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Metformin promotes induction of lipoprotein lipase in skeletal muscle through activation of adenosine monophosphate–activated protein kinase

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Abstract

Metformin is known to increase lipoprotein lipase (LPL) mass level in serum. Lipoprotein lipase is produced by adipose tissue and skeletal muscles. This study aimed to examine the effect of metformin on LPL production in adipocytes and skeletal muscle cells and to investigate the mechanism by which metformin enhances LPL production. 3T3-L1 preadipocytes and L6 skeletal muscle cells were incubated with metformin or 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR). Lipoprotein lipase activity, LPL protein expression, and LPL messenger RNA (mRNA) expression were measured. Metformin increased LPL activity only in skeletal muscle cells. To clarify the mechanism of this phenomenon, AICAR, which is well known as an activator of adenosine monophosphate–activated protein kinase (AMPK), was used. Metformin and AICAR enhanced phosphorylated AMPK in skeletal muscle cells by Western blot analysis. Like metformin, AICAR increased LPL activity only in skeletal muscle cells. Both metformin and AICAR also enhanced LPL protein and LPL mRNA expressions in skeletal muscle cells but not in adipocytes. Phosphorylated AMPK protein expression was decreased when AMPK signaling was interfered by AMPKα small interfering RNA. Lipoprotein lipase activity and LPL expression, which were enhanced by 1 μmol/L metformin, were reduced by AMPKα small interfering RNA. These results suggest that metformin increases LPL activity, LPL protein expression, and LPL mRNA expression through activation of AMPK in skeletal muscle cells but not in adipocytes.

1. Introduction

Metformin, an insulin-sensitizing biguanide, is widely used to reduce blood glucose in patients with type 2 diabetes mellitus. This drug has been reported to exert its effects primary in the liver by inhibiting gluconeogenesis and reducing hepatic glucose output [1-3]. Metformin has also been reported to increase glucose uptake in skeletal muscle and adipose tissue [1]. Recent reports have shown that metformin has other direct actions on adipocytes and skeletal muscle. For example, metformin inhibits plasminogen activator inhibitor–1 in adipocytes [4] and stimulates adenosine monophosphate–activated protein kinase (AMPK) in skeletal muscle [5].

Previous reports indicate that metformin decreases very low-density lipoprotein (VLDL) levels [6], but the mechanism for this effect is not fully understood. We have reported that serum lipoprotein lipase (LPL) mass level, which reflects total LPL production in the whole body [7,8], was increased by metformin administration [9]. It is possible that metformin may increase LPL production in adipocytes and skeletal muscle, and promote VLDL catabolism. The mechanism of increasing LPL by metformin has not been reported before.

This study was conducted to test the hypothesis that metformin acts on adipocytes and/or skeletal muscle and enhances LPL production, and to examine the mechanism by which metformin enhances LPL production.

2. Materials and methods

2.1. Culture of 3T3-L1 preadipocytes and L6 skeletal muscle cells

3T3-L1 preadipocytes and L6 skeletal muscle cells were cultured in Dulbecco modified Eagle minimal essential medium supplemented with 10% (vol/vol) delipidated fetal

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calf serum/dilapidated calf serum mixture, 2 mmol/L L-glutamine, and 40 μg/mL gentamicin (growth medium) at 37°C under 5% CO_2. 3T3-L1 preadipocytes (2.5×10^4) seeded in 12-well plates were incubated for 2 days and then incubated in growth medium supplemented with dexamethasone (0.25 μmol/L), insulin (10 μg/mL), and 3-isobutyl-1-methylxanthine (0.5 mmol/L) for 4 days. 3T3-L1 cells were cultured with growth medium with 10 μg/mL insulin for 2 days and incubated with only growth medium for another 2 days. Differentiation was complete in 8 days. L6 skeletal muscle cells (2.5×10^4) were seeded in 12-well plates and incubated for 4 days. The extent of 3T3-L1 adipocytes differentiation was confirmed by oil red O stain.

When the culture was 50% confluent, metformin (a gift from Nihon Shinyaku, Kyoto, Japan) or 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) (Sigma, St Louis, MO) was added. 5-Aminoimidazole-4-carboxamide ribonucleoside is an activator of AMPK. Metformin was added to the culture medium at a final concentration of 1, 5, or 10 μmol/L; and AICAR, at a final concentration of 0.1, 1, or 10 μmol/L. Both metformin and AICAR were dissolved in growth medium.

