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Metadoxine, an ion-pair of pyridoxine and L-2-pyrrolidone-5-carboxylate, blocks adipocyte differentiation in association with inhibition of the PKA-CREB pathway

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ABSTRACT

Adipogenesis is orchestrated by the expression of master adipogenic regulators. In particular, phosphorylation of cAMP response element binding protein (CREB) by protein kinase A promotes CREB nuclear translocation, thereby inducing expression of the adipogenic regulators and resulting in adipogenic maturation. Although metadoxine, an ion-pair of pyridoxine and L-2-pyrrolidone-5-carboxylate, has been shown to inhibit lipid accumulation in the liver, its effect on adipocyte differentiation has never been explored. This study investigated the effects of metadoxine on the differentiation of 3T3-L1 preadipocytes and the molecular mechanism. Metadoxine treatment did not inhibit mitotic clonal expansion, but inhibited late-stage cell differentiation, suggesting that metadoxine may block the differentiation step of preadipocytes. Metadoxine inhibited CREB phosphorylation and binding to the cAMP response element, thereby repressing CCAAT/enhancer-binding protein β during hormone-induced adipogenesis. Overall, metadoxine inhibits adipogenic differentiation in association with the inhibition of CREB/cAMP response element-dependent CCAAT/enhancer-binding protein β induction in the protein kinase A-CREB pathway.
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Introduction

Obesity has become a widespread issue in modern society. When energy input exceeds energy expenditure, adipose tissue mass increases by adipocyte hyperplasia (e.g., increase in cell number) and hypertrophy (e.g., increase in cell size) [1]. In order to manage the health concerns associated with obesity, it is necessary to understand its development and regulation. At the cellular level, obesity is considered as a hypertrophic disease resulting from an increase in adipogenesis (e.g., increase in fat cell volume and size) [2].

Adipocyte growth and differentiation can be modeled using the 3T3-L1 preadipocyte cell line. Hormones stimulate differentiation in the 3T3-L1 preadipocyte model, which includes synchronous reentrance of cells into the cell-cycle, mitotic clonal expansion and adipogenic differentiation [3,4]. This process is characterized by a rapid and transient increase in CCAAT/enhancer-binding protein β (C/EBPβ)[1] [5]. DNA-binding by C/EBPβ occurs after phosphorylation by cellular kinases, especially mitogen activated protein kinases (MAPKs) [6], cells then reenter the cell cycle and begin terminal differentiation. It has been well established that C/EBPα and peroxisome proliferator-activated receptor γ (PPARγ) coordinate the expression of genes that create and maintain the adipocyte phenotype [4].

cAMP response element binding protein (CREB) is constitutively expressed in preadipocytes prior to the induction of adipogenesis and throughout the differentiation process [7,8]. CREB is activated by differentiation-inducing agents, such as insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX). CREB binds to the putative cAMP response element (CRE) in the promoters of several adipocyte-specific genes and regulates their transcription [8,9]. Several lines of evidence have implicated that CREB activation is necessary and sufficient to induce adipogenesis [8,9]. For the promotion of CREB activity, protein kinase A

[1] *Abbreviations used:* ACC, acetyl CoA carboxylase; AMPK, AMP-activated protein kinase; CDK, cyclin-dependent kinase; C/EBP, CCAAT/enhancer-binding protein; CRE, cAMP response element; CREB, cAMP response element binding protein; FAS, fatty acid synthase; IBMX, 3-isobutyl-1-methylxanthine; LAP, liver-enriched transcriptional activating protein; LIP, liver-enriched transcriptional inhibitory protein; MAPK, mitogen activated protein kinase; MDI, 3-isobutyl-1-methylxanthine, dexamethasone and insulin; PBS, phosphate-buffered saline; PKA, protein kinase A; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR response elements; RXR, retinod X receptor; SREBP, sterol regulatory element binding protein.

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(PKA), as the cellular target of cAMP, catalyzes phosphorylation of CREB at serine 133 and thereby activates transcription of target genes [10,11]. Activated CREB increases the expression of C/EBPβ at an early time point in the adipogenic differentiation process. CREB activates C/EBPβ, which triggers a cascade of transcriptional events and consequently promotes activation of the C/EBPα and PPARγ genes. Then, C/EBPα and PPARγ enhance the transcription of the set of genes that give rise to the adipocyte phenotype [9].

Metadoxine, or pyridoxol L-2-pyrrolidone-5-carboxylate (the ion-pair between pyrrolidone carboxylate and pyridoxine) (Fig. 1A), has been studied as a treatment for alcoholic liver disease [12,13]. In an animal model, metadoxine treatment increased the clearance of alcohol and acetaldehyde, reduced the damaging effect of free radicals, and enabled cells to restore cellular ATP and glutathione levels [14]. Moreover, it has been clinically used to treat alcoholic liver disease [13]. In a previous study, we showed that the combined treatment of metadoxine and garlic oil efficaciously abrogates alcoholic steatosis and CYP2E1 induction in rat liver with restoration of AMP-activated protein kinase (AMPK) activity [12]. In addition, it has been recognized that metadoxine may be useful for treating non-alcoholic steatohepatitis partly because the agent inhibits oxidative damage [15]. Although metadoxine was shown to inhibit lipid accumulation in the liver, the effect of this agent on adipocyte growth and differentiation has never been explored.

This study investigated whether metadoxine regulates adipocyte growth and differentiation, and if so, by what molecular mechanism. First, we examined the effects of metadoxine on the mitotic clonal expansion and differentiation of 3T3-L1 preadipocytes and found that it has an inhibitory effect on adipocyte differentiation, but not proliferation. In addition, our observation indicated that the metadoxine blockade of adipocyte differentiation might be associated with inhibition of the PKA-CREB pathway and repression of CREB/CRE-dependent C/EBPβ induction.

