Indirubin promotes adipocyte differentiation and reduces lipid accumulation in 3T3-L1 cells via peroxisome proliferator-activated receptor γ activation

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Abstract. The peroxisome proliferator-activated receptor γ (PPARγ) plays an important role in insulin sensitivity and adipocyte differentiation. It is known as ligand-receptor that improves insulin sensitivity in type 2 diabetes mellitus. Several kinds of indigo plant have been already used to treat diabetes in oriental traditional medicine, but its mechanism has not been clarified yet. To investigate the effect of indirubin, which is a component of *Polygonum tinctorium* on the cell differentiation and adipprocess in 3T3-L1 cells, 3T3-L1 cells were cultured to determine the effect of cell differentiation and glucose uptake with indirubin. As a result, Indirubin compound enhanced adipocyte differentiation in 3T3-L1 cells similar to rosiglitazone. This effect was terminated by cotreatment with GW9662, a PPARγ antagonist. In mature 3T3-L1 adipocytes, the lipid droplet size and accumulation were reduced by this compound. The basal and insulin-stimulated glucose uptakes were also significantly increased. In addition, indirubin treatment significantly enhanced estrogen level by 1.64-fold with mature adipocytes which can be attributed to its aromatase activity. Conclutionaly, this finding suggested that indirubin is a potential anti-diabetic compound for type 2 diabetes mellitus by promoting adipocyte differentiation and glucose uptake via PPARγ.

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Abbreviations: PPARγ, peroxisome proliferator-activated receptor γ; LD, lipid droplet; C/EBP, CCAAT-enhancer-binding protein; GLUT, glucose transporter; TG, triglyceride; AMPK, AMP-activated protein kinase

Key words: indirubin, *polygonum tinctorium*, insulin resistance, diabetes mellitus, PPARγ, 3T3-L1 cells, lipid accumulation, glucose uptake, estrogen

Introduction

Insulin resistance is implicated in the development of metabolic syndromes such as obesity, type 2 diabetes mellitus and hyperlipidemia (1). Lipid metabolism disorders result in excessive lipid accumulation and adipocyte hypertrophy. Typically, adipocytes increase insulin sensitivity and maintain glucose homeostasis (2). However, as shown in Fig. 1, insulin resistance caused by adipocyte dysfunction impairs glucose homeostasis, further enhancing insulin resistance through a feedback loop (3). Accordingly, an increase in the production of insulin-sensitive adipocytes promotes mature adipocyte differentiation.

The peroxisome proliferator associated receptor γ (PPAR γ) is a ligand-activated transcription factor that belongs to the nuclear receptor superfamily, and is associated with adipocyte cell differentiation and glucose and lipid metabolism (4). As PPARγ regulates the differentiation of pre-adipocytes, it also increases the number of small insulin-sensitive adipocytes. Moreover, the activation of PPARγ in mature adipocytes regulates several target genes involved in the insulin signaling cascade and glucose and lipid metabolism (5). Consequently, PPARγ serves as a therapeutic target for antidiabetic drugs regulating glucose and lipid metabolism.

The thiazolidinediones, including rosiglitazone and pioglitazone, are a group of anti-diabetic drugs that strongly activate PPARγ (6). The activation of PPARγ leads to the trapping of free fatty acids in adipocytes and increased hormone secretion, which both contribute to insulin sensitivity (7). However, while thiazolidinediones exhibit excellent antidiabetic effects, they are also associated with undesirable side effects, such as heart failure, weight gain and osteopenia, which restrict their clinical use (8).

Polygonum tinctorium, a widely-known indigo plant, is an annual herbaceous plant belonging to the Polygonaceae family. It has been used in traditional Chinese medicinal and as an indigo-blue dye since ancient times in Asian countries. Extracts from *Polygonum tinctorium* have been reported to exert biological effects, including anti-inflammatory, antibacterial and antitumor activities. Furthermore, this plant has also been reported to improve hyperlipidemia (9). This activity may contribute to the enhancement of insulin sensitivity through

the modulation of adipocyte function by PPARγ. The present study investigated the effect of indirubin on adipocyte differentiation and glucose regulation in mature 3T3-L1 adipocytes.