2.2. Measurements of LPL activity

For the assay of LPL activity, 3T3-L1 preadipocytes and L6 skeletal muscle cells were harvested, dissolved in 0.1 mol/L Tris buffer (pH 7.4), and sonicated on ice for 30 seconds using an Ultrasonic Generator model US-50 (Nissei, Tokyo, Japan); and the homogenates were centrifuged at 2000*g* for 10 minutes (Himac CF 15D2; Hitachi, Tokyo, Japan). The supernatants were used for the enzyme assay.

Lipoprotein lipase activity was measured using triolein (Sigma) as a substrate. The substrate solution was prepared as follows: 100 mg of triolein and 7.5 mL of 0.2% Triton X-100 in a final volume of 7.5 mL of 1 mol/L Tris-HCl (pH 8.0) were sonicated on ice for 10 minutes. For the measurement of LPL activity, the reaction mixture contained 50 μL of substrate solution, 25 μL of 20% fatty acid–free bovine serum albumin (pH 8.0, Sigma), 5 μL of high-density lipoprotein (3 mg protein per milliliter) as apolipoprotein C-II, and an appropriate amount of sample (170 μL). After incubation for 120 minutes at 37°C, the enzyme reaction was terminated by the addition of 10 μL of diisopropyl fluorophosphate. The amount of free fatty acids released in the mixture was measured at 4°C using an enzymatic method (Nescauto NEFA V2; Azwell, Osaka, Japan).

2.3. Western blot analysis of LPL protein, phosphorylated (Thr^{172}) AMPK protein, and AMPKα (α1 and α2) protein from 3T3-L1 preadipocytes and L6 skeletal muscle cells

Lipoprotein lipase protein, phosphorylated (Thr^{172}) AMPK protein, and AMPKα (α1 and α2) protein were detected by Western blot analysis. Cells were suspended in a lysis buffer containing 10 mmol/L Tris-HCL (pH 7.5), 150 mmol/L NaCl, 0.5% Triton X-100, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L EDTA for 4 hours at 4°C. After centrifugation at 12 000*g*, the protein concentration in the supernatant was measured using the Bio-Rad (Hercules, CA) protein assay. Samples were diluted 1:1 with electrophoresis sample buffer containing 100 mmol/L Tris (pH 6.8), 10% sodium dodecyl sulfate, 10% glycerol, 0.1% bromophenol blue, and 5% *b*-mercaptoethanol; boiled for 5 minutes; and electrophoresed on 10% sodium dodecyl sulfate–polyacrylamide gel. The proteins were transferred onto Hybond-ECL nitrocellulose membrane (Amersham Biosciences, Pittsburgh, PA). After blotting, the membrane was washed with Tris buffer saline (TBS; 100 mmol/L Tris [pH 7.5] and 0.9% NaCl), blocked with 5% bovine serum albumin in TBS, and then briefly washed in TBS with 0.1% Tween-20 (TTBS). The membrane was incubated with rabbit antiserum against rat LPL (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:1000, rabbit polyclonal antibodies that recognize AMPK only when phosphorylated with Thr172 (Cell Signaling Technology, Boston, MA), or rabbit polyclonal antibodies against AMPKα (α1 and α2 (Cell Signaling Technology) at a dilution of 1:1000 for 75 minutes at room temperature. After a wash with TTBS, the blot was incubated with peroxidase-labeled affinity purified antibody to rabbit immunoglobulin G at a dilution of 1:1000 for 1 hour at room temperature, washed with TTBS, and incubated with diluted (1:3000) biotinylated horseradish peroxidase streptavidin complex for 1 hour at room temperature. All antibodies have reactivity with rat and mouse antigens. Visualization of the antigen-antibody complex was performed by photodetection. The reaction products were quantified with digital scanning and Scion Image software (Frederick, MD).