Materials and methods

Materials

Metadoxine was provided from Pharma-King Pharmaceutical Co (Seoul, Korea). IBMX, dexamethasone, and insulin were obtained from Sigma–Aldrich (St. Louis, MO). Antibodies directed against PPARγ, C/EBPα, C/EBPβ, sterol regulatory element binding protein-1 (SREBP1), p21, p27, and cyclin A were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies recognizing CREB, p-CREB (Ser133), acetyl coenzyme A carboxylase (ACC), ERK, p-ERK (Thr202/Tyr204), JNK, p-JNK(Thr183/Tyr185), p38 kinase, and p-p38 kinase (Thr180/Tyr182) were obtained from Cell Signaling Technology (Beverly, MA). Anti-fatty acid synthase (FAS) antibody was supplied from BD Biosciences Pharmingen (San Jose,

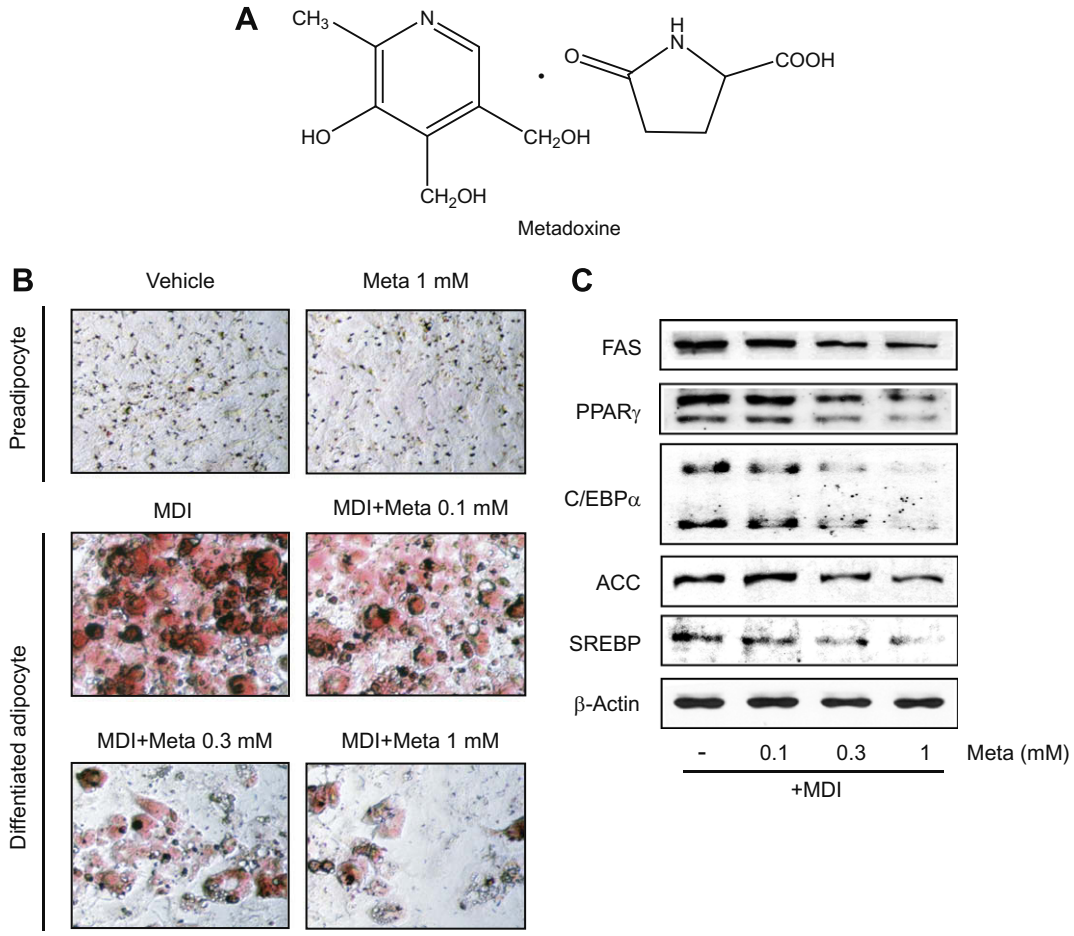

Fig. 1. The anti-adipogenic effect of metadoxine (Meta). (A) The chemical structure of metadoxine (pyridoxol L-2-pyrrolidone-5-carboxylate). (B) Oil Red O staining of cytoplasmic triglycerides. 3T3-L1 preadipocytes were treated with 0.1–1 mM metadoxine in a medium containing MDI. 3T3-L1 preadipocytes incubated in a medium lacking MDI were used as a negative control. Microphotographs show the cells fixed in 10% formalin and stained with Oil Red O at day 8. (C) Immunoblottings for adipogenic marker proteins. The cell lysates were subjected to immunoblottings for FAS, PPARγ, C/EBPα, ACC, and SREBP. Equal loading of proteins was verified by probing the replicate blot for β-actin. Results were confirmed by three separate experiments and representative blots are shown.

CA). Anti-β-actin antibody was provided from Sigma–Aldrich (St. Louis, MO). [Methyl-^{3}H]thymidine was purchased from Amersham Biosciences (Buckinghamshire, UK).

Cell culture

The 3T3-L1 preadipocyte cell line was obtained from ATCC (Rockville, MD). The cells were maintained in growth medium containing Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 50 U/ml penicillin, and 50 μg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2.

Adipocyte differentiation

3T3-L1 preadipocytes were induced to differentiate as previously described [16]. In brief, 2 day-postconfluent preadipocytes (day 0) were cultured in growth medium containing 0.5 mM IBMX, 1 μM dexamethasone, and 1 μg/ml insulin (MDI) for 2 days. The cells were further incubated in growth medium containing 1 μg/ml insulin for additional 2 days, and thereafter medium was replaced every 2 days. At day 8, the cells were stained with Oil Red O or harvested for immunoblot analyses. If necessary, the cells were counted after harvest on a hemocytometer under light microscopy.

Oil Red O staining

In order to determine the state of adipocyte differentiation, the cells were washed with phosphate-buffered saline (PBS), fixed with 10% formalin for 1 h, and stained with Oil Red O prior to visual inspection.

[^{3}H]Thymidine incorporation

The rate of DNA synthesis was measured using [^{3}H]thymidine incorporation according to the previously published method [17]. Post-confluent cells were incubated with metadoxine, insulin, and/or the MDI for 2 days. The cells were pulse-labeled with 1 μCi/ml [^{3}H]thymidine for 8 h. Chromosomal DNA was precipitated with trichloroacetic acid and extracted with 0.5 N NaOH/0.5% sodium dodecyl sulfate. The radioactivity was quantitated using a liquid scintillation counter.

Luciferase reporter gene assay

We used the Dual-Luciferase Reporter Assay System (Promega). Briefly, 3T3-L1 cells were seeded in six-well plates overnight, serum starved for 3 h, and transiently transfected with PPAR response element (PPRE) promoter-luciferase construct and pRL-SV plasmid (a plasmid that encodes for Renilla luciferase and is used to normalize transfection efficacy) in the presence of Lipofectamine Plus Reagent (Life Technologies, Inc. Gaithersburg, MD) for 3 h. Transfected cells were incubated in Eagle's minimal essential medium containing 1% fetal bovine serum for 3 h and exposed to metadoxine for 48 h at 37 °C. Firefly and Renilla luciferase activities in cell lysates were measured using a Luminoskan luminometer (Thermo Labsystems, Helsinki, Finland). The activity of firefly luciferase was measured by adding Luciferase Assay Reagent II (Promega) according to the manufacturer's instructions, and, after quenching the reaction, the Renilla luciferase reaction was initiated by adding Stop & Gloreagent (Promega). The relative luciferase activity was calculated by normalizing firefly luciferase activity to that of Renilla luciferase.