Materials and methods

Chemicals and Reagents. The antibiotic-antimycotic solution was obtained from Sigma-Aldrich Co., Ltd. Indirubin was purchased from Beijing Putian Tongchuang Biotechnology Co., Ltd. All other chemicals were purchased from Wako Pure Chemical Industry Ltd. unless otherwise stated.

PPARγ binding assay. Cyclic AMP response element binding protein (CREB) binding protein (CBP) was immobilized in plastic wells at 37˚C for 1 h. PPARγ was incubated for 24 h at 4˚C after preincubation of 5% skim milk for 1 h at 37˚C as a blocking reagent. Pioglitazone and indirubin were added to an individual well and incubated for 1 h at 37˚C. PPARγ antibody (rabbit polyclonal, Bio-Rad) and alkaline phosphatase conjugated IgG antibody (goat anti-rabbit IgG, Bio-Rad) were diluted and incubated for 1 h at 37˚C, respectively. *p*-Nitrophenyl phosphate (SIGMA*FAST*™ *p*-Nitrophenyl phosphate tablets; Sigma-Aldrich Co., LLC.) was used as the substrate for alkaline phosphatase. After the developing process (shaking at 600 rpm in dark site), the absorbance of the solution in each well was measured at 405 nm. When the absorbance of the sample well was greater than that of the blank well (no sample well), the sample was deemed to contain PPARγ agonist. The indirubin and pioglitazone were dissolved in 100% DMSO and diluted with the D-PBS to obtain the each concentrations. The final concentration of DMSO was equal (0.5%) in all groups.

Cell culture. The 3T3-L1 adipocytes were purchased from the JCRB Cell Bank (Osaka, Japan) and were cultured at 37˚C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL) and antibiotic-antimycotic solution (100 IU/ml of penicillin and 25 µg/ml of amphotericin B). After the preadipocytes reached confluence, differentiation for adipocyte was induced with or without samples in a mixture containing 0.25 mM isobutyl-methylxanthine, 1 µM dexamethasone, and 1.7 µM insulin. The indirubin and rosiglitazone were dissolved 100% DMSO and diluted with the culture medium to obtain the needed each reaction concentrations (10 µM-1 nM). The final concentration of DMSO was equal (0.5%) in all groups. When the PPARγ antagonist GW9662 and samples (indirubin and rosiglitazone) were treated together, the samples was added after treatment with its antagonist.

Cell viability assay. Cell viability was determined by MTT assay. The 3T3-L1 cell suspension 100 µl ($5.00x10^3$ cells) was added to each well of a 96-well plate and the medium was preincubated in a CO_2 incubator for 24 h. Each well was added with 10 µl of each concentration of the prepared indirubin and rosiglitazone and incubated the medium for 24 h in CO_2 incubator. Each plate was added 10 µl of Cell Count Reagent SF (Nacalai tesque) and incubated the medium for 1 h in CO_2 incubator. Reduction of MTT to formazan was measured by a microplate reader (Immuno Mini NJ-2300; Biotech Ltd.) at 450 nm. Viability was calculated considering vehicle control (vehicle with cells) as a control. Fig. S1 shows that native control and vehicle control have the same background.

Oil-Red-O staining. After differentiation in 5 days with indirubin and rosiglitazone, the 3T3-L1 cells were harvested. Then, cells were washed with D-PBS(-) and immobilized with 10% formaldehyde for 1 h at room temperature. Cells were stained with the ORO working solution (3 mg/ml in 60% (v/v) 2-propanol) for 1 h at 37˚C and examined by an optical microscope x200 (Nikon ECLIPSE Ts2; Olympus CKX31) to evaluate lipid accumulation. Lipid droplet (LD) were extracted from the cells using 2-propanol for 5 min and quantified using a microplate reader at 510 nm. The results were expressed relative to the differentiated cell control group. In addition, PPARγ is required for mature adipocyte-survival, therefore adipocytes only survive for a few days after selective ablation of PPARγ in mature adipocytes of mice (10). GW9662 would inhibit mature adipocytes-survival completely, followed by these cell death. Thus, in this experiment using mature cell, we used only the samples without GW9662.

