoproterenol significantly elevated intracellular cAMP level by ~48% ($P < 0.05$). However, isoproterenol failed to promote cAMP content in adipocytes that were pretreated with metformin ($P < 0.001$). The inhibitory effect of metformin on cAMP level was consistent with and parallel to its antilipolytic action in adipocytes by reducing the level of glycerol (Fig. 2B).

Metformin restricted the elevation of PKA activity on isoproterenol stimulation

Elevated intracellular cAMP level causes activation of PKA, which activates subsequent signalings in the lipolytic cascade, therefore conferring a full lipolytic response (Londos *et al.* 1999). The corrected PKA activity ratio more faithfully reflects the true activity of cAMP-dependent PKA upon lipolytic or antilipolytic stimulations (Honnor *et al.* 1985*a,b*). We examined the alteration in corrected PKA activity ratio in the cytosol fraction from adipocytes in response to isoproterenol, metformin, or both (Honnor *et al.* 1985*a*). Adipocytes were pretreated for 16 h with or without 500 μ M metformin, then washed and stimulated for 15 min by 1 μ M isoproterenol. Isoproterenol increased the PKA activity ratio by threefold ($P < 0.01$) in adipocytes. By contrast, pretreatment with metformin did not alter the basal PKA activity ratio but completely suppressed the isoproterenol-promoted PKA activity ratio in adipocytes ($P < 0.001$, Fig. 3A). In parallel to the inhibitory effect of metformin on activating PKA with isoproterenol stimulation, the level of glycerol released in the incubation medium was reduced, which indicated that metformin attenuated isoproterenol-stimulated lipolysis (Fig. 3B).

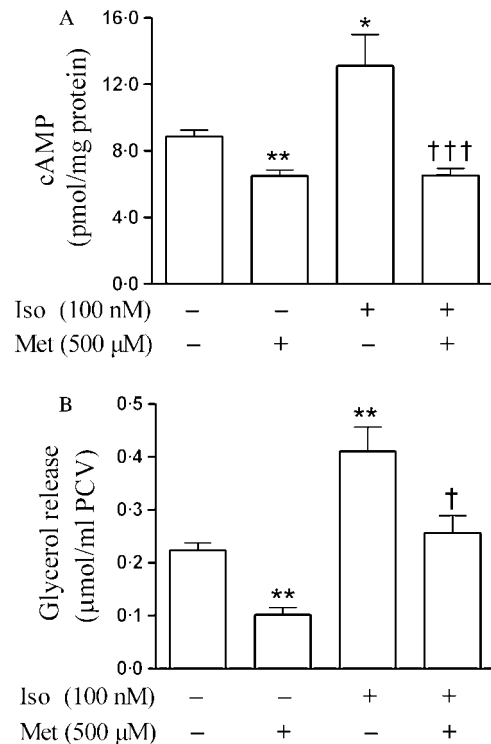


Figure 2 Effects of isoproterenol and metformin on intracellular cAMP levels and lipolysis. Primary rat adipocytes were preincubated for 16 h in the presence or absence of 500 μ M metformin (Met), then washed, and stimulated with 100 nM isoproterenol (Iso) under fresh medium for 15 min prior to the 125 I-RIA assay of cellular cAMP content (A) or for 30 min prior to the measurement of glycerol (B). Results (means \pm S.E.M.) are representative of three separate experiments performed six times (A) or three times (B). *, $P < 0.05$ and **, $P < 0.01$ versus control; †, $P < 0.05$ and †††, $P < 0.001$ versus Iso alone.

Besides the cAMP/PKA cascade, the activation of MAPK1/3 signaling is another important event during catecholamine-stimulated lipolysis (Greenberg *et al.* 2001, Robidoux *et al.* 2006). We performed immunoblotting analysis to examine whether metformin affected the phosphorylation of MAPK1/3 in primary adipocytes. Adipocytes were preincubated for 2 h with 500 μ M metformin and then stimulated for 5 min with 100 nM isoproterenol. Exposure of adipocytes to isoproterenol promoted MAPK1/3 phosphorylation by ~41% ($P < 0.01$); metformin remarkably attenuated isoproterenol-mediated MAPK1/3 phosphorylation ($P < 0.01$; Fig. 4A and B). Neither isoproterenol nor metformin altered the level of total MAPK3 proteins (Fig. 4A).