Immunoblot analysis

Cell lysates were prepared according to previously published methods [18]. Briefly, the cells were lysed in buffer containing 10 mM Tris–HCl (pH 7.1), 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Triton X-100, 0.5% Nonidet P-40, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonylfluoride, supplemented with a protease inhibitor cocktail (Calbiochem, La Jolla, CA). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblot analyses were performed as described previously [18]. Immunoreactive protein was visualized by the ECL chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK) or the SuperSignal West Pico chemiluminescent substrate kit (Pierce, Rockford, IL). Equal loading of proteins was verified by immunoblotting for β-actin. At least three separate experiments were performed with different samples to confirm changes in protein levels.

Preparation of nuclear extracts

Cells were washed with ice cold PBS, scraped from the surface and transferred to microtubes. Swelling was induced by adding 50 μl of lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonylfluoride] followed by vortexing to disrupt cell membranes. The samples were then incubated for 10 min on ice and centrifuged for 5 min at 4 °C. Next, pellets containing crude nuclei were resuspended in 25 μl of the extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride, and then incubated for 30 min on ice. Finally, the samples were centrifuged at 10,000*g* for 10 min to obtain the supernatant containing nuclear extracts.

Gel shift assay

A double-stranded DNA probe for the consensus sequence of CREB (5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′) or C/EBP (5′-TGCAGATTGCGCAATCTGCA-3′) was used for gel shift analyses after end labeling of the probes with [γ-^{32}P]ATP and T4 polynucleotide kinase. The reaction mixtures contained 2 μl of 5× binding buffer containing 20% glycerol, 5 mM $MgCl_2$, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM dithiothreitol, 0.25 mg/ml poly dI-dC, 50 mM Tris–HCl (pH 7.5), 10 μg of nuclear extracts and sterile water in a total volume of 10 μl. Reactions were initiated by the addition of 1 μl probe (10^6 cpm) following 10 min preincubation and continued for 20 min at room temperature. The specificity of protein binding to the DNA was confirmed by competition reactions in which a 20-fold molar excess of unlabeled oligonucleotides was added to each reaction mixture before the addition of radiolabeled probe. In some experiments, the specificity of CREB binding to the DNA consensus sequence was confirmed by supershift analysis using specific antibodies (2 μg each). Samples were loaded onto 4% polyacrylamide gels and run at 140 V. The gels were removed, fixed and dried, followed by autoradiography.

Protein kinase A (PKA) assay

PKA assay was performed using a commercially available kit (Upstate Biotechnology, Lake Placid, NY), according to the manufacturer's instructions. The reaction mixture consisted of 20 μM cAMP, 1 mM kemptide, protein kinase C/Ca^{2+}-calmodulin-dependent protein kinases inhibitor cocktail and PKA inhibitor peptide in the dilution buffer containing 100 mM MOPS (pH 7.2), 125 mM β-glycerophosphate, 25 mM EGTA, 5 mM sodium orthovanadate, and 5 mM dithiothreitol. Reaction was initiated by adding cell lysates and magnesium/ATP cocktail containing [γ-^{32}P]ATP

(3000 Ci/mmol) to the buffer solution, and was continued for 10 min at 30 °C. The aliquots of reaction mixture were blotted onto P81 paper. The paper was washed three times with 0.75% phosphoric acid, and once with 90% ethanol. The radioactivity of phosphorylated substrate was measured using a β-counter (Wallac, Gaithersburg, MD).

Data analysis

Scanning densitometry of the immunoblots was performed with the Image Scan & Analysis System (Alpha-Innotech Corporation, San Leandro, CA). The area of each lane was integrated using the software AlphaEase™ version 5.5, followed by background subtraction.

Results

The anti-adipogenic effect of metadoxine

We first determined whether metadoxine blocks differentiation of preadipocytes. 3T3-L1 preadipocytes were induced to differentiate with MDI for 8 days in the presence or absence of metadoxine in growth medium containing 10% fetal bovine serum. Hormone treatment allowed preadipocytes to be well differentiated, as evidenced by the accumulation of cytoplasmic triglycerides stained with Oil Red O, whereas simultaneous metadoxine treatment notably inhibited adipogenic differentiation in a dose-dependent manner. 3T3-L1 preadipocytes cultured in a medium lacking MDI were used as a negative control (Fig. 1B). We also determined the expression levels of major transcription factors and adipogenic markers, including FAS, PPARγ, C/EBPα, ACC, and SREBP to verify biochemical changes in adipogenesis. As expected, each of the adipogenic marker proteins was virtually abolished in cells treated with metadoxine in a dose-dependent manner (Fig. 1C).

The effect of metadoxine on cell proliferation

When growth-arrested 3T3-L1 preadipocytes are stimulated by hormonal inducers, they reenter the cell cycle and undergo approximately two rounds of cell division (e.g., clonal expansion) [1,19]. Whether the anti-adipogenic effect of metadoxine was due to arresting cell-cycle progression was determined. When 2 day post-confluent cells were incubated in medium containing MDI, the cell number increased during the clonal expansion phase as a function of time up to 3 days. In the metadoxine-treated cells, there was no change in the relative cell number as compared with the MDI-treated control (Fig. 2A). The rate of DNA synthesis was also measured by [^{3}H]thymidine incorporation assay, which was not affected by metadoxine treatment (Fig. 2B). Next, we measured the expression of cyclin-dependent kinase (CDK) inhibitors including p27, p21, and cyclin A, which are associated with cell-cycle progression [20]. The protein levels of p27, p21, and cyclin A, all distinctly changed after MDI stimulation (Fig. 2C). Concomitant metadoxine treatment did not alter the MDI-induced patterns of expression. Our results demonstrate that inhibition of preadipocyte differentiation by metadoxine may not be associated with alteration in cell-cycle progression.