Glucose uptake assay. Completely differentiated 3T3-L1 adipocytes in 96-well plates were incubated in the presence of 4 concentrations of indirubin (1 µM-1 nM) and rosiglitazone (1 µM-1 nM) for 4 days. After the cells were washed by D-PBS(-), they were incubated with DMEM for another 3 h and determined the glucose concentrations at 3 points over time. The glucose concentrations in the cell culture medium were determined by the Glucose Assay kit (Cell Biolabs, Inc.).

Measurement of estradiol in adipocytes. Fully differentiated 3T3-L1 adipocytes in 96-well plates were incubated in the presence of 4 concentrations of indirubin (1 µM-1 nM) and rosiglitazone (1 µM-1 nM) for 4 days. Supernatants were taken from these plates on the 4th day of differentiation. The estradiol levels were determined by the Estradiol ELISA kit (Cayman Chemical, Inc.) and the concentrations were calculated following the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed by one-way ANOVA followed by Dunnett's test for multiple group comparisons using Sigma Stat statistical software ver. 2.03 (SPSS, Inc.). All data are presented as the mean ± standard deviation. Experiments were repeated three times. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PPARγ ligand activity. The present study investigated whether indirubin served as a PPARγ ligand. Compared with 50 nM pioglitazone, 5 and 50 nM indirubin exhibited a 1.35- and 1.80-fold increased activity, respectively ($P<0.001$; Fig. 2A), indicating that indirubin exerted a stronger PPARγ ligand-binding activity than pioglitazone. In addition, the effects of a PPARγ antagonist, GW9662, on indirubin-binding activity were examined. As shown in Fig. 2B, treatment with 10 nM GW9662 significantly suppressed the activity of indirubin in a dose-dependent manner. Therefore, these results suggested that indirubin was a PPARγ agonist by directly binding to the PPARγ ligand-binding domain.

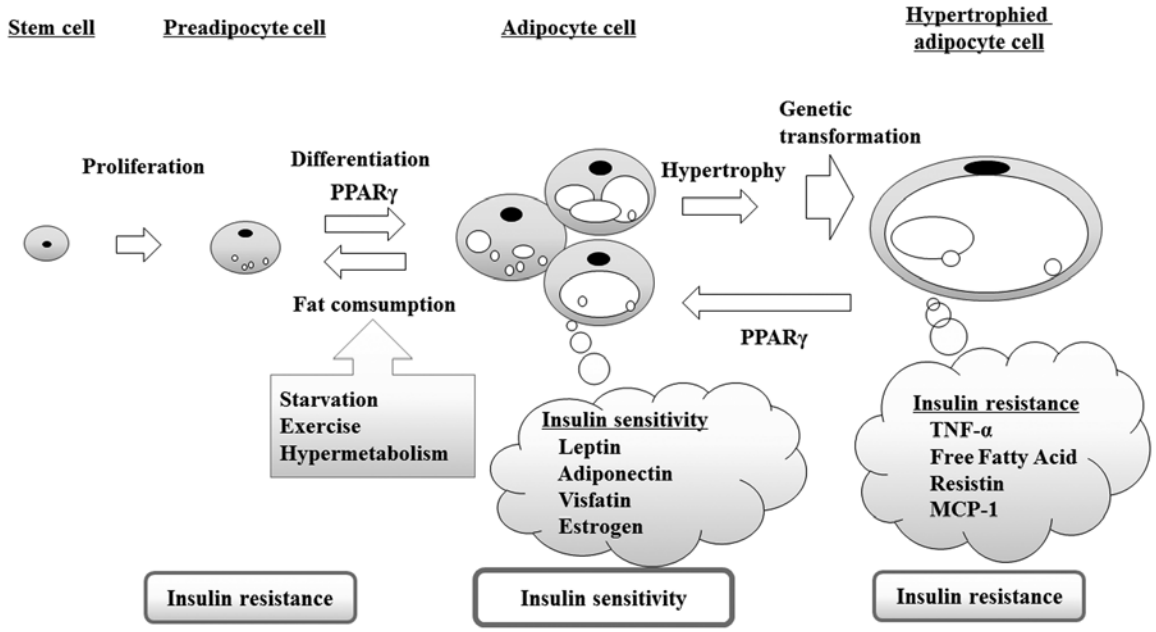

Figure 1. Differentiation process of adipocytes and insulin sensitivity. PPARγ, peroxisome proliferator-activated receptor γ; TNF-α, tumor necrosis factor-α; monocyte chemoattract protein-1.