Metformin decreased perilipin phosphorylation on isoproterenol stimulation

Perilipin proteins coat the surface of the lipid droplets to function as a barrier to protect the triglycerides in

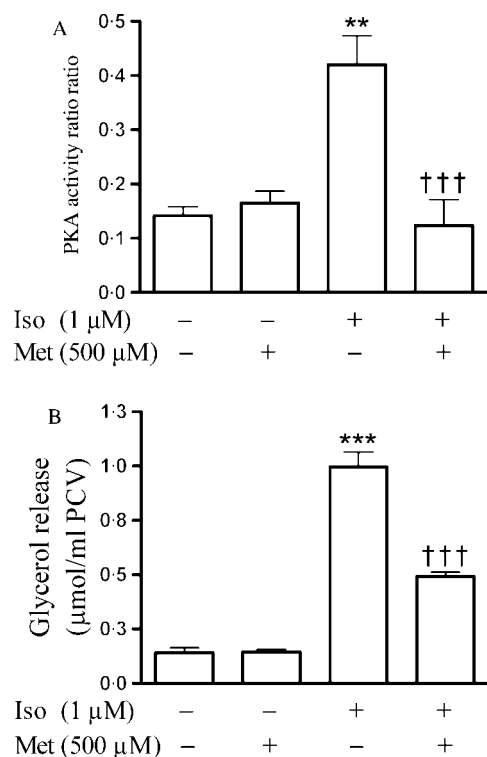


Figure 3 Metformin restricts the elevation of PKA activity on isoproterenol stimulation. Isolated primary adipocytes were preincubated for 16 h with or without 500 μ M metformin (Met), and then washed, and medium was changed to fresh medium. (A) Adipocytes were stimulated with 1 μ M isoproterenol (Iso) for 15 min, and then homogenized to prepare the cytosolic fraction. PKA activity in each cytosol extract was assayed six times under three separate conditions, and the corrected PKA activity ratio was calculated as described in Methods. Values are expressed as means \pm S.E.M. of three separate experiments performed six times. (B) Glycerol released in the incubation medium was assayed in parallel. **, $P < 0.01$ and ***, $P < 0.001$ versus control; †††, $P < 0.001$ versus Iso.

the droplet from hydrolysis by cellular lipases (Londos *et al.* 2005). When perilipin is phosphorylated by PKA, its barrier function ceases and the lipolysis reaction is initiated (Sztalryd *et al.* 2003, Londos *et al.* 2005, He *et al.* 2006). We examined the phosphorylation status of perilipin in adipocytes in response to isoproterenol, metformin, or both. Adipocytes were preincubated for 12 h in the presence or absence of 500 μ M metformin and then stimulated for 5 min with 1 μ M isoproterenol. Adipocytes were lysed and protein extracts underwent immunoblotting with primary antibodies against perilipin. The addition of isoproterenol to adipocytes caused a migration shift of perilipin bands from 65 to \sim 67 kDa on a low-bis concentration of SDS-polyacrylamide gel (Fig. 5); this electrophoretic shift pattern was previously shown to result from the phosphorylation of perilipin proteins mediated by PKA (Egan *et al.* 1990) or non-PKA stimulation of adipocytes (Sengenès *et al.* 2003,

