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RESEARCH PAPER

Metformin inhibits P-glycoprotein expression via the NF-κB pathway and CRE transcriptional activity through AMPK activation

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BACKGROUND AND PURPOSE
The expression of P-glycoprotein (P-gp), encoded by the multidrug resistance 1 (MDR1) gene, is associated with the emergence of the MDR phenotype in cancer cells. We investigated whether metformin (1,1-dimethylbiguanide hydrochloride) down-regulates MDR1 expression in MCF-7/adriamycin (MCF-7/adr) cells.

EXPERIMENTAL APPROACH
MCF-7 and MCF-7/adr cells were incubated with metformin and changes in P-gp expression were determined at the mRNA, protein and functional level. Transient transfection assays were performed to assess its gene promoter activities, and immunoblot analysis to study its molecular mechanisms of action.

KEY RESULTS
Metformin significantly inhibited MDR1 expression by blocking MDR1 gene transcription. Metformin also significantly increased the intracellular accumulation of the fluorescent P-gp substrate rhodamine-123. Nuclear factor-κB (NF-κB) activity and the level of IκB degradation were reduced by metformin treatment. Moreover, transduction of MCF-7/adr cells with the p65 subunit of NF-κB induced MDR1 promoter activity and expression, and this effect was attenuated by metformin. The suppression of MDR1 promoter activity and protein expression was mediated through metformin-induced activation of AMP-activated protein kinase (AMPK). Small interfering RNA methods confirmed that reduction of AMPK levels attenuates the inhibition of MDR1 activation associated with metformin exposure. Furthermore, the inhibitory effects of metformin on MDR1 expression and cAMP-responsive element binding protein (CREB) phosphorylation were reversed by overexpression of a dominant-negative mutant of AMPK.

CONCLUSIONS AND IMPLICATIONS
These results suggest that metformin activates AMPK and suppresses MDR1 expression in MCF-7/adr cells by inhibiting the activation of NF-κB and CREB. This study reveals a novel function of metformin as an anticancer agent.

Abbreviations
ACC, acetyl-CoA carboxylase; aicar, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, adenosine 5′-monophosphate-activated protein kinase; CRE, cAMP-responsive element; CREB, cAMP-responsive element binding protein; DN-AMPK, dominant negative AMPK; GSK-3β, glycogen synthase kinase-3β; MDR1, multidrug resistance 1; NF-κB, nuclear factor-κB; P-gp, P-glycoprotein; PKA, protein kinase A; Rh-123, rhodamine 123; TNF-α, tumour necrosis factor α

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Introduction

Chemotherapy is a common and effective treatment for tumours. However, some tumours are inherently resistant to the majority of chemotherapeutic agents (i.e. intrinsic resistance), and many other tumours exhibit broad-spectrum or multidrug resistance (MDR) after several bouts of chemotherapy (i.e. acquired resistance) (Ambudkar *et al.*, 1999; Krishna and Mayer, 2000). P-glycoprotein (P-gp) is encoded by the MDR gene family, which contains two members (MDR1 and MDR2) in humans (Chen *et al.*, 1986) and three (mdr1a, mdr1b and mdr2) in rodents (Gros *et al.*, 1986). MDR arises by several mechanisms in cancer, but is frequently associated with enhanced drug efflux via broad-specificity, ATP-dependent, ATP-binding cassette (ABC) family transporters (herein termed multidrug transporters) (Gottesman *et al.*, 2002). Multidrug transporters confer upon cancer cells the ability to resist lethal doses of certain cytotoxic drugs by actively pumping the drugs out of the cells, thus reducing cytotoxicity. Another model has been proposed in which P-gp acts as a flippase, carrying its substrate from the inner layer of the lipid bilayer to the outer layer (Gottesman and Pastan, 1993; van Helvoort *et al.*, 1996) In tumour cells, the overexpression of P-gp is responsible for the efflux of various hydrophobic chemotherapeutic agents from cells, which results in low chemotherapy efficacy (Krishna and Mayer, 2000). The P-gp demonstrates broad substrate specificity towards *Vinca* alkaloids, anthracyclines, taxanes and epipodophylotoxins, and is responsible for intrinsic and acquired drug resistance in numerous human cancers. P-gp-mediated drug resistance can be effectively overcome by either blocking its drug-pump function or inhibiting its expression (Gottesman *et al.*, 2002).

Nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) is a protein complex that acts as a signal-induced transcription factor. In tumour cells, NF-κB is activated by mutations in genes that encode NF-κB or that control NF-κB activity, such as IκB genes. Phosphorylation of IκB by IκB kinase, which is essential for NF-κB activation, induces IκB ubiquitination and degradation by the 26S proteasome, releasing the NF-κB subunits, which then translocate to the nucleus (Ashkenazi and Dixit, 1998; Perkins, 2007). Thus, inhibition of NF-κB signalling can be induced either by blocking proteasome-mediated degradation with chemical proteasome inhibitors or by using mutant IκBs that cannot be phosphorylated. Blocking NF-κB causes tumour cells to stop proliferating and die, or to become more sensitive to the action of antitumour agents (Escárcega *et al.*, 2007). Previous studies have demonstrated that MDR1 activation occurs through NF-κB activation (Thévenod *et al.*, 2000; Kuo *et al.*, 2002).

AMP-activated protein kinase (AMPK) is a key sensor of cellular energy, because it phosphorylates and regulates enzymes such as acetyl-CoA carboxylase (ACC) and HMG-CoA reductase (Carling *et al.*, 1989). AICAR (an AMPK activator) increased glycogen synthase kinase-3β (GSK-3β), AMPK, and ACC phosphorylation, but decreased the phosphorylation of cAMP response element binding protein (CREB) in HepG2 cells (Horike *et al.*, 2008). Furthermore, overexpression of the dominant-negative CREB mutant KCREB in primary rat hepatocytes repressed intrinsic and misoprostol-induced mdr1b promoter activity, through either a direct or an indirect effect (Ziemann *et al.*, 2006).

Metformin (1,1-dimethylbiguanide hydrochloride) is a biguanide commonly used to treat type 2 diabetes mellitus (Musi *et al.*, 2002; Zou *et al.*, 2004; Vazquez-Martin *et al.*, 2009). Metformin has various beneficial metabolic effects, including antihyperglycaemic activity and reduction of hyper-triglyceridaemia. Recently, metformin was reported to inhibit breast cancer cell growth (Zakikhani *et al.*, 2006), overcome breast cancer chemoresistance (Vazquez-Martin *et al.*, 2009), and inhibit NF-κB activation (Hattori *et al.*, 2006; Huang *et al.*, 2009).

In the present study, we investigated whether metformin down-regulates MDR1 by inhibiting NF-κB activation in MCF-7/adriamycin (MCF-7/adr) cells. Furthermore, we demonstrated that CRE transcriptional activity was inhibited by the up-regulation of AMPK, a novel regulated signalling molecule, providing evidence that this protein plays an important role in the regulation of MDR1 expression by metformin in MCF-7/adr cells.

Methods

Cell culture

MCF-7 human breast adenocarcinoma cells and the multidrug-resistant subline MCF-7/adr were provided by Dr Marilyn E. Morris (Buffalo University, NY, USA). The cells were grown in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum. Both cell lines were cultured at 37°C in a humidified CO_2 incubator. The cells were cultured for 2 weeks in drug-free medium prior to use in experiments.