Indirubin promotes cell differentiation of 3T3-L1 cells via PPARγ. An MTT assay was performed to evaluate the cytotoxicity of indirubin and rosiglitazone in pre-adipocytes. As shown in Fig. 3, >10 μM of either indirubin or rosiglitazone impaired cell viability. Therefore, a concentration range of 1 nM-1 μM was used for further experimentation. As indirubin and rosiglitazone exhibited PPARγ ligand activity, their effect on adipogenesis and cell differentiation was investigated. 3T3-L1 pre-adipocytes were exposed to indirubin and rosiglitazone in the presence or absence of insulin for seven days. Accumulated lipid droplets were subsequently detected by Oil Red O (ORO) staining and microscopy. 3T3-L1 pre-adipocytes become mature adipocytes via PPARγ signaling, and accumulate lipid droplets. Therefore, mature cells are stained red following ORO staining.

Effects of indirubin-treatment on pre-adipocytes. As shown in Fig. 4A, indirubin-treated cells in the presence or absence of insulin were stained red compared with the control cells. However, clear dose-dependency was not observed. Furthermore, in the presence of insulin, indirubin-treated pre-adipocytes, exhibited a morphological change from spindle-like to a more rounded shape following differentiation. Moreover, it was observed that in the differentiated adipocytes, large LD fragments had dispersed into smaller micro LD. In the absence of insulin, indirubin promoted adipocyte differentiation compared with the control group, but to a lesser extent than the indirubin-treated cells in the presence of insulin. This result suggested that indirubin promoted the differentiation of pre-adipocytes.

In the presence of GW9662, the effects of indirubin-treatment on pre-adipocytes. 3T3-L1 pre-adipocytes were induced to differentiate for seven days by culturing them in 1 μM indirubin in the absence and presence of GW9662 (1 μM-1 nM). In the presence of insulin, the addition of 1 μM GW9662 significantly reduced lipid accumulation and adipocyte differentiation compared with the control group for indirubin-treated cells. Furthermore, a dose-dependent effect in cells treated with or without insulin was observed.

Effects of rosiglitazone-treatment on pre-adipocytes. As shown in Fig. 4B, rosiglitazone-treated cells in the presence or absence of insulin were stained red compared with the control cells. However, clear dose-dependency was not observed. Furthermore, in the presence of insulin, rosiglitazone-treated pre-adipocytes, exhibited a morphological change from spindle-like to a more rounded shape following differentiation as with indirubin. Moreover, it was observed that in the differentiated adipocytes, large LD fragments had dispersed into smaller micro LD. In the absence of insulin, rosiglitazone promoted adipocyte differentiation compared with the control group, but to a lesser extent than the indirubin-treated cells.

In the presence of GW9662, the effects of rosiglitazone-treatment on pre-adipocytes. In the presence of insulin, the addition of 1 μM GW9662 significantly reduced lipid accumulation and adipocyte differentiation compared with the control group for rosiglitazone-treated cells. Furthermore, undifferentiated pre-adipocytes were observed in the absence of insulin. Insulin activates the PI3K/Akt signaling pathway, which plays an important role in cell differentiation and lipid metabolism. Therefore, the presence or absence of insulin affects the regulation of PPARγ, which is downstream of this pathway. The lipid accumulation rate was higher in the presence of insulin compared with cells cultured without insulin. This suggested that the selectivity of the target gene affected by indirubin may be different depending on the presence or absence of insulin. In other words, the presence of insulin may activate lipid metabolism-related genes while the absence of