The effect of metadoxine on adipocyte differentiation

We continued to examine the effect of metadoxine on 3T3-L1 preadipocyte differentiation with the purpose of identifying the inhibitory time-period of metadoxine on hormone-induced adipogenesis. In this set of experiments, the effects of metadoxine treatment duration or time of treatment were tested. We found that metadoxine treatment from day 4 through day 8 was required for inhibition of MDI-induced preadipocyte differentiation (Fig. 3A, experiments #3, #4, and #5). In contrast, preadipocytes exposed to metadoxine only from day 0 through day 4 were able to fully differentiate to adipocytes (experiment #2). 3T3-L1 preadi-

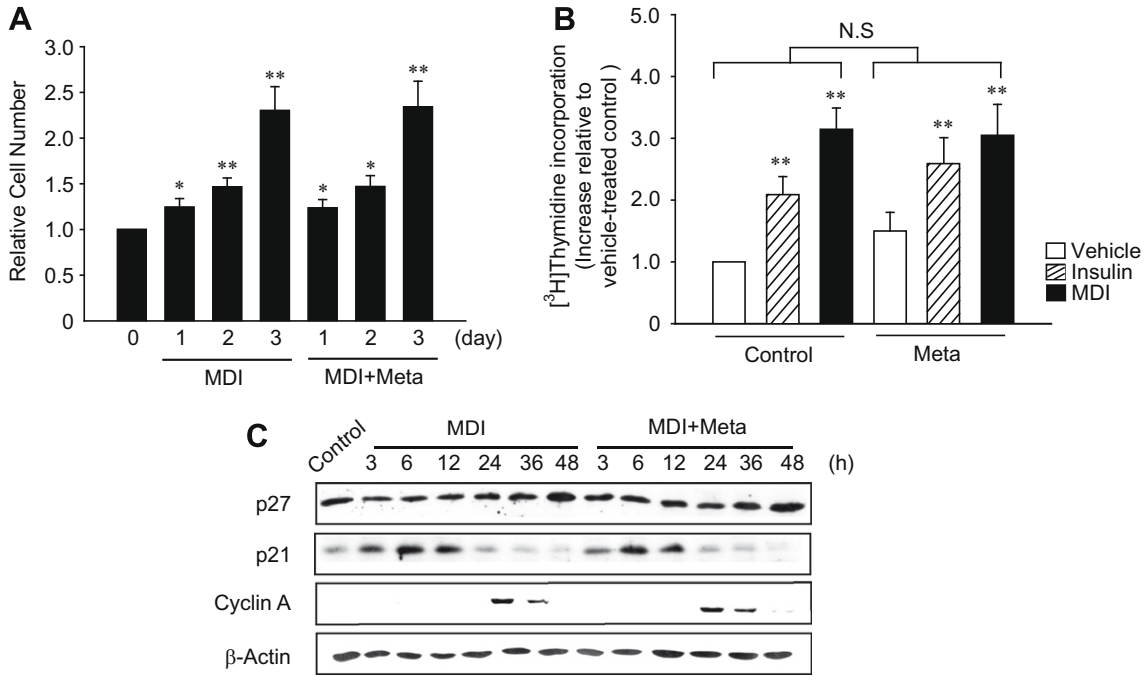

Fig. 2. The effects of metadoxine (Meta) on cell proliferation and cell-cycle-regulating proteins. (A) Relative cell numbers. Values represent the mean ± S.E. from three independent experiments ($^{*}p < 0.05$, $^{**}p < 0.01$, significant compared with control). (B) [^{3}H]Thymidine incorporation. Post-confluent preadipocytes were treated with 5 μg/ml insulin or MDI in the presence or absence of 1 mM metadoxine for 48 h. Data represent the mean ± S.E. of three independent experiments (each performed in triplicate; $^{**}p < 0.01$, significant compared with vehicle; N.S., not significant). (C) Immunoblottings. The cell lysates were subjected to immunoblottings for p27, p21, and cyclin A. Equal loading of proteins was verified by probing the replicate blot for β-actin. Results were confirmed by three separate experiments.

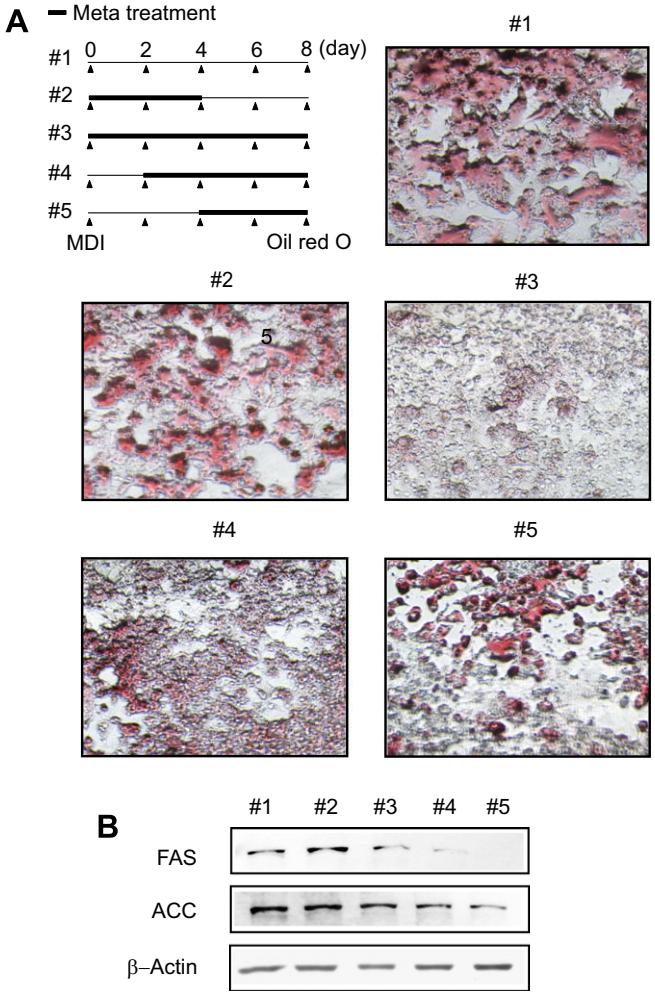

Fig. 3. The time-course effect of metadoxine (Meta) on adipocyte differentiation. (A) Oil Red O staining of cytoplasmic triglycerides. 3T3-L1 preadipocytes were incubated with 1 mM metadoxine for the indicated time periods during the MDI-induced differentiation program. Thick lines indicate metadoxine treatment. Microphotographs show the cells fixed in 10% formalin and stained with Oil Red O at day 8. (B) Immunoblottings. The cell lysates were subjected to immunoblottings for FAS and ACC. Equal loading of proteins was verified by probing the replicate blot for β-actin. Results were confirmed by three independent experiments.

pocytes incubated with metadoxine only from day 2 or day 4 through day 8 differentiated to adipocytes to much less extents (experiments #4 and #5), suggesting that metadoxine may have an inhibitory effect during the late stage of MDI-induced adipogenic differentiation. The inhibitory effect was confirmed by changes in the expression of adipogenic markers such as FAS and ACC (Fig. 3B). Our results showing the inhibitory effect of metadoxine on adipogenesis at later time periods support the possibility that metadoxine has the ability to inhibit the preadipocyte differentiation program at the late differentiation phase, but not the early proliferation phase.