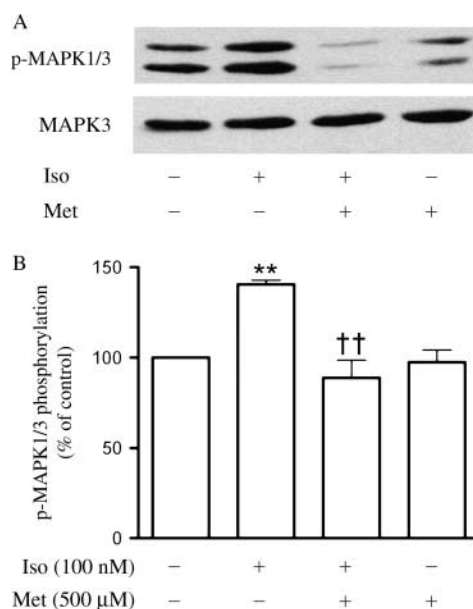


Figure 4 Inhibition of MAPK1/3 activation by metformin during isoproterenol-stimulated lipolysis. Adipocytes were preincubated for 2 h with 500 μ M metformin (Met), then stimulated with 100 nM isoproterenol (Iso) for 5 min. Adipocytes were lysed and proteins underwent immunoblotting with use of primary antibodies against phosphorylated-MAPK1/3 (p-MAPK1/3) and HRP-conjugated secondary antibodies. To detect total MAPK1, blots were stripped and reprobbed with primary anti-MAPK3 antibodies (MAPK3). The phosphorylated-MAPK1/3 levels were densitometrically quantitated against total MAPK3 levels. The data are from three separate experiments and expressed as percentage of the control. **, $P < 0.01$ versus control; ††, $P < 0.01$ versus Iso alone.

Aboulaich *et al.* 2006, He *et al.* 2006). Preincubation with metformin did not alter the status of basal perilipin phosphorylation in unstimulated cells but inhibited the isoproterenol-mediated perilipin phosphorylation, as evidenced by the appearance of a weak 65/67 kDa doublet (Fig. 5). Complete abolishment of PKA activity (Fig. 3A) but incomplete abrogation of perilipin phosphorylation (Fig. 5) by metformin implicates that

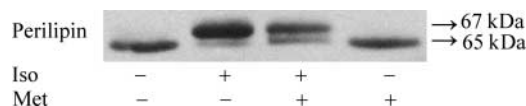


Figure 5 Metformin inhibited perilipin phosphorylation on isoproterenol stimulation. Isolated adipocytes were preincubated for 12 h in the presence or absence of 500 μ M metformin (Met), then washed and stimulated for 5 min with 1 μ M isoproterenol (Iso) under fresh medium. Adipocytes were lysed and proteins were separated on a low-bis concentration of SDS-polyacrylamide gel, which provides better resolution of proteins in the 60–70 kDa range. The proteins transferred on nitrocellulose membranes were immunoblotted with primary antibodies against rat perilipin and HRP-conjugated secondary antibodies. The electrophoretic shift of native perilipin at 65 kDa to its hyperphosphorylated form at \sim 67 kDa was a result of isoproterenol stimulation. The blot shown is representative of three separate experiments.

perilipin could be also phosphorylated, as yet not clarified, in a PKA-independent manner in isoproterenol-stimulated adipocytes.

Effect of metformin on lipase activity, translocation, and protein expression

The last step of lipolysis activation by PKA stimulation with isoproterenol is the process of hydrolysis of triglycerides by two major cellular lipases, HSL and adipose triglyceride lipase (ATGL; Londos *et al.* 1999, Schweiger *et al.* 2006). Therefore, we investigated the influence of metformin on total activity of adipocyte lipases. Isolated adipocytes were preincubated for 12 h with or without 500 μM metformin, and then stimulated for 20 min with 100 nM isoproterenol.

Isoproterenol promoted total lipase activity by $\sim 96\%$ compared with that in controls ($P < 0.01$, Fig. 6A). Metformin preincubation did not affect basal lipase activity in unstimulated adipocytes but greatly reduced isoproterenol-activated lipase activity (Fig. 6A).