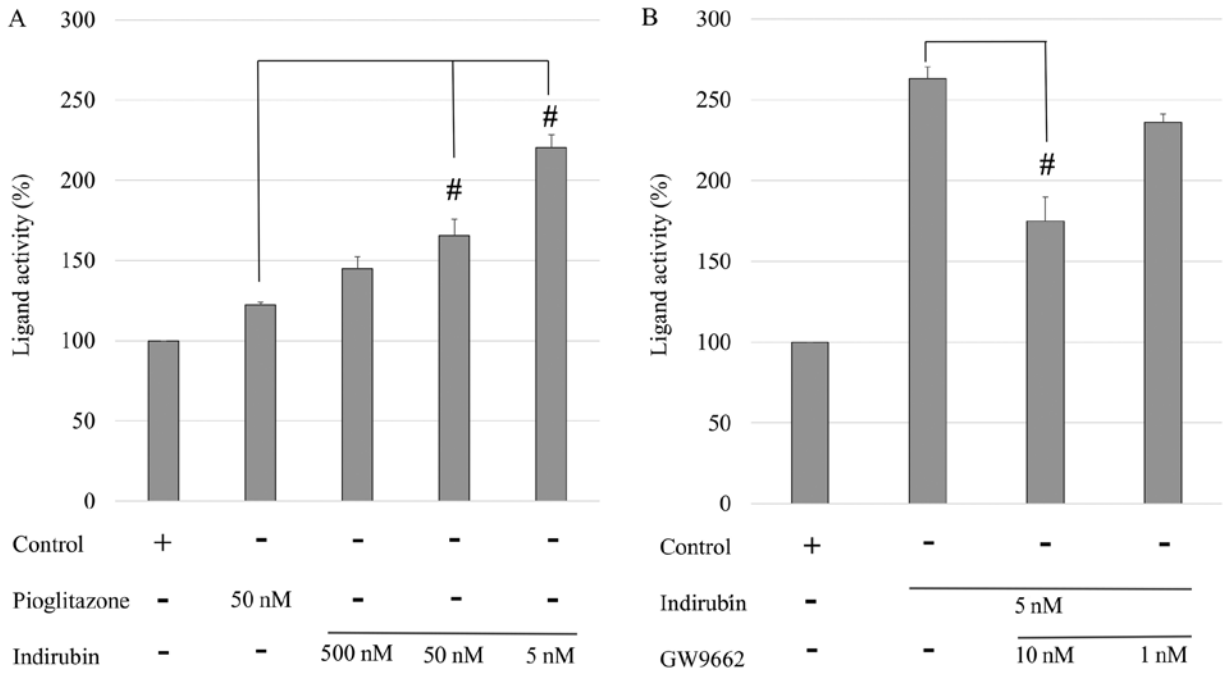

Figure 2. PPARγ ligand activity of indirubin. (A) PPARγ ligand-binding activity of indirubin. (B) Ligand activity of indirubin was suppressed in a dose-dependent manner by the PPARγ antagonist GW9662. #P<0.001. All values are presented as the mean ± SD. n=3. PPARγ, peroxisome proliferator-activated receptor γ.

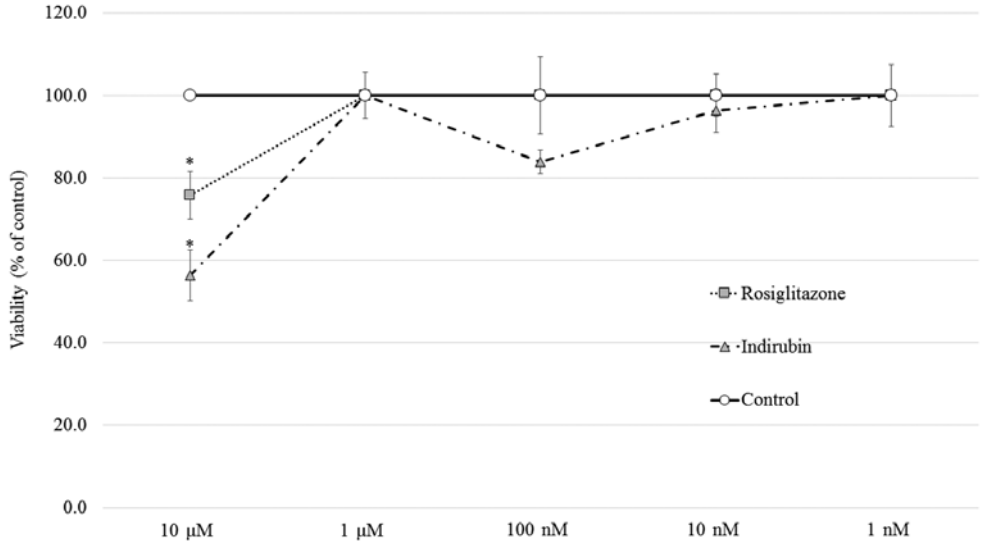

Figure 3. Cytotoxicity of indirubin and rosiglitazone in 3T3-L1 preadipocytes. The cells were treated with samples (10 μM-1 nM) for 24 h. All values are presented as the mean ± SD. n=3. *P<0.05 vs. control.

insulin may result in the activation of genes that promote cell differentiation. Therefore, indirubin may regulate the expression of PPARγ to optimize cell growth.