The effect of metadoxine on PPARγ gene transactivation

Among all of the PPARs, PPARγ is the most adipocyte-specific and expressed at the highest level in adipose tissue and adipocyte cell lines [21]. PPARγ forms a heterodimer complex with retinoid X receptor (RXR) and binds to the PPREs for regulation of the gene transcription [22]. To functionally determine whether the metadoxine-induced decreases in lipid droplet accumulation obtained in Oil Red O staining occurred as a result of change in PPAR binding to the target promoter, luciferase gene transactivation was monitored in 3T3-L1 preadipocytes cultured and differentiated by MDI for 2 days. Metadoxine treatment reduced luciferase activity in differentiating 3T3-L1 cells transfected with the PPRE reporter construct in a concentration-dependent manner (Fig. 4A). To determine whether metadoxine directly antagonizes PPRE-driven gene transactivation, we also monitored the effect of metadoxine on PPARγ and RXRα-dependent reporter activity in HEK293 cells. Metadoxine treatment failed to change the PPRE luciferase activity in the cells transfected with the plasmids encoding PPARγ and RXRα (Fig. 4B). Hence, the change in PPRE activity during the adipocyte differentiation program may result from inhibition of cellular differentiation, but not that of PPRE-dependent gene transcription.

Inhibition of CREB activation by metadoxine

CREB is constitutively active as a transcription factor in preadipocytes and throughout the differentiation process, and therefore serves as a potential regulator of proliferation and differentiation in preadipocytes [7,8]. Induction of the constitutively active form of CREB was sufficient to initiate adipogenesis, as determined by triglyceride storage, change in cell morphology, and the induction

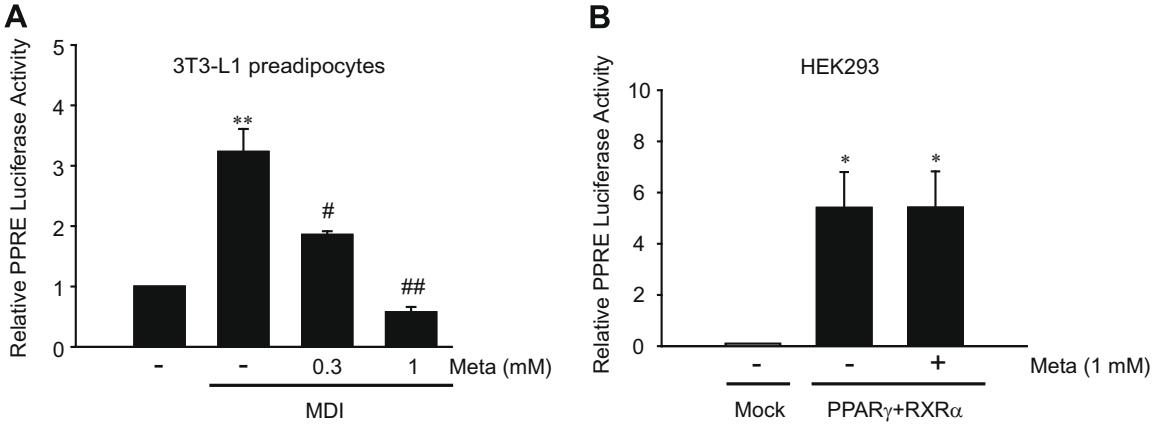

Fig. 4. The effect of metadoxine (Meta) on PPARγ reporter activity. (A) The relative PPRE reporter activity in 3T3-L1 preadipocytes. The effect of metadoxine on PPRE luciferase activity was determined in 3T3-L1 preadipocytes subjected to the MDI-induced differentiation program for 48 h. Values represent the mean ± S.E. from at least three independent experiments ($^{**}p < 0.01$, significant compared with control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, significant compared with MDI alone). (B) The relative PPRE luciferase activity in HEK293 cells. PPARγ activity was measured in HEK293 cells treated with 1 mM metadoxine for 48 h. Values represent the mean ± S.E. from at least three independent experiments ($^{*}p < 0.05$, significant compared with mock transfection).

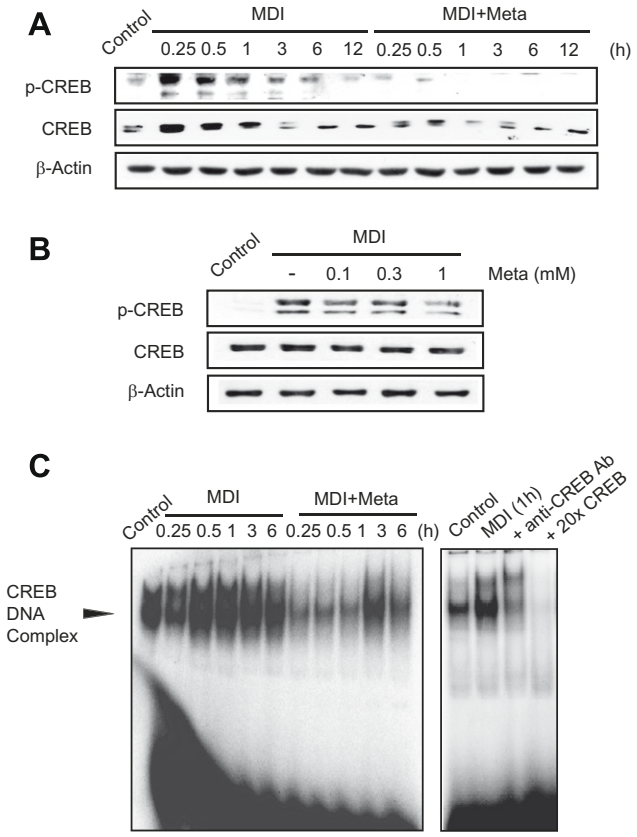

Fig. 5. Inhibition of CREB phosphorylation by metadoxine (Meta) during the MDI-induced differentiation program. (A) Immunoblottings for CREB. Phosphorylated (Ser133) and total CREB levels were assessed in 3T3-L1 preadipocytes treated with vehicle or 1 mM metadoxine for the indicated time-period. Immunoblotting for β-actin confirms equal loading of proteins. (B) The dose–response effect. Phosphorylated and total CREB levels were measured in 3T3-L1 preadipocytes treated with 0.1–1 mM metadoxine for 30 min. (C) Gel shift analysis. The CREB–DNA-binding activity was measured in 3T3-L1 preadipocytes treated with vehicle or 1 mM metadoxine. Nuclear extracts were incubated with CREB-binding oligonucleotide, and binding specificity was confirmed by excess probe competition or supershift assay. Results were confirmed by three independent experiments.