2.4. Subcloning of LPL messenger RNA fragment by reverse transcriptase–polymerase chain reaction

Total cellular RNA was extracted from 3T3-L1 preadipocytes and L6 skeletal muscle cells using the RNeasy kit (Qiagen, Courtaboeuf, France). The concentration of RNA was determined by measuring the absorbance at 260 nm. Reverse transcriptase–polymerase chain reaction (RT-PCR) was carried out using 1 μg of reverse transcribed total RNA (complementary DNA). The expression of a house keeping gene, *β*-actin messenger RNA (mRNA), was considered as an internal standard. The LPL primers were sense 5′-CTCGATCCAGCTGGACCTAA-3′ and antisense 5′-TCCAAGTCCTCTCTCTGCAA-3′, and the *β*-actin primers were sense 5′-CTCTTCCAGCCTTCCTTCCT-3′ and antisense 5′-AGCACTGTGTTGGCGTACAG-3′. Polymerase chain reaction was run on a Gene Amp PCR System 9700 (Applied Biosystems, Foster city, CA) for 35 cycles both for LPL and *β*-actin. Denaturation, annealing, and extension were performed at 94°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, respectively. The PCR products were electrophoresed on 1% agarose gels, stained with ethidium bromide, and visualized under UV irradiation. The images were photographed with an Olympus digital camera (Tokyo, Japan) and analyzed with

Scion Image software. The levels of LPL mRNA were expressed as the ratio of LPL to β-actin.

2.5. Knockdown of AMPKα by RNA interference

To reduce the level of endogenous AMPKα, L6 skeletal muscle cells were transfected with small interfering RNA (siRNA) for AMPKα: CUA ACG UCA UUG AUG AUG AdTdT (nucleotides 861-879, accession no. NM023991). A negative control siRNA (UCU GGC UAA UAU CUA AAG GdTdT) was used in all experiments. Confluent monolayers of L6 skeletal muscle cells (plated and grown as described in the cell culture section) were transfected 5 days after plating. Small interfering RNA (10 nmol/L) was suspended in a serum- and antibiotic-free medium prepared for transfection with RiboJuice siRNA transfection reagent (Novagen, Darmstadt, Germany) following the instructions of the manufacturer. The siRNA was added to the cells and incubated for 2 to 3 hours at 37°C. After transfection, 1 μmol/L of metformin was added. Specific interference of AMPK protein expression was confirmed by Western blot analysis using antibody against phosphorylated AMPK protein and AMPKα (α1 and α2) protein (as described above).

2.6. Statistical analysis

The software used was Stat View J 5.0 (SAS, Cary, NC). Data are expressed as mean ± SD. A *t* test was used for 2-group comparisons. *P* values less than .05 were considered significant.

3. Results

3.1. Effect of metformin on LPL activity in 3T3-L1 preadipocytes and L6 skeletal muscle cells

Lipoprotein lipase activity was measured in 3T3-L1 preadipocytes and L6 skeletal muscle cells in the presence of metformin. In 3T3-L1 preadipocytes, LPL activity was not changed by the addition of metformin (Fig. 1A). However, LPL activity in skeletal muscle cells was increased by the addition of metformin; and significant increases were observed at metformin concentrations of 1 μmol/L and above. Especially, 1 μmol/L of metformin increased LPL activity by approximately 2.5-fold against control (Fig. 1B).

3.2. Western blot analysis of phosphorylated (Thr^{172}) AMPK protein and AMPKα protein in 3T3-L1 cells and L6 skeletal muscle cells

To clarify the mechanism by which metformin increases LPL activity only in skeletal muscle cells, the effect of metformin on AMPK expression in adipocytes and skeletal muscle cells was investigated by Western blot analysis. In 3T3-L1 preadipocytes, phosphorylated (Thr^{172}) AMPK protein expression was significantly increased by metformin or AICAR (Fig. 2A). In skeletal muscle cells, phosphorylated AMPK protein expression was significantly enhanced not only by metformin (final concentrations of 1 and 5 μmol/L for 72 hours) but also by AICAR (final concentrations of 1 and 10 μmol/L for 72 hours) (Fig. 2B). The AMPKα protein