During lipolysis activated by PKA, the translocation of HSL from the cytosol to the lipid droplet surface is a key factor in lipolysis modulation (Londos *et al.* 1999, Sztalryd *et al.* 2003). We next differentiated rat preadipocytes to mature adipocytes and performed immunofluorescent staining of HSL. Immunofluorescent signals for HSL were dispersed throughout the cytoplasm in unstimulated differentiating adipocytes but translocated to the surface (ring loop) of the intracellular lipid droplets after 5 min stimulation with 1 μM isoproterenol (Fig. 6B). Short or prolonged pretreatment with 500 μM metformin

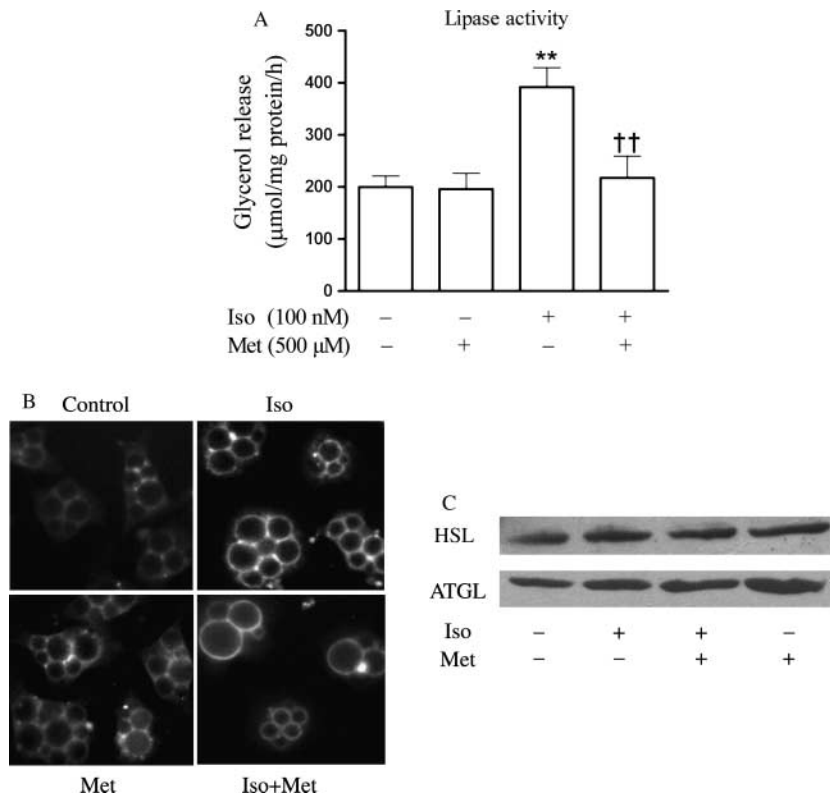


Figure 6 Effect of metformin on lipase activity, translocation, and protein expression. (A) Lipase activity. Primary rat adipocytes were preincubated for 12 h with or without 500 μM metformin (Met), then stimulated with 100 nM isoproterenol (Iso) for 20 min. The cellular lipase activity in the cytosolic fraction of adipocytes was determined. Results are means \pm s.e.m. of three individual experiments performed in triplicate. **, $P < 0.01$ versus control; ††, $P < 0.01$ versus Iso. (B) Immunofluorescence of HSL. Rat preadipocytes were isolated and differentiated into adipocytes. On day 5, differentiated adipocytes were incubated with or without 500 μM metformin (Met) for 24 h, then washed and stimulated for 5 min with 1 μM isoproterenol (Iso) under fresh medium. Immunostaining was performed by use of anti-HSL antibody and FITC-conjugated second antibody. (C) Immunoblot. Primary rat adipocytes were incubated with 500 μM metformin for 24 h, then stimulated with 1 μM isoproterenol for 30 min. Protein extracts of the lysed adipocyte underwent immunoblotting with primary antibodies against rat HSL or ATGL and HRP-conjugated secondary antibodies. The blots shown are representative of three separate experiments.

for 12 (data not shown) or for 24 h (Fig. 6B) seemed to slightly increase the assembly of HSL to the lipid droplet periphery, but did not inhibit the HSL translocation induced by isoproterenol (Fig. 6B). Finally, immunoblotting results indicated that metformin did not alter the protein levels of the two major cellular lipases, ATGL and HSL, in primary adipocytes (Fig. 6C).

Discussion

The major goals of the present study were to elucidate the pharmacological characteristics and cellular basis of the inhibitory effect of metformin on adrenergic-stimulated lipolysis in primary rat adipocytes.