of adipocyte marker genes, whereas a dominant-negative mutant of CREB effectively blocked the process [9]. We determined whether metadoxine has the ability to reduce the level of phosphorylated CREB induced by MDI. Anti-p-CREB antibody recognizes phosphorylated CREB at Ser133, the site specifically phosphorylated by PKA. Immunoblot analysis revealed that metadoxine (1 mM) decreased MDI-induced CREB phosphorylation (Fig. 5A). In addition, metadoxine inhibited the CREB phosphorylation in a dose-dependent manner (Fig. 5B). Moreover, the expression of total CREB was also down-regulated, which might result from decrease in its own regulation [23]. Gel shift analysis was performed to assess protein binding to the CREB binding site. As expected, the ability of proteins in nuclear extracts to bind with the CREB consensus oligonucleotide was reduced in cells treated with metadoxine most distinctly for 15 or 30 min (Fig. 5C), which paralleled the result of the immunoblot analyses.

The effect of metadoxine on PKA-dependent CREB phosphorylation

Next, we determined the effect of metadoxine on PKA activation induced by MDI. Exposure of 3T3-L1 preadipocytes to MDI for 30 min resulted in an increase in PKA activity, which was completely prevented by metadoxine pretreatment (Fig. 6A). To verify whether metadoxine had the ability to reduce CREB phosphorylation, we examined its effect on CREB phosphorylation in preadipocytes that had been incubated in a medium containing agents that increase cAMP via the PKA-dependent pathway. When 3T3-L1 preadipocytes, incubated in a medium lacking differentiating hormones and serum mitogens, were treated with either 8-Br-cAMP or forskolin, the level of phosphorylated CREB increased. As expected, this phosphorylation was almost completely inhibited by simultaneous metadoxine treatment (Fig. 6B and C). Moreover, cotreatment with H89, a chemical inhibitor of PKA, abolished MDI-induced CREB phosphorylation (Fig. 6D). These results demonstrate that metadoxine-induced inhibition of CREB phosphorylation may result from the inhibition of the PKA-adenylate cyclase pathway.

Decrease in C/EBPβ-LAP expression by metadoxine

C/EBPβ is an early regulator of preadipocyte differentiation [24]. Because C/EBPβ is a target gene of CREB and plays a key role in adipogenesis, we next measured the levels of C/EBPβ-liver-enriched transcriptional activating protein (LAP) and C/EBPβ-liver-enriched transcriptional inhibitory protein (LIP) after MDI hormonal stimulation. C/EBPβ transcriptionally activates two genes encoding for major adipogenic transcription factors, C/EBPα and PPARγ, and thus appears to serve dual functions: an initiator of mitotic clonal expansion and a mediator of differentiation [25]. Metadoxine treatment effectively inhibited C/EBPβ-LAP expression 24 h after hormonal stimulation (Fig. 7A, upper). The relative LAP intensities were plotted as a function of time after scanning densitometry of the immunoblots (Fig. 7A, lower left). The LAP/LIP ratio during the time-period of 24 or 48 h post-metadoxine treatment was much smaller than that in vehicle-treated control (Fig. 7A, lower right), indicating that metadoxine may increase formation of a negative transcription factor complex. Gel shift analysis showed that the ability of proteins in nuclear extracts of cells treated with MDI to bind with C/EBP consensus oligonucleotide was decreased by metadoxine treatment compared to that of MDI treatment alone (Fig. 7B).

Phosphorylation is an important post-translational modification of C/EBPβ that leads to the acquisition of its DNA-binding function as preadipocytes traverse the G1-S check-point at the onset of mitotic clonal expansion [26]. A number of cellular kinases phosphorylate C/EBPβ, including MAPKs, protein kinase A, protein kinase C, Ca^{2+}-calmodulin-dependent kinase II, cyclin-dependent kinase 2, and glycogen synthase kinase 3β [26–29]. The activation status of C/EBPβ may be affected by Akt-dependent pathway [30]. As MAPK is the major and first kinase initiating phosphorylation, the inhibition of MAPK activity might also contribute to the anti-adipogenic effect of metadoxine. Therefore, we assessed the phosphorylation levels of Akt, ERK, JNK, and p38 kinase after induction of adipogenic differentiation. The levels of phosphorylated Akt and MAPKs were all increased by hormone stimulation, which was not changed by metadoxine as compared to control (Fig. 7C). Our data showed that metadoxine did not change the status of Akt and MAPK phosphorylations activated by MDI hormones, which excludes the possibility of MAPK inhibition by metadoxine in its anti-adipogenic effect.

Discussion

Obesity causes many pathological disorders, especially, type 2 diabetes mellitus, coronary heart disease, cancer, respiratory complications, and osteoarthritis [31]. Obesity results from the proliferation and differentiation of adipocyte, which is not only important for energy storage, but also an endocrine cell that regulates energy homeostasis [32]. 3T3-L1 preadipocyte cell line is a best-characterized cell model for the study of adipocytes. In this study, we inves-