Measurement of cell viability

The cells were plated in 48-well plates, and cell viability was determined by the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay and lactate dehydrogenase (LDH) assay. After a 24-h incubation, various metformin concentrations were added to the wells, and the plates were incubated at 37°C for 24 h. The cell supernatant was used for LDH determination at 490 nm using a microplate reader (Varioskan; Thermo Electron, Waltham, MA, USA). The cells were treated with MTT solution for 1 h, the dark blue formazan crystals that formed in intact cells were solubilized with dimethyl sulphoxide, and the absorbance was measured at 570 nm with a microplate reader. Percentage cell viability was calculated based on the absorbance of the metformin-treated cells relative to the absorbance of the control vehicle-treated cells.

RNA isolation and RT-PCR analysis

RT-PCR or real-time PCR were performed to evaluate MDR1 mRNA expression after metformin treatment. Total RNA was isolated with an RNA isolation kit (Takara Shuzo, Kyoto, Japan), according to the manufacturer's protocol, and the RNA quality was confirmed by an optical density measurement of A260/A280 >1.8. PCR product formation was monitored continuously using Sequence Detection System software, version 1.7 (Applied Biosystems, Foster City, CA, USA). The cDNA was amplified by 25 PCR cycles of 94°C

for 30 s, 56°C for 30 s and 72°C for 45 s, using Taq DNA polymerase and the following primers: 5′-GCCTGGC AGCTGGAAGACAAATACACAAAATT-3′ and 5′-CAGACAG CAGCTGACAGTCCAAGAACAGGACT-3′ for MDR1, and 5′-GATGATATCGCCGCGCTCGTCGTCGAC-3′ and 5′-AGCC AGGTCCAGACGCAGGATGGCATG-3′ for β-actin. The PCR products were electrophoresed in 1.5% agarose gels, visualized by ethidium bromide staining, and photographed under ultraviolet light. The primers for real-time PCR were the same as those for RT-PCR. Accumulated PCR products were detected directly by monitoring the increase in the reporter dye (SYBR) Green. The quantity of each transcript was calculated as described in the instrument manual and normalized to the amount of β-actin.

Western blot analysis

After treatment, the cells were collected and washed with phosphate-buffered saline (PBS). The harvested cells were lysed on ice for 30 min in 100 μL of lysis buffer (120 mM NaCl, 40 mM Tris, pH 8, 0.1% Nonidet P-40) and centrifuged at 14 000× *g* for 30 min. The supernatants were collected, and protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). Aliquots of the lysates (40 μg of protein) were boiled for 5 min and electrophoresed in 10% SDS-polyacrylamide gels. The separated proteins were transferred onto nitrocellulose membranes, which were incubated with antibodies against MDR1, phospho-IκB-α, phospho-AMPK, AMPK, phospho-CREB, CREB, phospho-GSK-3β, GSK-3β and β-actin, followed by incubation with secondary anti-mouse or anti-rabbit antibodies.

Preparation of nuclear and cytosolic extracts

Nuclear extracts were prepared with a commercial kit according to the manufacturer's instructions (Active Motif, Carlsbad, CA, USA). All steps were performed on ice or at 4°C, unless stated otherwise. Protease inhibitors (10 μg·mL^{-1} aprotinin and 10 μg·mL^{-1} leupeptin) and reducing agents (1 mM dithiothreitol and 1 mM phenylmethylsulphonyl fluoride) were added to each buffer just prior to use. Briefly, cells were incubated in five volumes of hypotonic Buffer A (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl) on ice for 15 min and homogenized. Nuclei were recovered by centrifugation at 900× *g* for 15 min, and the supernatant was collected as the cytoplasmic extract. The nuclei were washed once using a nuclei wash buffer (10 mM HEPES, pH 7.9, 0.2 mM $MgCl_2$, 10 mM KCl) and extracted using Buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$) for 30 min on ice. Insoluble material was removed by centrifugation at 14 000× *g* for 10 min, and the supernatant was used as the nuclear extract.

Transient transfection and luciferase assay

We used a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) to determine promoter activity. Briefly, cells were plated in 24-well plates at a density of 10^5 cells per well and transiently co-transfected overnight with the hMDR1-Luc, CRE-Luc, or NF-κB-Luc construct and pRL-SV (Renilla luciferase expression for normalization) (Promega) using LipofectAMINE™ 2000 reagent (Invitrogen). The cells were then exposed to metformin, tumour necrosis factor (TNF)-α, H89, forskolin, or compound C for 24 h. Luciferase activities in cell lysates were measured using a luminometer (TD-20; Turner Designs, Sunnyvale, CA, USA). The MDR1 or NF-κB promoter-driven firefly luciferase activities were normalized to *Renilla luciferase* activity.

Preparation of plasmids and transfection of MCF-7/adr cells

Construction of the dominant-negative AMPK plasmid (DN-AMPK), p65-GFP plasmid, and control plasmid (pcDNA 3.1) has been described previously (Lee *et al.*, 2003). Cells were transfected with DN-AMPK, p65-GFP, or pcDNA 3.1 in complete medium and cultured for 48 h prior to experimentation.

RNA interference

Small interfering RNA (siRNA) was synthesized by Genolution (Seoul, Korea) with the following sequences: AMPKα, 5′-GGT TGG CAA ACA TGA ATT G-3′. Cells, grown to 50% confluence, were transfected utilizing oligofectamine transfection reagent (Invitrogen, Carlsbad, CA, USA) with AMPKα siRNA or non-specific control siRNA according to the manufacturer's instructions.

Rhodamine-123 accumulation assay

MCF-7/adr cells were seeded onto 24-well plates at a density of 10^5 cells per well. The cells were pretreated with 0.5 to 10 mM metformin and 20 μM verapamil for 48 h. Verapamil was used as a positive control for MDR inhibition (Toffoli *et al.*, 1995). After pretreatment, the cells were incubated with 5 μM Rhodamine-123 (Rh-123) in culture medium in the dark at 37°C in 5% CO_2 for 90 min. The subconfluent monolayer of cells was trypsinized, and the cells were collected by centrifugation, washed twice with ice-cold PBS, and suspended in 1 mL of PBS. To determine intracellular Rh-123 accumulation, fluorescence intensity was measured using a Varioskan reader at 488-nm excitation and 530-nm emission wavelengths.

Statistical analysis

All experiments were repeated at least 3 times. For quantitative analysis, the sum of the density of bands corresponding to protein blotting with the antibody under study was calculated, and the amount of β-actin normalized. One-way ANOVA was used to determine the significance of differences between treatment groups. The Newman–Keuls test was used for multigroup comparisons. Statistical significance was accepted for P-values < 0.01.

Materials

Metformin, verapamil, rhodamine, adriamycin, MG-132 and compound C were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium, foetal bovine serum and trypsin were purchased from Gibco-BRL (Grand Island, NY, USA). Antibodies against phospho-IκB-α, phospho-AMPK, AMPK, phospho-CREB, CREB, phospho-GSK-3β, and GSK-3β, and horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against β-actin, MDR1, p65 and lamin B were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The

MTT was purchased from USB Corp. (Cleveland, OH, USA). The cytotoxicity detection kit used to measure lactate dehydrogenase release was from Roche Applied Science (Indianapolis, IN, USA). All other chemicals were of the highest purity available. Drug and many molecular targets are named according to the nomenclature of *British Journal of Pharmacology*'s Guide to Receptors and Channels (Alexander *et al.*, 2009).