The therapeutic concentrations of metformin are between 10 and 2000 μM in human or diabetic animals (Pentikainen 1986, Bailey & Turner 1996, Wang *et al.* 2003). In the present study, metformin exhibited concentration- and time-dependent antilipolytic effects in primary rat adipocytes. Incubation with metformin at 500 μM but not 250 μM effectively reduced basal lipolysis and inhibited FFA efflux from unstimulated adipocytes, whereas at 250–500 μM , it largely attenuated FFA and glycerol release from isoproterenol-stimulated adipocytes. These antilipolytic effects occurred early, at 4 h after the addition of metformin. Previous studies indicated that low concentrations of metformin (2–4 mg/l, equivalent to 12–24 $\mu\text{mol/l}$) failed to inhibit norepinephrine-stimulated lipolysis in isolated human adipose tissue (Cigolini *et al.* 1984); by contrast, perfusion of metformin at high concentrations (1 mM) directly into abdominal adipose tissues of obese and diabetic subjects inhibited basal and adrenergic-stimulated glycerol release (Flechtner-Mors *et al.* 1999). Moreover, our previous study demonstrated that metformin at 500 μM effectively inhibited the lipolysis response to TNF or excess glucose in primary rat adipocytes (Ren *et al.* 2006). These data confirm that adipose tissue is another target in addition to muscle and liver for metformin to act on. Therefore, the administration of relatively high-dose metformin in diabetic patients may sufficiently restrict the basal and stimulated lipolysis response to catecholamine or TNF. This antilipolytic action, which decreases FFA efflux from adipose cells, could be the cellular basis by which metformin reduces serum FFA levels and hence increases insulin sensitivity. In addition, the lipolytic effect of catecholamines is more pronounced in visceral than s.c. fat tissues (Arner *et al.* 1990), which leads to more FFA efflux to the portal venous system and directly provides a substrate for hepatic lipoprotein metabolism or glucose production. The reduction of the catecholamine-induced lipolytic rate by metformin may prevent the ectopic lipid accumulation and, hence, further improve insulin resistance.

Catecholamines are the most important lipolytic hormones. The early events for catecholamine-stimulated lipolysis in adipocytes are cAMP elevation and PKA activation (Londos *et al.* 1999), as confirmed by the present study. In unstimulated adipocytes, metformin alone inhibited basal lipolysis but only slightly decreased basal cAMP level and did not alter PKA activity. However, metformin significantly inhibited cellular cAMP elevation, suppressed PKA activation, and thus attenuated the lipolysis response to isoproterenol stimulation in adipocytes. Besides activation of PKA, that of MAPK1/3 is required for maximal lipolytic activity on adrenergic stimulation (Greenberg *et al.* 2001, Robidoux *et al.* 2006). Metformin attenuated the basal and isoproterenol-mediated MAPK1/3 phosphorylation without affecting the total MAPK3 protein level; these effects are similar to our previous observations of metformin inhibiting MAPK1/3 phosphorylation and lipolysis mediated by TNF in adipocytes (Ren *et al.* 2006). In addition to MAPK1/3, there are two other mitogen-activated protein kinases (MAPKs), mitogen-activated protein kinase 8 (MAPK8), and p38 MAPK. We observed that the phosphorylated MAPK8 was rarely detectable in primary rat adipocytes, and the phosphorylation of p38 MAPK was not apparently affected by isoproterenol (unpublished data) but was modestly promoted by metformin (Ren *et al.* 2006). Therefore, inhibition of MAPK1/3 activation on isoproterenol stimulation may be another basis for the antilipolytic action of metformin.