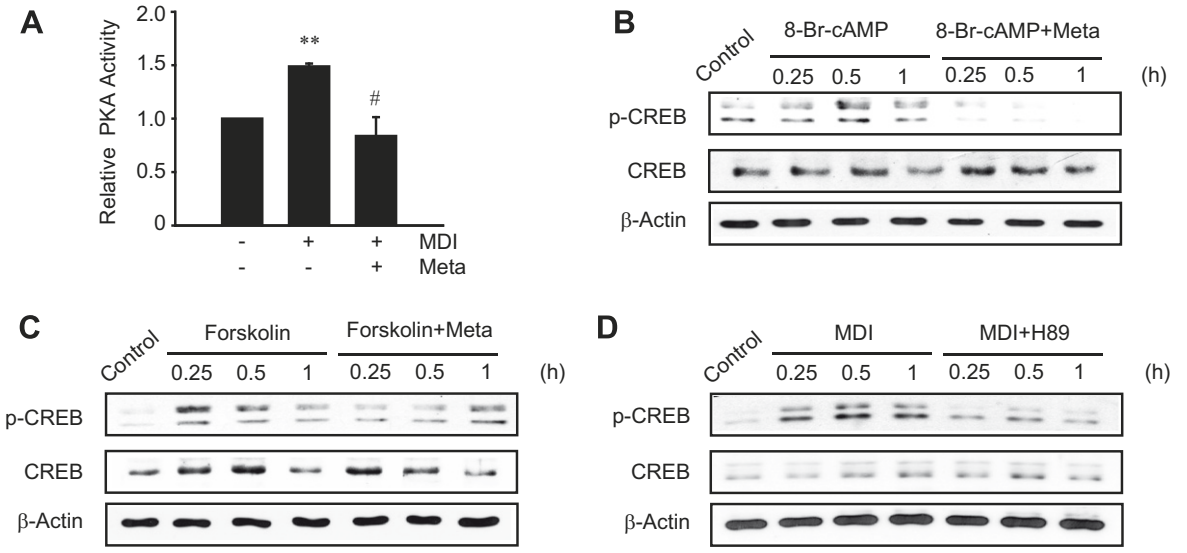

Fig. 6. Inhibition of PKA-dependent CREB phosphorylation by metadoxine (Meta). (A) The kinase activity of PKA. PKA activities were measured in lysates of cells that had been treated with 1 mM metadoxine for 10 min and subsequently exposed to MDI for 30 min. Values represent the mean ± S.E. of three independent experiments ($^{**}p < 0.01$, significant compared with control; $^{\#}p < 0.05$, significant compared with MDI alone). (B) The effect of metadoxine on CREB phosphorylation induced by 8-Br-cAMP. The phosphorylation of CREB at Ser133 was assessed in 3T3-L1 preadipocytes treated with 100 μM 8-Br-cAMP in combination with 1 mM metadoxine for 0.25–1 h. (C) The effect of metadoxine on forskolin-induced CREB phosphorylation (Ser133). 3T3-L1 preadipocytes were treated with 10 μM forskolin with or without metadoxine for 0.25–1 h. (D) The inhibitory effect of 10 μM H89, a PKA inhibitor, on CREB phosphorylation in 3T3-L1 preadipocytes exposed to MDI for 0.25–1 h. Results were confirmed by three repeated experiments and representative blots are shown.

tigated the effect of metadoxine on adipocyte differentiation and its underlying mechanism. Our results demonstrated that metadoxine had the ability to block 3T3-L1 preadipocyte differentiation by regulating integral molecules of the adipogenesis, specifically PPARγ and C/EBPα. We first examined whether metadoxine affects cell-cycle progression in the proliferation stage. It has been demonstrated that CDK inhibitor expression is up-regulated during cellular proliferation and differentiation *in vitro* and *in vivo*, suggesting that these cell-cycle inhibitors may play a universal role in regulating the exit from the cell cycle and maintenance of the irreversible growth arrest and terminal differentiation [20]. However, we additionally found that metadoxine treatment does not affect the expression of CDKs and their inhibitors, p27, p21, and cyclin A. Likewise, both the relative cell number and the rate of [^{3}H]thymidine incorporation were not significantly changed in cells treated with metadoxine compared with control. Our data suggests that metadoxine has no effect on the mitotic clonal expansion phase of 3T3-L1 preadipocytes. In order to confirm that metadoxine has an anti-adipogenic effect on the differentiation phase and identify metadoxine's inhibitory time-period on hormone-induced adipogenesis, 3T3-L1 preadipocytes were exposed to metadoxine with different treatment duration or time of treatment. We found that metadoxine treatment from day 4 through day 8 caused inhibition of MDI-induced preadipocyte differentiation. These results raised the possibility that metadoxine has an inhibitory effect on adipogenesis at the late differentiation phase.

Adipogenesis requires a number of transcription factors, signaling pathways, and the induced expression of genes required for the development of the adipocyte phenotype [1]. In particular, CREB is known as a potential regulator of cellular proliferation and differentiation [7–9]. Both the phosphorylation and the transcriptional activity of CREB were stimulated by agents that induce adipocyte differentiation such as IBMX and insulin via PKA and ERK1/2 pathways, respectively [11]. PKA is known as the kinase important for regulating adipogenesis [33]. We found the ability of metadoxine to decrease the activity of PKA as well as the level of CREB phosphorylations induced by 8-Br-cAMP, forskolin (PKA activators), and MDI. As expected, they were decreased in the early time-period after metadoxine treatment. The ERK1/2 MAPK pathway is also indispensable for adipogenesis and is transiently activated following the induction of preadipocyte differentiation [6]. Our observation indicated that metadoxine did not affect the Akt and MAPK phosphorylations induced by MDI during the early stage, implying that the inhibition of adipogenesis by metadoxine may not be associated with alterations in the Akt and MAPK pathways.

CREB, expressed immediately after exposure of growth-arrested preadipocytes to adipogenic inducers [7–9], regulates C/EBPβ induction. C/EBPβ then enhances PPARγ and C/EBPα expression through its interactions with the DNA response elements located in the target gene promoters [25]. Once expressed, C/EBPα and PPARγ serve as transcriptional activators for a group of adipogenic genes whose protein products give rise to the terminally differentiated adipocyte phenotype [25,34]. Thus, C/EBPβ plays an important role in mitotic clonal expansion of preadipocytes as well as adipogenic differentiation phase [35]. Our results indicated that the expression of C/EBPβ induced by hormonal stimulation was inhibited by concomitant metadoxine treatment. We previously found that C/EBPβ was rapidly induced and the maximal protein level was detected within 3 h after differentiation induction [16].

C/EBPβ is expressed as at least three isoforms, full length 38 kDa, 35 kDa (LAP), and 20 kDa (LIP) proteins. LIP lacks the N-terminal transactivation domain and thus has been proposed to function as a dominant-negative regulator of C/EBPβ-induced transcription [36]. Thus, adipogenesis is regulated by the ratio of LAP to LIP. In this study, the expression of LAP decreased after metadoxine treatment as a function of time. Our observation showed that the LAP/LIP ratio during the time-period of 24 or 48 h was smaller in metadoxine-treated than in vehicle-treated cells, which suggests that formation of a negative C/EBPβ–DNA-binding complex might be increased by metadoxine. In this study, we confirmed that metadoxine indeed decreased C/EBPβ–DNA complex formation. Moreover, our preliminary data indicated that metadoxine did not change the translocation of RNA-binding protein (i.e., cytoplasmic translocation of CUG repeat-binding protein-1 and calreticulin RNA-binding proteins) from the nucleus (data not shown).