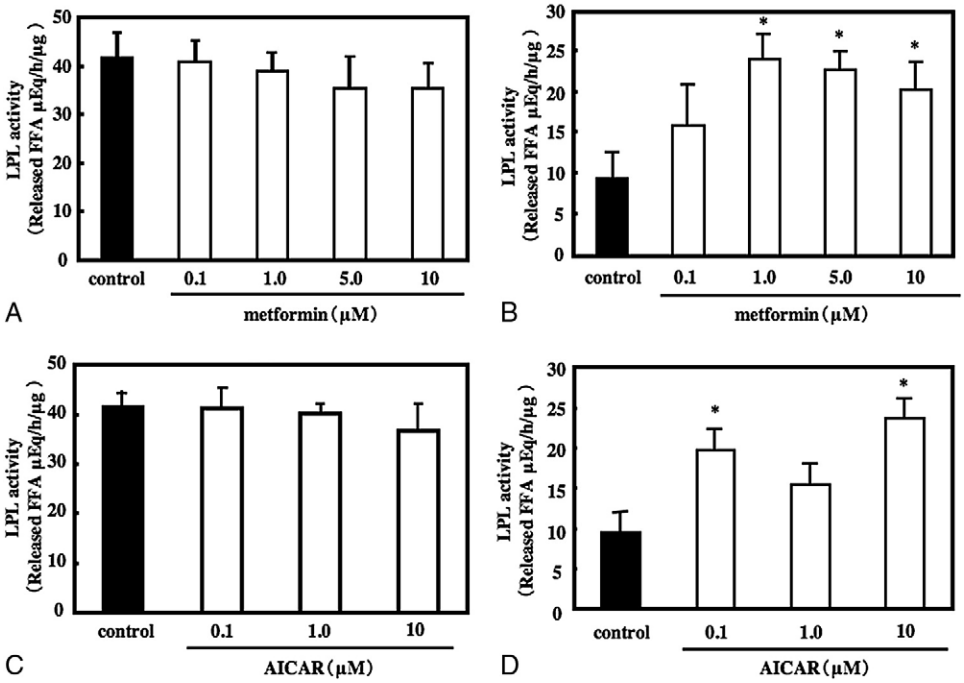

Fig. 1. Effects of metformin on LPL activity in 3T3-L1 preadipocytes (A) and L6 skeletal muscle cells. (B). Effects of AICAR on LPL activity in 3T3-L1 preadipocytes (C) and L6 skeletal muscle cells (D). These experiments were performed 5 times, and data are presented as means ± SD. *$P < .05$ vs control.

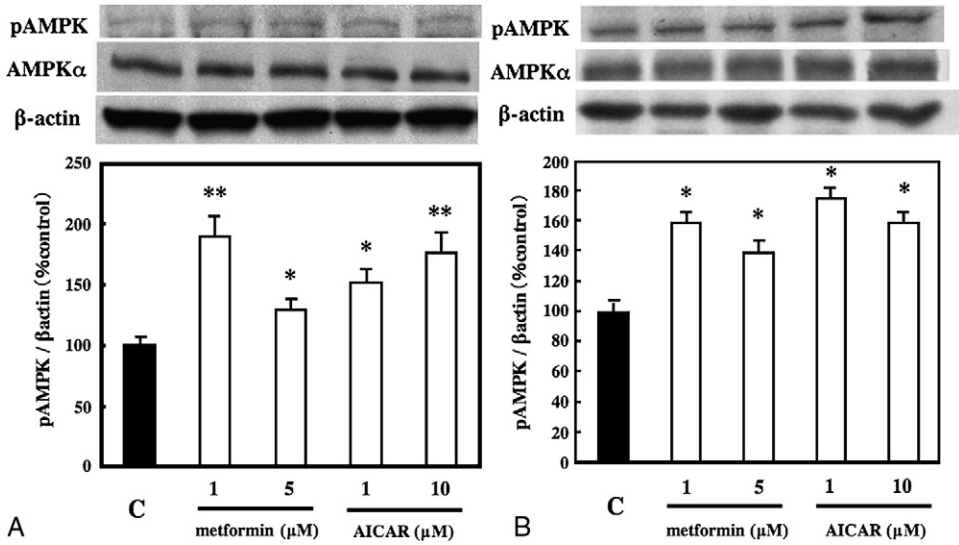

Fig. 2. Western blot analysis of phosphorylated (Thr[172]) AMPK protein and AMPKα (α1 and α2) protein in 3T3-L1 preadipocytes (A) and L6 skeletal muscle cells (B). Lower graphs show the quantitative results obtained by using Scion Image software. Phosphorylated AMPK protein level is expressed as the relative ratio of the density of phosphorylated AMPK protein against that of β-actin (internal control). These experiments were performed 5 times, and data are presented as means ± SD. *$P < .05$ vs control; **$P < .01$ vs control. C indicates control; pAMPK, phosphorylated (Thr[172]) AMPK.

expression was not changed in 3T3-L1 preadipocytes and L6 skeletal muscle cells (Fig. 2A, B).