Results

Characterization of MCF-7 and MCF-7/adr cells

To confirm the reported overexpression of MDR1 in MCF-7/adr cells (Fairchild *et al.*, 1987; Zyad *et al.*, 1994), the MDR1 expression level was compared between MCF-7/adr and MCF-7 cells by Western blot analysis, which showed that MDR1 was overexpressed in MCF-7/adr cells (Figure 1). MCF-7 and MCF-7/adr cells were exposed to various concentrations of adriamycin (0.01–40 μg·mL^{-1}) for 48 h. The cytotoxicity of adriamycin was higher in MCF-7 cells than in MCF-7/adr cells (Figure 1B), further confirming MDR1 overexpression in MCF-7/adr cells.

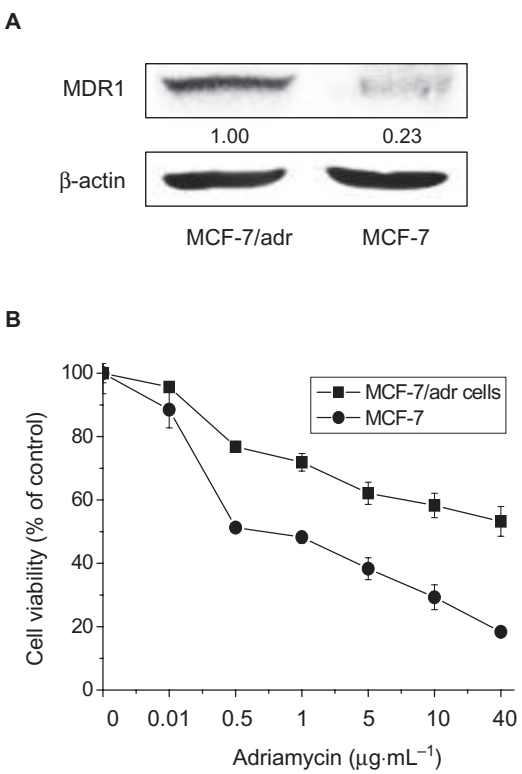

Figure 1

Characterization of MCF-7 and MCF-7/adriamycin (MCF-7/adr) cells. (A) The protein product of the multidrug resistance 1 (MDR1) gene in MCF-7/adr and MCF-7 cells was analysed by Western blotting. MDR1 was overexpressed in the resistant (MCF-7/adr) cells. (B) Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after adriamycin treatment in MCF-7 (sensitive) and MCF-7/adr (resistant) cells for 48 h. Each point shows the mean ± SD of three independent experiments, performed in triplicate. (A, B) Representative results are shown for experiments that were repeated independently 3 times.

Metformin increases adriamycin toxicity in MCF-7/adr cells

First, the cytotoxicity of metformin (0.3–10 mM) against MCF-7/adr cells treated for 24 h was determined using the MTT assay (Figure 2A). Next, as some studies have reported that adriamycin causes MDR in breast cancer cells (Smith and Zilfou, 1995), a possible effect of metformin on adriamycin cytotoxicity was examined in MCF-7/adr and MCF-7 cells. The cells were pretreated with metformin and then incubated with various concentrations of adriamycin for an additional 48 h. Cell viabilities were analysed by the MTT assay. As shown in Figure 2B and C, metformin enhanced the cytotoxicity of adriamycin against MCF-7/adr cells, but did not affect the cytotoxicity of adriamycin against MCF-7 cells (Figure 2D and E). These results indicate that metformin selectively increased the cytotoxic effect of adriamycin in MCF-7/adr cells.

Metformin decreases MDR1 mRNA and protein expression in MCF-7/adr cells

Overexpression of MDR1 mRNA and protein has been associated with the MDR phenotype (Gottesman *et al.*, 2002). RT-PCR and real-time PCR were performed to detect the change in MDR1 mRNA levels upon treatment with metformin. As shown in Figure 3A, the MDR1 mRNA level decreased in a dose- and time-dependent manner following metformin treatment. Real-time quantitative PCR confirmed these results (Figure 3B). The changes in MDR1 protein expression in MCF-7/adr cells after incubation with various metformin concentrations for 24 h or with 10 mM metformin for 3–48 h were determined by Western blot analysis. Metformin decreased the MDR1 protein level in a dose- and time-dependent manner (Figure 3C). Thus, metformin suppresses MDR1 expression in MCF-7/adr cells.

Metformin enhances intracellular Rh-123 accumulation in MCF-7/adr cells

The efflux of fluorescent Rh-123 is P-gp-dependent and has consequently been used extensively to determine efflux from drug-resistant cell lines expressing P-gp (Lugo and Sharom, 2005). Rh-123 enters cells by passive diffusion and is actively transported out of cells by P-gp. The accumulation of Rh-123 in MCF-7/adr cells was measured to determine whether the observed changes in MDR1 expression were correlated with changes in P-gp function. Following pretreatment with various concentrations of metformin or 20 μM verapamil, MCF-7/adr cells were incubated with 5 μM Rh-123 for 90 min. Figure 3D shows a significant accumulation of intracellular Rh-123 in MCF-7/adr cells after treatment with metformin, reflecting the decreased MDR1 expression (Figure 3C).

Metformin suppresses MDR1 expression by inhibiting NF-κB activation

NF-κB activation is a regulatory mechanism for MDR1 gene transcription (Deng *et al.*, 2001). To elucidate the effects of