Perilipin is the most abundant protein coating the surface of the lipid droplets in adipocytes, which may function as a barrier to limit triacylglycerol hydrolysis by lipases (Londos *et al.* 1999). Also, phosphorylation of perilipin is associated with the localization of HSL on the lipid droplet (Granneman *et al.* 2007). Upon catecholamine stimulation, PKA-phosphorylated perilipin is required for HSL translocation from the cytosol to the lipid droplets, thus facilitating lipolysis (Sztalryd *et al.* 2003). Moreover, PKA-independent hyperphosphorylation of perilipins is sufficient to confer a full lipolytic reaction without inducing HSL translocation in adipocytes, whereas lipolysis ceases with inhibited perilipin hyperphosphorylation (He *et al.* 2006). In our study, metformin inhibited perilipin phosphorylation on lipolytic stimulation with isoproterenol. This effect could mainly result from metformin inhibiting isoproterenol-promoted PKA activity. Surprisingly, when metformin inhibited PKA activation and perilipin phosphorylation and efficiently attenuated increase of the lipase activity, it failed to eliminate isoproterenol-induced HSL translocation to the lipid droplets in adipocytes. Perhaps, this finding was due to metformin being unable to completely abrogate the PKA phosphorylation of perilipin; hence HSL translocation remained. Conversely, MAPK1/3 activation, which promotes HSL activity but does not induce HSL

translocation, was found to be responsible for adrenergic-stimulated lipolysis, of ~30%, in a PKA-independent manner (Greenberg *et al.* 2001, Robidoux *et al.* 2006). As well, inhibition of MAPK1/3 was found to diminish lipolysis by 50% but did not prevent HSL translocation to the lipid droplet (Robidoux *et al.* 2006). Therefore, if MAPK1/3 inhibition accounts for a greater proportion of the mediation of the antilipolytic action of metformin, metformin could inhibit lipolysis without altering the HSL translocation. Metformin inhibited MAPK1/3 activation and also largely suppressed the cellular lipase activity elevated by isoproterenol. By contrast, neither isoproterenol nor metformin affected the protein levels of HSL and ATGL. HSL, together with ATGL, constitutes more than 95% of triglyceride hydrolase activity in white adipocytes; ATGL is not phosphorylated by PKA but is assumed to contribute most of the non-HSL activity during catecholamine-stimulated lipolysis (Zimmermann *et al.* 2004, Schweiger *et al.* 2006). The phosphorylation of perilipin is required for ATGL-catalyzed lipolytic activity (Miyoshi *et al.* 2007); thus, the inhibition of perilipin phosphorylation by metformin may account for the decrease in the non-HSL lipase activity. The lipase triacylglycerol hydrolase mainly regulates basal but not stimulated lipolysis (Wei *et al.* 2007); whether the inhibitory effect of metformin on basal lipolysis is related to triacylglycerol hydrolase requires further investigation.

Protein kinase, AMP-activated, alpha 1 catalytic subunit (AMPK) is a major metabolic modulator that can be activated by catecholamines and biguanide. The activation of AMPK regulates metformin-mediated glucose and lipid metabolism (Zhou *et al.* 2001). The possible role of AMPK in lipolysis regulation remains controversial. One study suggested that AMPK phosphorylation is responsible for maximal lipolysis in differentiated 3T3-L1 adipocytes (Yin *et al.* 2003). Other investigators contrarily propose that AMPK is an antilipolytic modulator, because promotion of AMPK activity by phenformin or 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) inhibited isoproterenol-induced lipolysis in primary rat adipocytes (Sullivan *et al.* 1994, Daval *et al.* 2005); by contrast, when AMPK is decreased either by a dominant-negative form or in AMPK-null mice, lipolysis is increased (Daval *et al.* 2005). However, we failed to detect the protein band of phosphorylated or total AMPK in primary rat adipocytes in the presence of metformin and/or isoproterenol (data not shown); thus we were unable to access whether AMPK mediates the antilipolytic action of metformin.

In conclusion, the present study provides direct evidence that metformin inhibits lipolysis through multiple mechanisms by attenuating cAMP-PKA and MAPK1/3 signaling, inhibiting perilipin

phosphorylation, and decreasing lipase activity in primary adipocytes in response to catecholamine stimulation. Despite the 50-year use of metformin as an effective antidiabetic drug, its antihyperglycemic effect remains a mystery. Our data reveal that adipose tissue is another important target for metformin to act on. Obviously, the antilipolytic action of metformin reduces FFA mobilization from adipose cells, which could be a cellular basis for metformin in lowering circulation FFA levels and ameliorating insulin sensitivity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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