3.3. LPL activity in 3T3-L1 preadipocytes and L6 skeletal muscle cells by AICAR

Lipoprotein lipase activity was increased by AICAR in skeletal muscle cells but not in 3T3-L1 preadipocytes (Fig. 1C, D). 5-Aminoimidazole-4-carboxamide ribonucleoside at concentrations of 0.1 and 10 μmol/L significantly increased LPL activity by approximately 2-fold compared with control.

3.4. Western blot analysis of LPL protein in 3T3-L1 cells and L6 skeletal muscle cells

Lipoprotein lipase protein expression in the presence of metformin (final concentrations of 1 and 5 μmol/L for 72 hours) or AICAR (final concentrations of 1 and 10 μmol/L for 72 hours) was studied by Western blotting. Lipoprotein lipase protein expression was also significantly increased by both metformin and AICAR only in skeletal muscle cells (Fig. 3). Metformin enhanced the expressions of phosphorylated AMPK protein and LPL protein more strongly at 1 μmol/L rather than at 5 μmol/L.

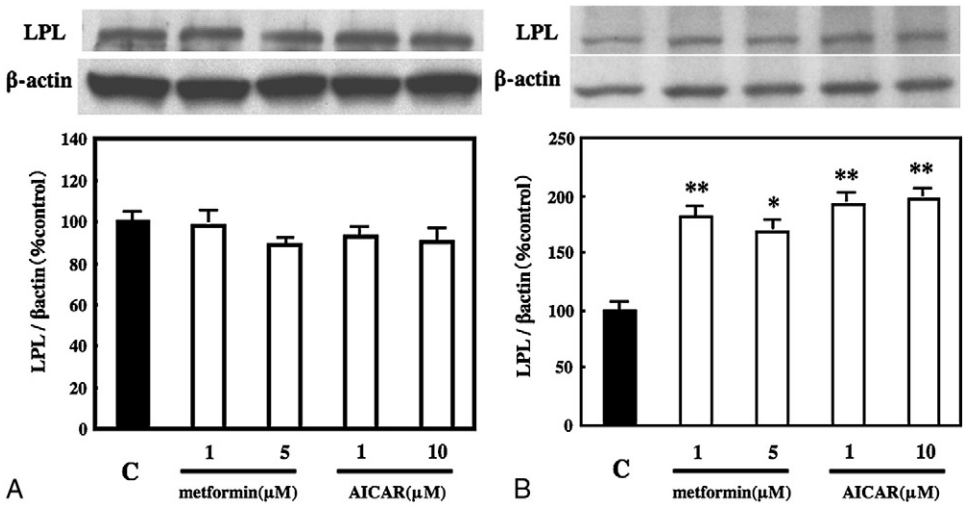

Fig. 3. Western blot analysis of LPL protein in 3T3-L1 preadipocytes (A) and L6 skeletal muscle cells (B). Lower graphs show the quantitative results obtained by using Scion Image software. Lipoprotein lipase protein level is expressed as the relative ratio of the density of LPL protein against that of β-actin (internal control). These experiments were performed 5 times, and data are presented as means ± SD. *$P < .05$ and **$P < .01$ vs control.