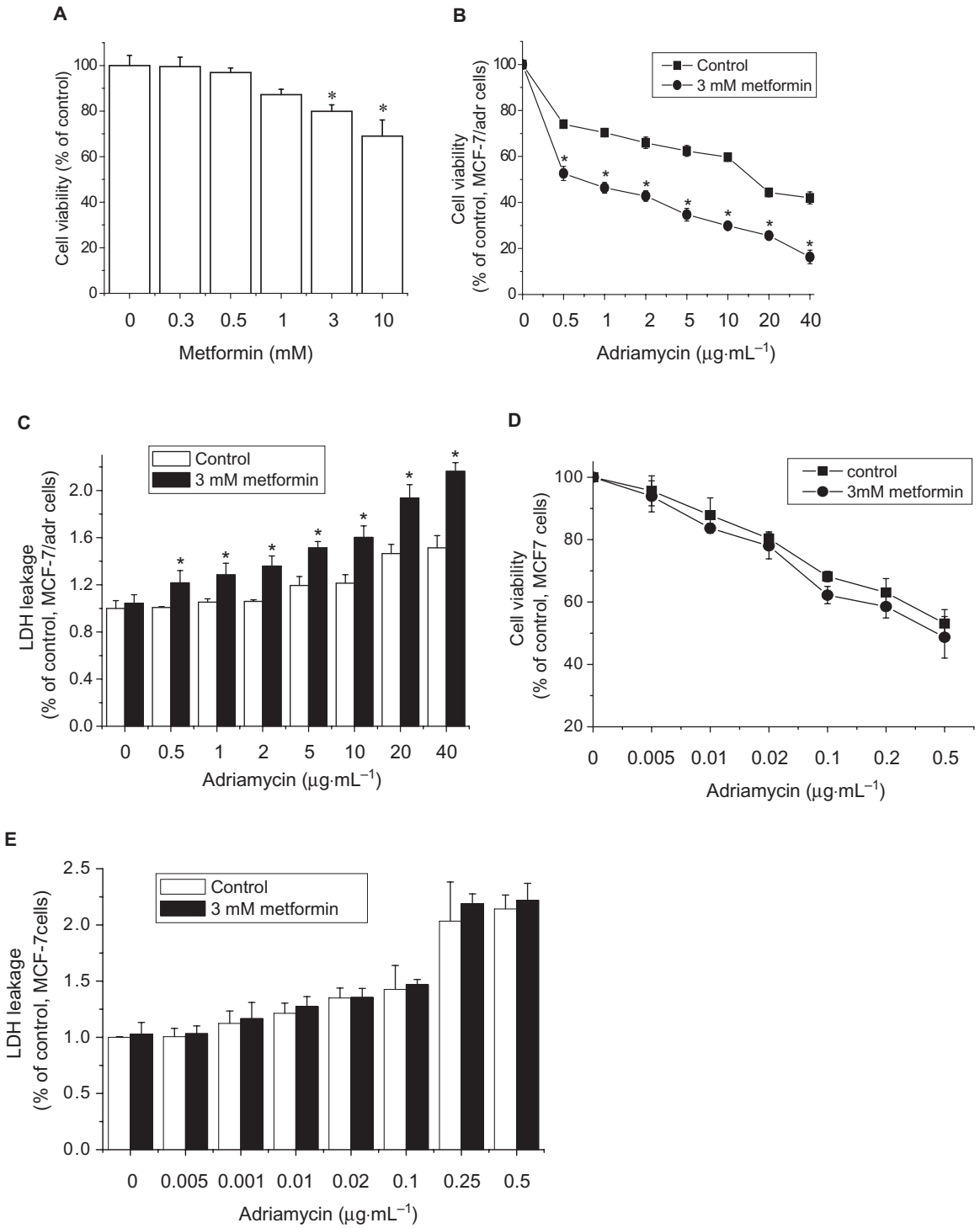

Figure 2

Metformin enhanced the cytotoxicity of adriamycin in MCF-7/adriamycin (MCF-7/adr) cells. (A) The cytotoxicity of metformin (0.5–10 mM) in MCF-7/adr was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Each column shows the mean ± SD of three independent experiments, performed in triplicate. *Significantly different from untreated cells ($P < 0.01$). (B–E) Effects of metformin and adriamycin in MCF-7/adr (B, C) and MCF-7 cells (D, E). Cells were pretreated with or without 3 mM metformin, followed by incubation with various concentrations of adriamycin for an additional 48 h. Cell viability was determined by MTT and lactate dehydrogenase (LDH) assays. Each column/point shows the mean ± SD of three independent experiments, performed in triplicate. *Significantly different from untreated cells ($P < 0.01$). (A–E) Representative results are shown for experiments that were repeated independently 3 times.

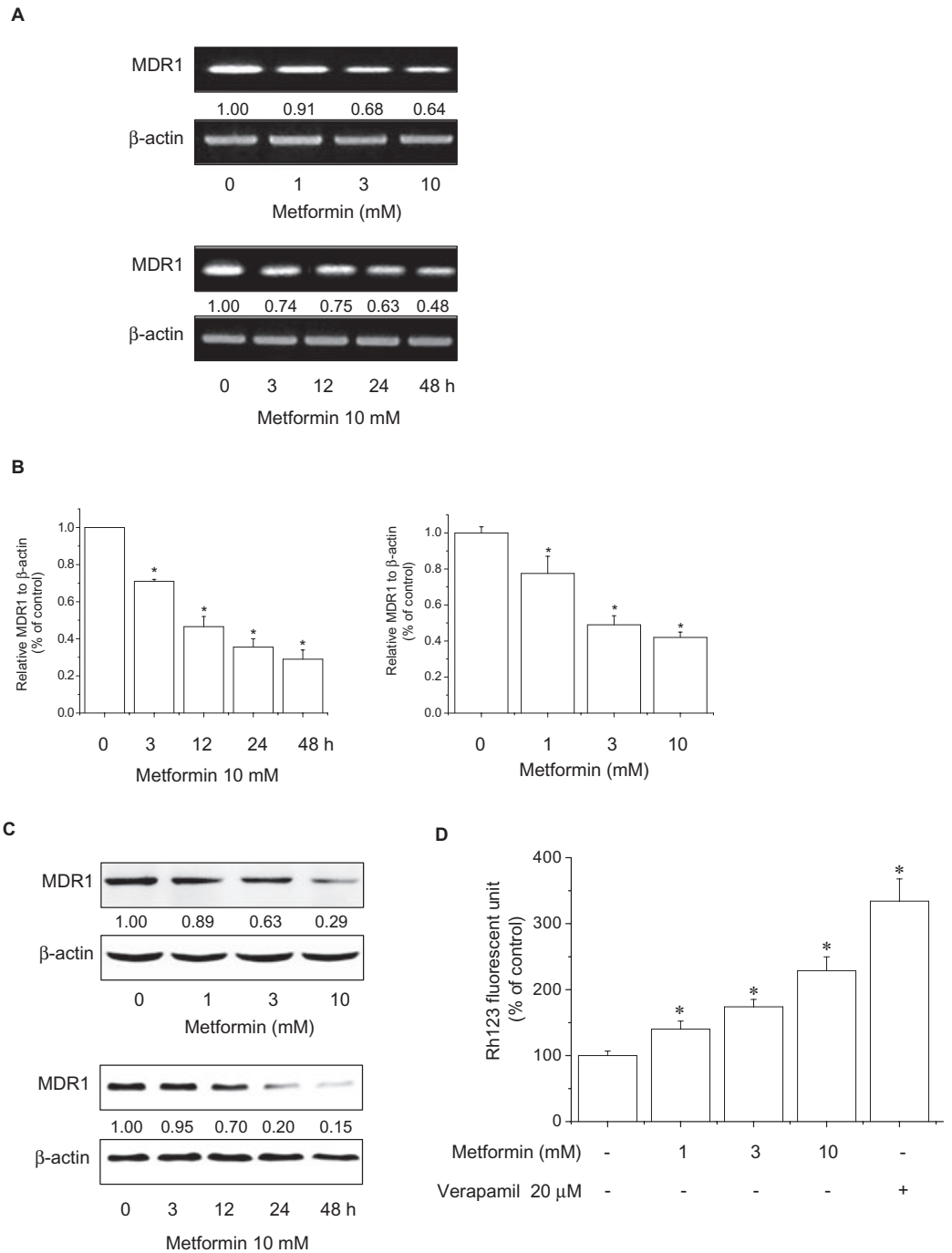

Figure 3

Effect of metformin on multidrug resistance 1 (MDR1) mRNA and protein expression in MCF-7/adriamycin (MCF-7/adr) cells. (A) Cells were treated with metformin (1–10 mM) for 24 h or with 10 mM metformin for 3–48 h. Total RNA was extracted. MDR1 expression was analysed by semiquantitative RT-PCR. The β-actin band confirms the integrity and equal loading of RNA. (B) Under identical conditions, cells were lysed, and total RNA was prepared to analyse MDR1 gene expression by real-time PCR. For normalization, β-actin was amplified in each sample. MDR1 mRNA expression was compared between metformin-treated and untreated cells by real-time PCR at each time point. Each column shows the mean ± SD of three independent experiments, performed in triplicate. *Significantly different from untreated cells ($P < 0.01$). (C) Lysates of cells treated with various concentrations of metformin for 24 h or with 10 mM metformin for 3–48 h were analysed by Western blotting with a MDR1-specific antibody. The β-actin band confirms the integrity and equal loading of protein. (D) Effect of metformin on intracellular rhodamine-123 (Rh-123) accumulation in MCF-7/adr cells. Cells were treated with vehicle, 1–10 mM metformin, or 20 μM verapamil (positive control) for 48 h and then exposed to 5 μM Rh-123 for 90 min. Intracellular Rh-123 accumulation was then measured. Each column shows the mean ± SD of three independent experiments, performed in triplicate. *Significantly different from untreated cells ($P < 0.01$). (A–D) Representative results are shown for experiments that were repeated independently 3 times.

metformin on NF-κB activation and MDR1 promoter activity, MCF-7/adr cells were transfected with reporter plasmids containing NF-κB responsive elements or the MDR1 promoter and were then treated with various concentrations of metformin and TNF-α. Metformin dramatically inhibited NF-κB activation and MDR1 promoter activity (Figure 4A and B). Furthermore, metformin dose-dependently suppressed adriamycin-activated NF-κB activation and MDR1 promoter activity.