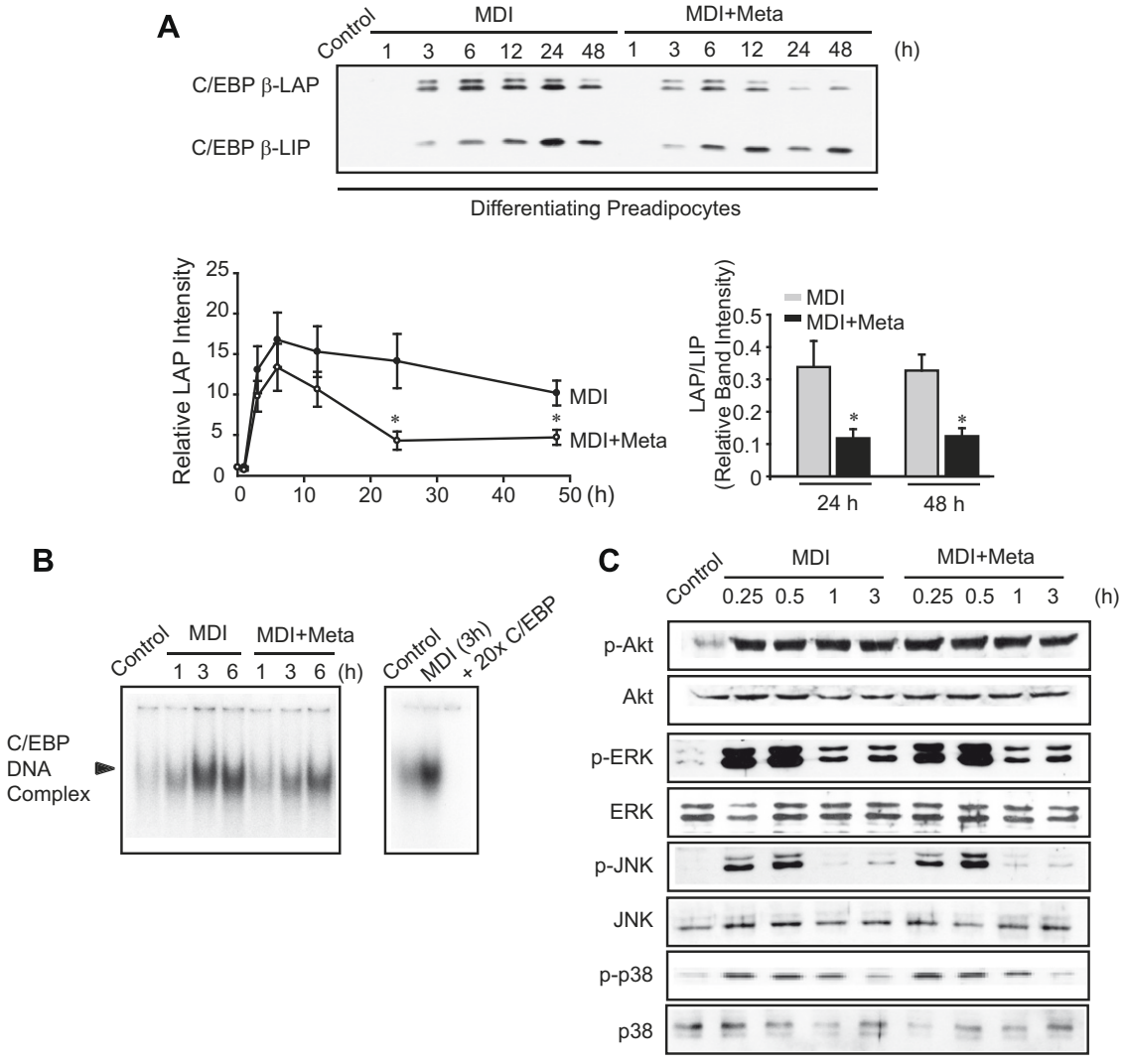

Fig. 7. The expression of C/EBPβ in 3T3-L1 preadipocytes treated with metadoxine. (A) The effect of metadoxine on C/EBPβ-LAP and C/EBPβ-LIP expression. C/EBPβ-LAP and C/EBPβ-LIP were immunoblotted in 3T3-L1 preadipocytes incubated in the medium containing MDI with or without metadoxine for 1–48 h. Results were confirmed by three repeated experiments. The relative intensities of LAP and LIP were assessed by scanning densitometry of the immunoblots. Lower right panel shows the ratio of LAP to LIP. Values represent the mean ± S.E. from three independent experiments ($^{*}p < 0.05$, significant compared with MDI alone). (B) Gel shift analysis of protein binding to the C/EBP binding site. Nuclear extracts were prepared from 3T3-L1 preadipocytes incubated with or without 1 mM metadoxine. Arrowhead indicates the DNA bound with C/EBP. For competition assays, 20-fold molar excess of unlabeled oligonucleotide was added to the nuclear extracts from cells incubated with MDI. Results were confirmed by three separate experiments. (C) The effect of metadoxine on Akt and MAPK phosphorylations. Immunoblottings for phospho-Akt (Thr308), Akt, phospho-ERK (Thr202/Tyr204), ERK, phospho-JNK (Thr183/Tyr185), JNK, phospho-p38 (Thr180/Tyr182) and p38 were performed in the lysates (20 μg each) of the cells. Results were confirmed by three repeated experiments and representative blots are shown.

The role of AMPK in adipogenesis has not been clarified yet. AICAR, a well-known AMPK activator, has been shown to block adipogenesis [37]. Hypoxia, an AMPK-activating condition, also inhibits adipogenic clonal expansion [38]. Recently, we reported that compound C, a well-known AMPK inhibitor, blocks adipogenesis as a consequence of inhibition of preadipocytes proliferation via increase in p21 content, which may not be associated with its AMPK inhibition [39]. In this study, we also explored the possible involvement of AMPK in the inhibition of adipogenesis by metadoxine, and found that metadoxine had no effect on AMPK activation/inactivation status (data not shown).

Obesity causes accumulation of fat in the liver and possibly progresses insulin resistance and non-alcoholic steatohepatitis [40]. Furthermore, individuals with non-alcoholic fatty liver disease are at a higher risk of developing Type 2 diabetes and cardiovascular disease since fat accumulation in the liver impedes glucose metabolism [40]. In this study, we demonstrate that metadoxine exhibits an anti-adipogenic effect through inhibition of the differentiation stage of adipogenesis. The aforementioned beneficial effect of metadoxine in the liver may be associated in part with the anti-adipogenic effect, which remains to be studied. Our finding holds a significant implication that inhibition of adipogenic differentiation may be controlled by pharmacological manipulation of PKA-dependent CREB activation.

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