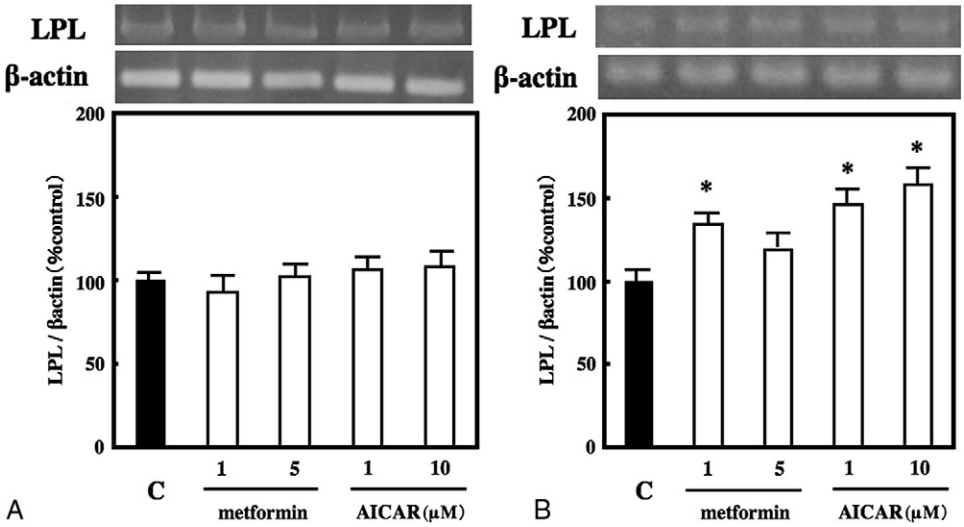

Fig. 4. Subcloning of LPL mRNA fragment by RT-PCR in 3T3-L1 preadipocytes (A) and L6 skeletal muscle cells (B). Lower graphs show the quantitative results obtained by using Scion Image software. Lipoprotein lipase mRNA level is expressed as the relative ratio of the density of LPL mRNA against that of β-actin (internal control). These experiments were performed 5 times, and data are presented as means ± SD. *$P < .05$ vs control.

3.5. Subcloning of LPL mRNA fragment by RT-PCR

Reverse transcriptase–polymerase chain reaction for LPL mRNA was performed. The analysis showed that LPL mRNA expression was enhanced by metformin and AICAR in skeletal muscle cells but not in 3T3-L1 preadipocytes (Fig. 4A, B). Lipoprotein lipase mRNA expression was significantly enhanced at 1 μmol/L metformin as well as 1 and 10 μmol/L AICAR. These results were the same as LPL activity and LPL protein expression.

3.6. Knockdown of AMPKα by RNA interference

To clarify the relationship between AMPK and LPL in skeletal muscle cells, we interfered AMPK signaling using AMPKα siRNA. The phosphorylated (Thr^{172}) AMPK protein expression was significantly enhanced in the presence of 1 μmol/L metformin, and this increase was abolished by the addition of AMPKα siRNA. The AMPKα protein expression was decreased by the addition of AMPKα siRNA (Fig. 5A). The AMPKα siRNA also