NF-κB is composed of p65 and p50 subunits, and inactive NF-κB dimers are sequestered in the cytosol in association with various inhibitory molecules of the IκB family. Stimulation of cells with TNF-α causes phosphorylation of the inhibitor IκBα, leading to its polyubiquitination and proteasome-mediated degradation and the release of active NF-κB (Ashkenazi and Dixit, 1998). Furthermore, Hattori *et al*. reported that metformin decreases TNF-α-induced NF-κB p65 in nuclear extracts, in a dose-dependent manner. Hence, we next examined whether metformin inhibition of NF-κB and MDR1 occurs by inhibiting IκB-α phosphorylation in MCF-7/adr cells. Lysates of the metformin-treated cells were analysed by Western blotting with anti-phospho-Ser32 of IκB-α and anti-p65 antibodies. Figure 4C shows that metformin partially inhibited phospho-IκB-α, in a dose-dependent manner. Moreover, the metformin-induced decrease in the level of phospho-IκB-α occurred in a time-dependent manner, with an observable decrease at 15 min (Figure 4D). Consistent with this, the amount of the nuclear p65 subunit of NF-κB was significantly decreased (Figure 4E). Furthermore, metformin inhibited the TNFα-induced protein and mRNA expression of the MDR1. Metformin inhibited the TNFα-induced NF-κB activation pathway (Supporting information). To further examine the involvement of NF-κB in the suppression MDR1 by metformin, MCF-7 and MCF-7/adr cells were transfected with p65-GFP prior to incubation with metformin for 24 h. MDR1 expression and activity were induced by p65-GFP, and this effect was reduced in the presence of metformin (Figure 4F and G). Also, MDR1 expression was induced by p65-GFP in MCF-7 cells. These data indicate that metformin suppressed MDR1 by inhibiting NF-κB activation.

MDR1 suppression by metformin involves the AMPK pathway

The treatment of MCF-7/adr cells with 3 mM metformin caused the time-dependent phosphorylation of AMPK and its downstream target ACC, whereas AKT phosphorylation was decreased (Figure 5A). Thus, metformin activated AMPK in MCF-7/adr cells, as reported previously in skeletal muscle and endothelial cells *in vitro* (Musi *et al*., 2002; Zou *et al*., 2004; Hattori *et al*., 2006). To address the role of AMPK activation in the inhibitory effect of metformin on MDR1 expression, we examined the effect of an AMPK inhibitor (compound C) on MCF-7/adr cells. As shown in Figure 5B and C, compound C blocked metformin-induced AMPK phosphorylation, and metformin inhibition of MDR1 expression was prevented by co-treatment with compound C, whereas compound C alone did not affect MDR1 expression. Similarly, MDR1 promoter activity was attenuated after co-treatment of metformin with compound C (Figure 5D). To confirm the role of AMPK in MDR1 suppression by metformin, we knocked down AMPK in MCF-7/adr cells using a siRNA. After transfection with siRNA for AMPK (or control) for 24 h, MCF-7/adr cells were treated with 10 mM metformin for 24 h; this treatment significantly reduced AMPK expression compared with the control (Figure 5E). As expected, knockdown of AMPK expression by siRNA modulated AMPK signals. Figure 5E showed that transfection with AMPK siRNA markedly decreased metformin-stimulated AMPK and AKT phosphorylation, compared with phosphorylation levels in control cells, and increased metformin-inhibited MDR1 expression, compared with controls. Furthermore, AMPK depletion by siRNA transfection significantly prevented metformin-induced inhibition of nuclear translocation of p65 compared with the control (Figure 5E). Additionally, the increased intracellular accumulation of Rh-123 induced by metformin was attenuated in AMPK siRNA transfected-cells (Figure 5F). These results indicate that metformin suppression of MDR1 transcription involves the AMPK pathway.

Metformin suppresses MDR1 via CREB transcriptional activity by up-regulating AMPK

Recently, Horike *et al*. reported that AICAR (an AMPK activator) increased GSK-3β, AMPK and ACC phosphorylation, but decreased CREB phosphorylation in HepG2 cells (Horike *et al*., 2008). We examined the role of AMPK-dependent CREB activity in MDR1 expression. In cells treated with metformin, AMPK, GSK-3β and ACC phosphorylation was increased significantly, whereas CREB phosphorylation was decreased (Figure 6A). To determine whether AMPK activation is required for CREB-dependent MDR1 suppression by metformin, MCF-7/adr cells were transfected with pcDNA 3.1 (control) or a dominant-negative form of AMPK (DN-AMPK) and incubated with metformin for 6 or 24 h. Transfection with DN-AMPK markedly attenuated metformin-stimulated AMPK, ACC, and GSK-3β phosphorylation, compared with phosphorylation levels in control cells, and increased metformin-induced inhibition of CREB phosphorylation and MDR1 expression, compared with controls (Figure 6B). Additionally, the increased intracellular accumulation of Rh-123 induced by metformin was prevented in metformin-treated cells transiently transfected with DN-AMPK (Figure 6C). Taken together, these results indicate that metformin inhibits MDR1 expression through AMPK-dependent CRE transcriptional activity.

Discussion

P-glycoprotein, encoded by MDR1, has attracted great interest because of its role in MDR in a variety of cancers (Gottesman and Pastan, 1993; Johnstone *et al*., 2000). P-glycoprotein is overexpressed in cancer cells that actively extrude chemotherapeutic agents (Gottesman and Pastan, 1993). P-glycoprotein affects patients with a variety of blood cancers and solid tumours, including breast, ovarian, lung and lower gastrointestinal tract cancers, and novel compounds that block MDR1 may enhance the efficacy of chemotherapy in these patients. Since the discovery in 1981 of the first P-gp inhibitor, verapamil, various agents, including cyclosporin A,