Indirubin reduces lipid droplet size and accumulation in mature adipocytes via PPARγ activation. Mature 3T3-L1 adipocytes were obtained by culturing pre-adipocytes in MDI medium for four days and maintenance medium for six days. The cells were subsequently cultured with various concentrations of indirubin and rosiglitazone for an additional five days. The effect of these treatments was examined using ORO staining. PPARγ affects the function of both pre-adipocytes and mature adipocytes. In addition to its role in cell differentiation and lipid metabolism, PPARγ is also important for regulating glucose and lipid metabolism, and increases the expression of glucose transporter 4 (GLUT4) and c-Cbl-associated protein (CAP). Moreover, PPARγ regulates the expression of various factors secreted from mature adipocytes, such as leptin, adiponectin and estrogen, which also affect insulin resistance. As shown in Fig. 5, indirubin and rosiglitazone interfered with adipocyte differentiation and lipid accumulation compared with the control group, as indicated by the decrease in ORO staining. It was also observed that larger LD had accumulated in treated cells compared with the control group. Although, treatment

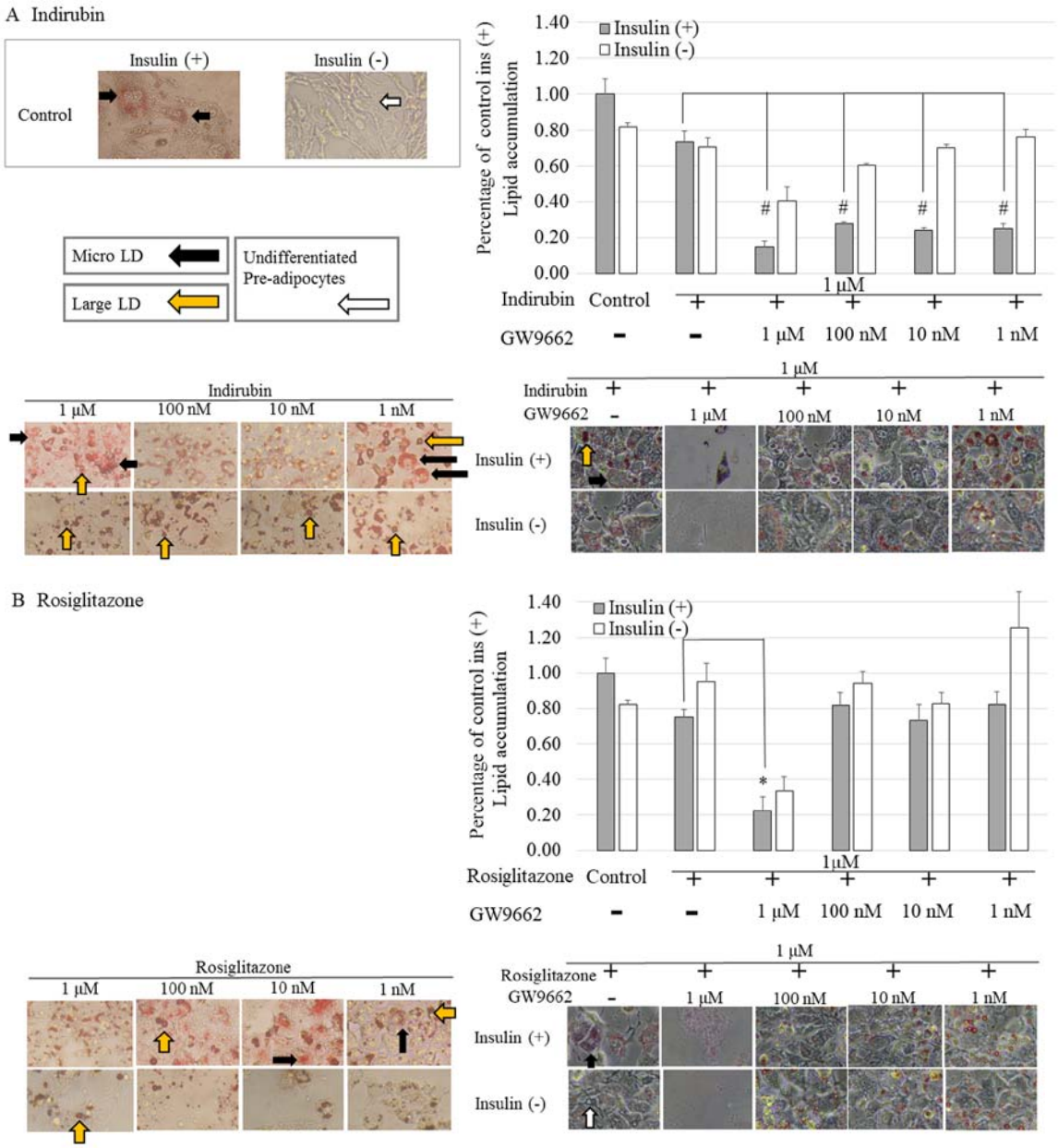

Figure 4. Effects of indirubin and rosiglitazone on adipocyte differentiation of 3T3-L1 preadipocytes. Preadipocytes were cultured in DMEM supplemented with 10% FBS, antibiotic-antimycotic solution, 0.25 mM isobutyl-methylxanthine, 1 μM dexamethasone and 1.7 μM insulin. Images of cells cultured with GW9662 were taken using Nikon ECLIPSE Ts2, and images of cells cultured without GW9662 were taken using the Olympus CKX31. (A) Treated group of 1 μM