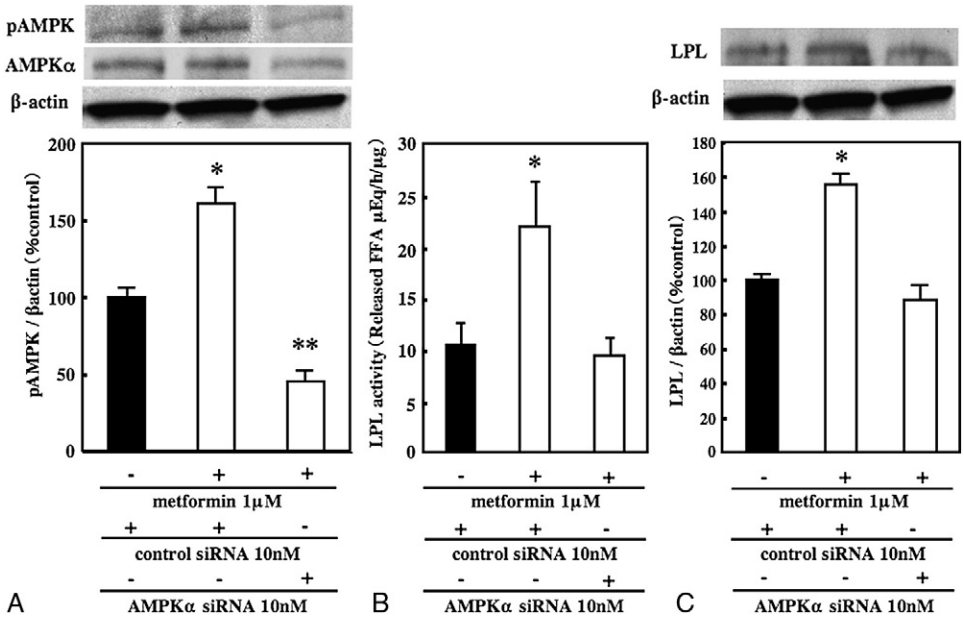

Fig. 5. Effects of interfering AMPK signaling by AMPKα siRNA on metformin-induced enhancement of phosphorylated AMPK protein expression and AMPKα (α1 and α2) protein expression (A), LPL activity (B), and LPL protein expression (C) in L6 skeletal muscle cells. Lower graphs of A and C show quantitative results obtained by using Scion Image software. Phosphorylated AMPK protein expression and LPL protein expression are expressed as the relative ratios of the density of phosphorylated AMPK and LPL protein, respectively, against that of β-actin (internal control). These experiments were performed 5 times, and data are presented as means ± SD. *$P < .05$ vs negative control; **$P < .01$ vs negative control.

abolished the metformin (1 μmol/L)-induced enhancement of LPL activity (Fig. 5B). Similarly, AMPKα siRNA also abolished the metformin-induced increase of LPL protein expression (Fig. 5C).

4. Discussion

Previous studies reported the effect of metformin in reducing VLDL [6], but the mechanism by which metformin improves serum lipid metabolism is not fully understood. In this study, metformin increased LPL activity, LPL protein expression, and LPL mRNA expression only in skeletal muscle cells. These results indicate that metformin increases LPL production in skeletal muscle cells and promotes catabolism of triglyceride-rich lipoprotein. We have reported that metformin administration increases serum LPL levels in diabetic patients [9]. Therefore, the in vitro effects of metformin demonstrated in this study may explain its in vivo effect in improving serum lipid metabolism.

Some reports have indicated that metformin has anti-atherogenic effect [10,11]. As for the mechanisms of the antiatherogenic effect of metformin, recent studies indicate that metformin significantly improves insulin resistance and decreases plasminogen activator inhibitor–1 [12], while also possessing antioxidant activity [13]. Lipoprotein lipase promotes the catabolism of triglyceride-rich lipoproteins that are well known to be atherogenic [14]. Induction of LPL by metformin may be one of the mechanisms of the antiatherogenic effect of this agent.

Metformin has been reported to stimulate AMPK in skeletal muscle [5]. Adenosine monophosphate–activated protein kinase is known to be a cellular energy charge sensor and has some effects on lipid metabolism such as stimulating fatty acid oxidation through phosphorylation and inactivation of acetyl–coenzyme A carboxylase [15,16]. Adenosine monophosphate–activated protein kinase increases cardiac LPL activity and recruitment of LPL to the coronary lumen [17]. We studied the relationship between AMPK and LPL because AMPK is activated by metformin. Metformin as well as AICAR enhanced LPL activity, LPL protein expression, and LPL mRNA in skeletal muscle cells. When AMPK signaling was interfered by AMPKα siRNA, metformin-induced increases in LPL activity and LPL protein expression were abolished in skeletal muscle cells. These results suggest that metformin acts on skeletal muscle cells and increases LPL production through activation of AMPK. It is reported that AICAR increases AMPK in adipocytes [18]. In this study, AMPK phosphorylation was also increased in 3T3-L1 adipocytes by AICAR. It is reported that LPL activity in 3T3-L1 adipocytes is increased when AMPK activation is decreased [19,20]. Lipoprotein lipase is not increased by activating AMPK in 3T3-L1 adipocytes.

The major regulatory factors of LPL reported include insulin and peroxisome proliferators–activated receptor–α and –γ [21]. These results in this study have not been reported before, so we propose a new pathway of LPL regulation through activation of AMPK. This pathway explains the effect of exercise in improving serum lipid metabolism because exercise enhances AMPK [22,23].

In summary, the present results indicate that metformin increases LPL activity, LPL protein expression, and LPL mRNA expression through activation of AMPK in skeletal muscle cells and not in adipocytes. These phenomena may contribute to the lipid metabolism improvement and antiatherogenic effects of metformin.

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