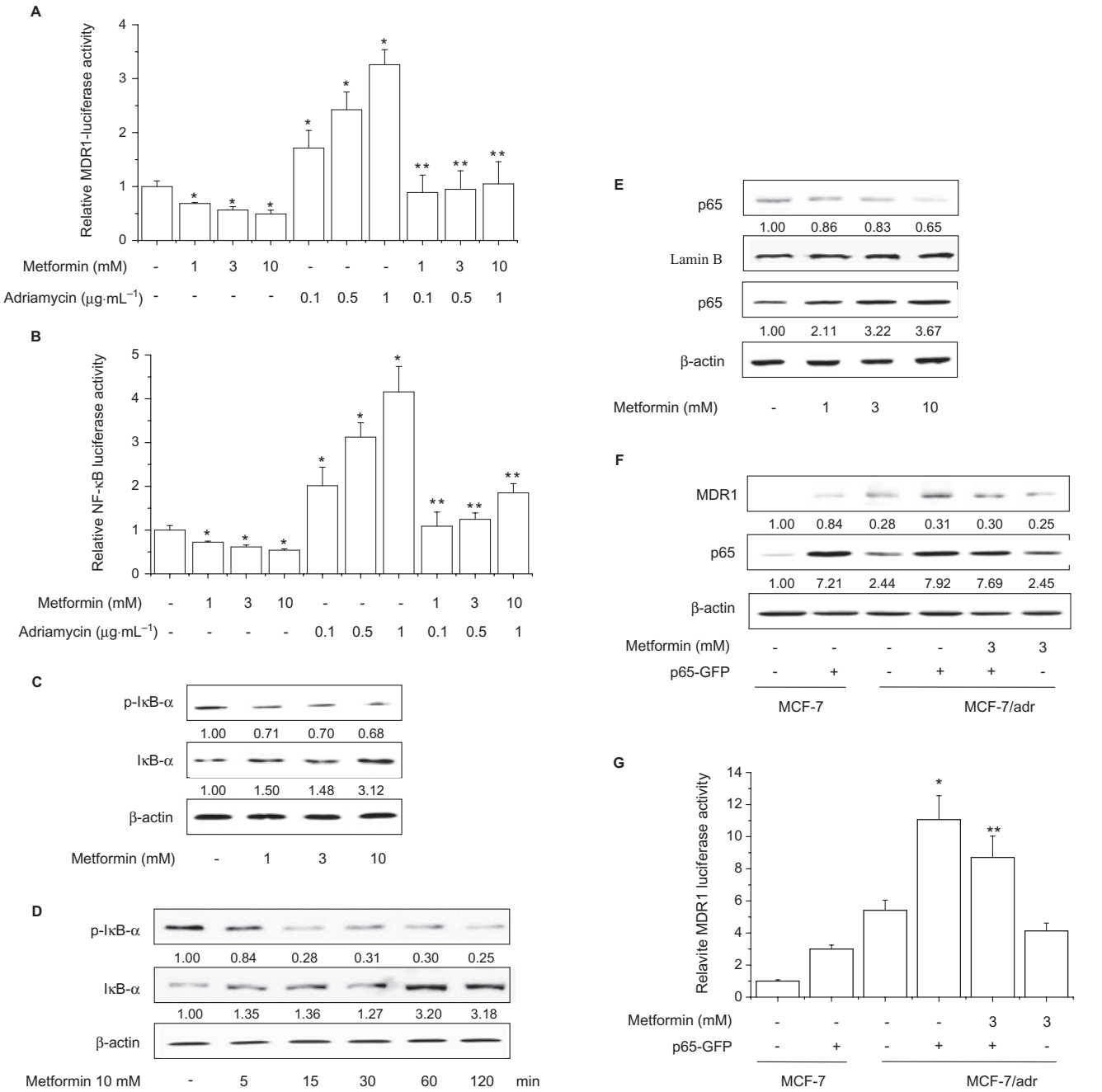

Figure 4

Metformin suppressed the multidrug resistance 1 (MDR1) gene through NF-κB activation. Cells were transiently transfected with plasmids harboring MDR1 and NF-κB reporter genes and treated with 1–10 mM metformin and 0.1–1 μg·mL^{-1} adriamycin for 24 h. The cells were lysed, and MDR1 (A) and NF-κB (B) activities were measured by luciferase assay. Each column shows the mean±SD of three independent experiments, performed in triplicate. *Significantly different from untreated cells ($P < 0.01$). **Significantly different from adriamycin–treated cells ($P < 0.01$). (C) Cells were incubated for 24 h with metformin (0.5–10 mM). The cells were lysed and subjected to Western blot analysis using anti-phospho-IκB-α, anti-IκB-α and anti-β-actin antibodies. Metformin dose-dependently inhibited the TNF-α-induced phospho-IκB-α level. (D) MCF-7/adriamycin (MCF-7/adr) cells were incubated with 10 mM metformin for 5–120 min. The cells were lysed and subjected to Western blot analysis using anti-phospho-IκB-α, anti-IκB-α and anti-β-actin antibodies. Metformin time-dependently inhibited phospho-IκB-α levels. (E) MCF-7/adr cells were incubated with metformin. The cells were lysed, and nuclear extracts were prepared for Western blot analysis using antibodies against nuclear p65 and lamin B. Under identical conditions, the cells were lysed and subjected to Western blot analysis using anti-p65 and anti-β-actin antibodies. (F, G) Cells were transfected with p65-GFP plasmid, incubated with metformin for 24 h, and lysed. MDR1 protein expression was determined by Western blot analysis, and MDR1 activity was determined by luciferase activity. Each column shows the mean ± SD of three independent experiments, performed in triplicate. *Significantly different from untreated MCF-7/adr cells ($P < 0.01$). **Significantly different from metformin-treated MCF-7/adr cells ($P < 0.01$). (A–G) Representative results are shown for experiments that were repeated independently 3 times.