indirubin without GW9662 or with GW9662 (1 μM-1 nM). After ORO staining, the ORO dye was eluted and quantified at 510 nm. Magnification, x200. (B) Treated group of rosiglitazone without GW9662 or with GW9662 (1 μM-1 nM). All values are presented as the mean ± SD. n=3. $^{\#}$P<0.001; *P<0.05. LD, lipid droplet; ORO, Oil Red O.

with 1 nM-1 μM indirubin reduced LD size, several micro LD were observed. The absorbance quantified at a wavelength of 510 nm also indicated that indirubin dose-dependently decreased lipid accumulation. Considering that these effects were likely mediated through distinct signaling pathways and targets, several signaling pathways may be involved in reducing lipid accumulation in adipocytes.

Indirubin increases insulin-stimulated glucose uptake in mature adipocytes. PPARγ is an important transcription factor in insulin sensitivity and adipogenesis. It has been reported that activation of this factor stimulates the expression of downstream genes involved in glucose metabolism. Therefore, the present study examined the effects of indirubin and rosiglitazone on glucose consumption in mature 3T3-L1 adipocytes. As shown in Fig. 6A, treatment with 1 μM indirubin resulted in a decrease in the medium glucose concentration, which was 13.7% lower than the control group. The 1 μM-10 nM rosiglitazone treated groups resulted in a decrease in the medium glucose concentration (Fig. 6B). However, there was no definite dose-dependence as insulin-stimulated glucose uptake was enhanced in mature adipocytes.

Indirubin promotes estrogen biosynthesis by activating aromatase in mature adipocytes. After menopause, adipocytes become the major source of estrogens. It is known that estrogen