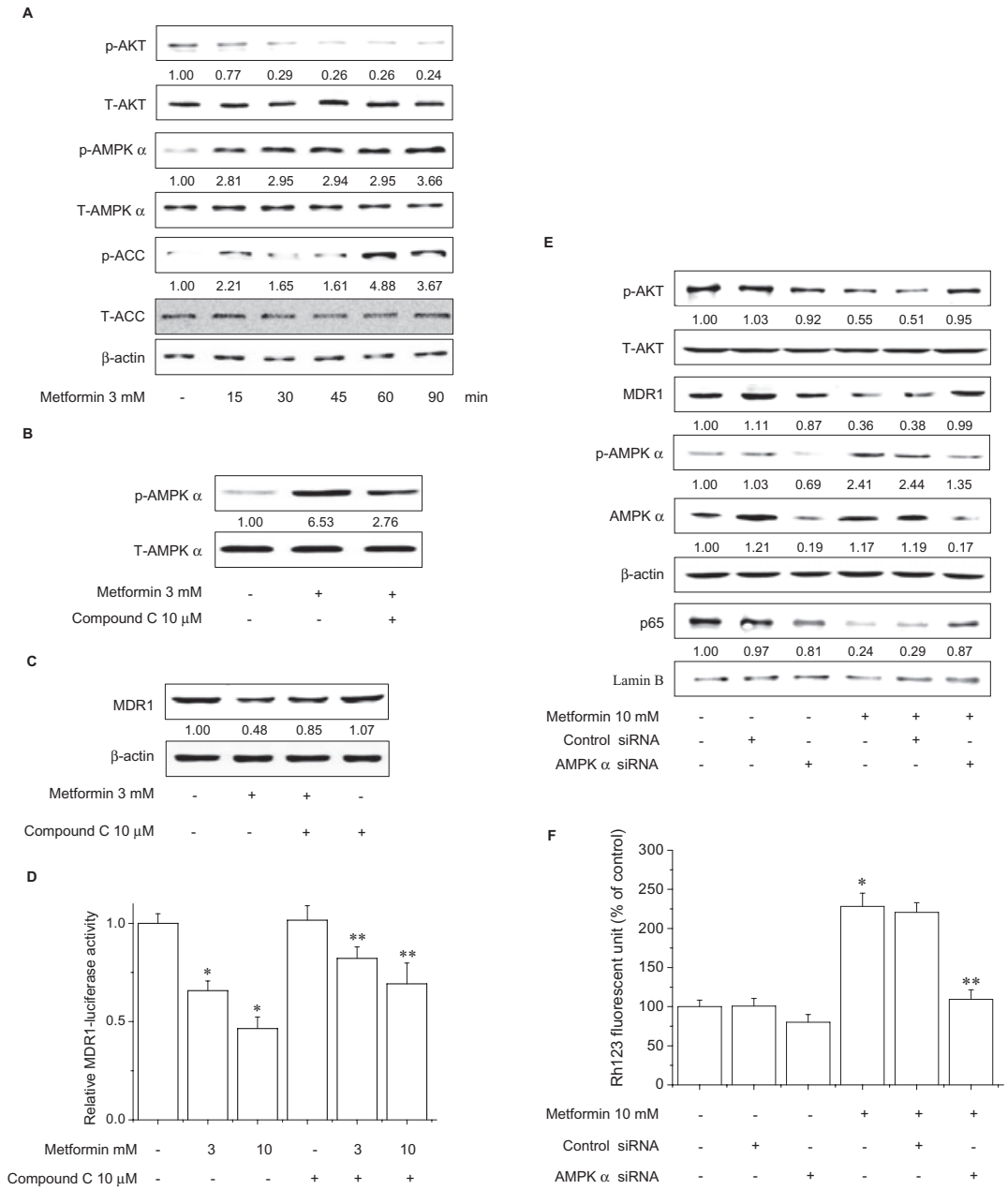

Figure 5

Involvement of the AMP-activated protein kinase (AMPK) pathway in multidrug resistance 1 (MDR1) expression induced by metformin. (A) Metformin stimulated AMPK and acetyl-CoA carboxylase (ACC) phosphorylation in MCF-7/adriamycin (MCF-7/adr) cells at the indicated times. (B) MCF-7/adr cells were treated for 30 min with 10 μM compound C (AMPK inhibitor) and then for 30 min with 3 mM metformin. Phosphorylated AMPK and AMPK were detected by Western blot analysis. (C) The effect of metformin and compound C on MDR1 expression in MCF-7/adr cells. Cells were incubated for 24 h with 3 mM metformin and 10 μM compound C, lysed, and subjected to Western blot analysis using anti-MDR1 and anti-β-actin antibodies. (D) Effect of metformin and compound C on MDR1 promoter activity in MCF-7/adr cells. Cells were transiently transfected with a MDR1 promoter reporter gene and then treated with 3 or 10 mM metformin and 10 μM compound C for 24 h. The cells were lysed, and luciferase activity was measured. Each column shows the mean ± SD of three independent experiments, performed in triplicate. *Significantly different from untreated cells ($P < 0.01$). **Significantly different from compound C-treated cells ($P < 0.01$). (E) MCF-7/adr cells were transfected with AMPK small interfering (siRNA) or control siRNA for 24 h. The transfected cells were treated with 10 mM metformin for 24 h. MDR1 expression and phosphorylation of AKT, AMPK, and MDR were measured by Western blot analysis. The cells were lysed, and nuclear extracts were prepared for Western blot analysis using antibodies against nuclear p65 and lamin B. (F) After transfection with AMPK siRNA or control siRNA for 24 h, cells were treated with 10 mM metformin for 48 h and then exposed to 5 μM rhodamine-123 (Rh-123) for 90 min. The intracellular Rh-123 accumulation was measured. Each column shows the mean ± SD of three independent experiments, performed in triplicate. *Significantly different from untreated cells ($P < 0.01$). **Significantly different from metformin-treated cells ($P < 0.01$). (A–F) Representative results are shown for experiments that were repeated independently 3 times.

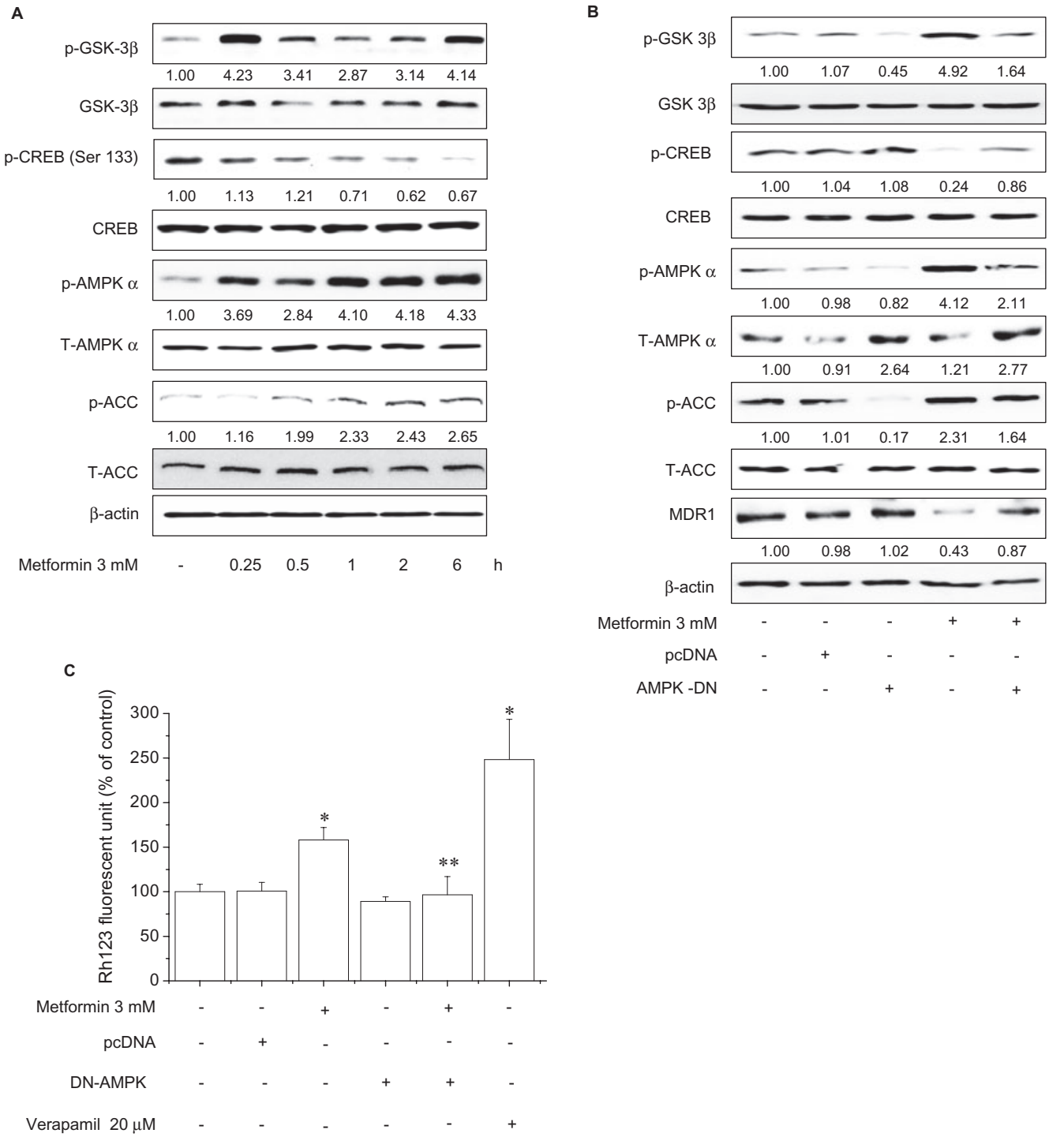

Figure 6

Metformin inhibited the multidrug resistance 1 (MDR1) gene via cAMP response element (CRE) transcriptional activity. (A) MCF-7/adriamycin (MCF-7/adr) cells were treated with 3 mM metformin for 15–360 min, and cell extracts were analysed by Western blotting with antibodies against phospho-AMP-activated protein kinase (AMPK), AMPK, phospho-acetyl-CoA carboxylase (ACC), ACC, phospho-glycogen synthase kinase (GSK)-3β, GSK-3β, phospho-cAMP-responsive element binding protein (CREB) (Ser 133), CREB and β-actin. (B) MCF-7/adr cells were transfected with dominant-negative AMPK (DN-AMPK) or a pcDNA 3.1 control for 6 h. The transfected cells were treated with 3 mM metformin for 6 or 24 h. MDR1 expression and phosphorylation of CREB, AMPK, ACC and GSK-3β were measured by Western blot analysis. (C) After transfection with DN-AMPK or the pcDNA 3.1 control for 6 h, cells were treated with 3 mM metformin or 20 μM verapamil for 48 h and then exposed to 5 μM rhodamine-123 (Rh-123) for 90 min. The intracellular Rh-123 accumulation was measured. Each column shows the mean ± SD of three independent experiments, performed in triplicate. *Significantly different from untreated cells ($P < 0.01$). **Significantly different from metformin-treated cells ($P < 0.01$). (A–C) Representative results are shown for experiments that were repeated independently 3 times.

have been reported to reverse MDR (Twentyman, 1988). However, because these drugs show dose-limiting toxicity, clinical trial results have been disappointing.

The aims of this study were to identify an effective MDR-reversing agent with fewer side effects and to gain insight regarding its molecular mechanism. Metformin (1,1-dimethylbiguanide hydrochloride) is a representative therapeutic agent for type 2 diabetes mellitus. Recently, metformin was reported to inhibit NF-κB activation (Hattori *et al.*, 2006; Huang *et al.*, 2009) and breast cancer cell growth (Vazquez-Martin *et al.*, 2009). However, the relationship between metformin and MDR in MCF-7/adr cells has yet to be firmly established. In the present study, we demonstrated that metformin inhibits MDR1 in MCF-7/adr cells.

Based on reports that it causes MDR in breast cancer cells, adriamycin was used to develop MDR in MCF-7 cells, producing drug-resistant MCF-7/adr cells (Fairchild *et al.*, 1987; Zyad *et al.*, 1994). Metformin enhanced adriamycin cytotoxicity in MCF-7/adr cells (Figure 2B), and the elevated levels of MDR1 mRNA and protein in MCF-7/adr cells were decreased significantly with metformin treatment. Metformin also dramatically inhibited the activity of the MDR1 promoter (Figure 4A). The reduction of MDR1 expression at both the transcriptional and translational levels has been proposed as one mechanism by which certain agents reverse the MDR phenotype (Krishna and Mayer, 2000). Furthermore, metformin increased the intracellular accumulation of Rh-123, presumably by inhibiting P-gp-dependent drug efflux (Figure 3D). The efflux of Rh-123 is known to be P-gp-dependent and has been used extensively to assess efflux from drug-resistant cell lines expressing P-gp (Lugo and Sharom, 2005).

MDR1 expression has been studied in cancer cells, including MCF-7/adr (Zyad *et al.*, 1994; Misra *et al.*, 2005), K562/ADM (Motomura *et al.*, 1998), A549, HCT 116, L1210 (Rappa *et al.*, 1993), HepG2/ADM (Zheng *et al.*, 2008) and Caco-2 cells (De Rosa *et al.*, 2008), and *in vivo* (Deng *et al.*, 2001). The molecular mechanism of induced MDR1 expression has been reported to correlate with NF-κB activation (Deng *et al.*, 2001; Kuo *et al.*, 2002), cyclooxygenases-2 (Fantappiè *et al.*, 2002), CYP3A4 (Lamba *et al.*, 2006), reactive oxygen species (Deng *et al.*, 2001), the mitogen-activated protein kinase pathway, phosphoinositide 3-kinase (Kuo *et al.*, 2002; Misra *et al.*, 2005) and protein kinase C (Zhan *et al.*, 2005). Among these, NF-κB activation is the most frequently mentioned molecular mechanism for inducing MDR. The generation of reactive oxygen species, the activation of IκB kinase, and the degradation of IκB-α and IκB-β result in NF-κB activation (Deng *et al.*, 2001), and previous studies have demonstrated that a NF-κB binding site located at nucleotide position –159 of the MDR1 promoter is involved in growth factor activation of MDR1 (Zhou and Kuo, 1997). Furthermore, the induction of MDR1 expression has been shown to be mediated by a NF-κB activating signal that requires a NF-κB binding site located distal to the MDR1 promoter (Kuo *et al.*, 2002). In the current experiments designed to assess a possible role for NF-κB activation in metformin-suppressed MDR1 expression, metformin was shown to inhibit NF-κB activation by inhibiting IκB-α degradation and p65 nuclear translocation (Figure 4). We found that metformin inhibited the constitutive phosphorylation of AKT. Interestingly, metformin inhibited the NF-kB-dependent MDR1 promoter activity, and this inhibition was attenuated by the transient expression of the constitutive p65. Furthermore, metformin-induced inhibition of AKT phosphorylation was completely abolished by inhibiting AMPK activity by siRNA for AMPK (Figure 5). These results suggest that the ability of metformin to reduce MDR1 expression in MCF-7/adr cells is closely associated with the inhibition of the AKT/NF-kB signalling through the up-regulation of AMPK. The ability of NF-κB activation to induce chemoresistance has previously been related to the down-regulation of apoptosis, but the NF-κB pathway is linked to many aspects of cell growth and apoptosis (Prasad *et al.*, 2009). Our results show that the inhibition of NF-κB activation may be involved in modulating intracellular chemotherapeutic drug accumulation and/or transport.

Until now, there has been no reported evidence for a correlation between MDR1 expression and the AMPK pathway. Several reports have demonstrated that GSK-3β phosphorylates CREB (Fiol *et al.*, 1994) and that a GSK-3β inhibitor increases MDR1 (Lim *et al.*, 2008). However, the suppression of MDR1 expression by GSK-3β has remained unclear. We considered the possibility that GSK-3β contributes to AMPK-induced MDR1 gene suppression. In our study, metformin treatment of MCF-7/adr cells increased AMPK, ACC and GSK-3β phosphorylation, but inhibited CREB phosphorylation (Figure 6A). In addition, an AMPK inhibitor (compound C) blocked metformin-induced AMPK phosphorylation. Similarly, transient transfection experiments and Western blot analysis provided further evidence that the metformin-induced decrease in MDR1 involved AMPK (Figure 5C and D). The transfection of cells with DN-AMPK prior to incubation with metformin increased the metformin-induced inhibition of CREB phosphorylation and MDR1 expression, compared with controls (Figure 6B). In addition, the increased intracellular accumulation of Rh-123 induced by metformin was abolished in metformin-treated cells transiently transfected with DN-AMPK or siRNA for AMPK (Figure 6C). These results suggest a new molecular mechanism for metformin reversal of MDR in MCF7-adr cells via CRE transcriptional activity through the up-regulation of AMPK (Zang *et al.*, 2004).

In conclusion, our data demonstrate that metformin inhibits MDR1 expression in MCF-7/adr cells by two mechanisms. First, the finding that drug efflux and accumulation was not affected in the parental cell line MCF-7 (MDR1 non-expressing) strongly indicates that the reversal of drug resistance by metformin is probably attributable to the inhibition of MDR1-mediated efflux. Second, metformin suppresses MDR1 expression by inhibiting CRE and NF-κB transcriptional activity through the up-regulation of AMPK. The antidiabetic drug metformin warrants further exploration as a potential chemotherapeutic agent in drug-resistant human breast cancer cells.

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Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Metformin suppressed the multidrug resistance 1 (MDR1) gene through NF-κB activation.

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