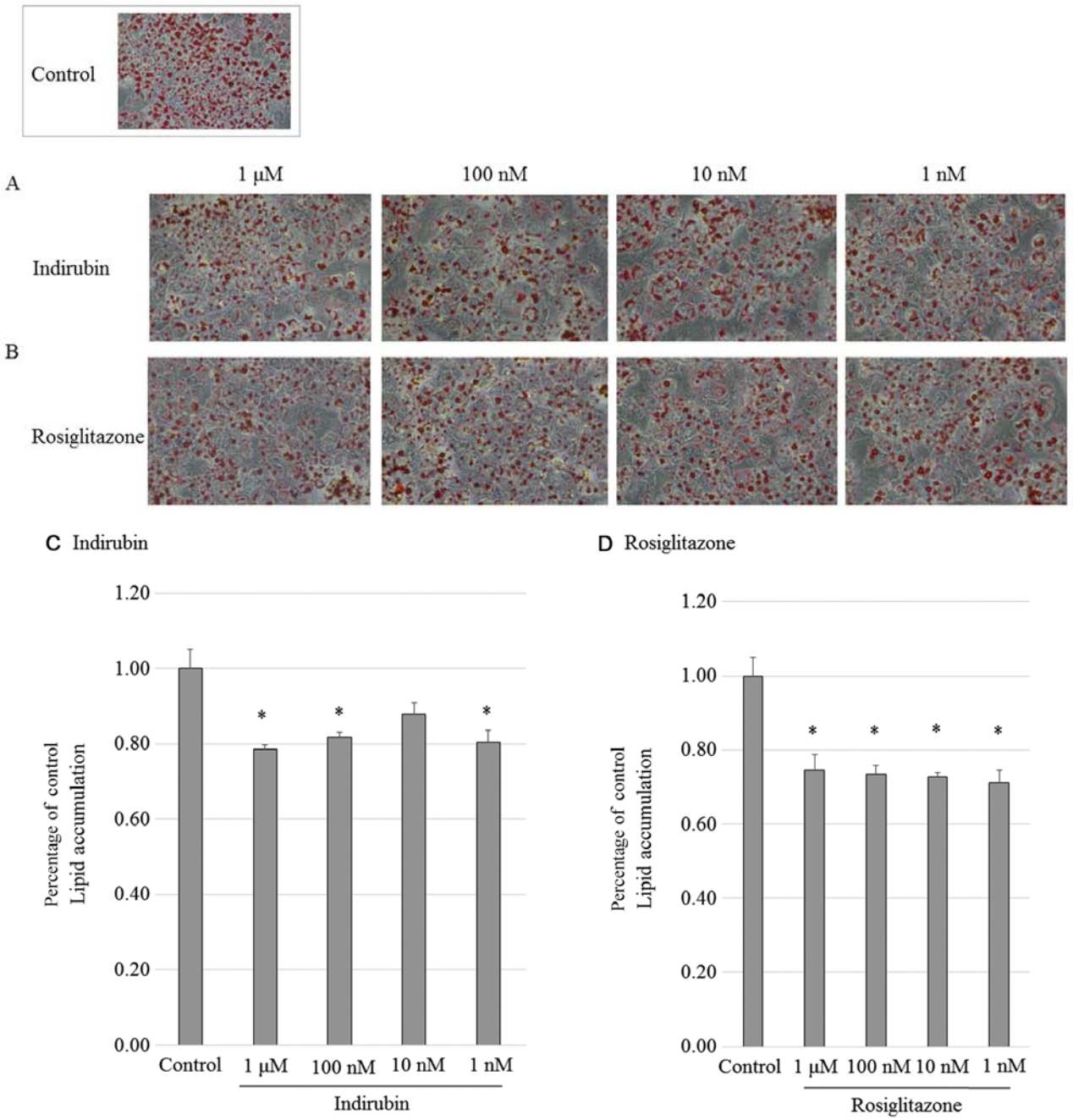

Figure 5. Indirubin and rosiglitazone reduced lipid droplet size and accumulation in mature adipocytes. After ORO staining, the ORO dye was eluted and quantified at 510 nm. (A) ORO staining of indirubin treated group. (B) ORO staining of rosiglitazone treated group. Magnification, x200. (C) Lipid accumulation of indirubin treated group. (D) Lipid accumulation of rosiglitazone treated group. All values are presented as the mean ± SD. n=3. *P<0.05. ORO, Oil Red O.

is biosynthesized from androgens by aromatase cytochrome P450 in mature adipocytes (11). Mature 3T3-L1 adipocyte cells were treated with 1 nM-1 μM indirubin to determine its effect on estrogen biosynthesis in adipocytes. As shown in Fig. 7, estrogen biosynthesis exhibited a 1.64-fold increase following treatment with 1 μM indirubin compared with the control group. However, this effect was not significantly different at 1-100 nM. Since undifferentiated cells do not participate in the biosynthesis of estrogen, the results observed in the present study suggested that the pre-adipocytes had differentiated into mature cells. Therefore, the 1 μM indirubin-treated group may have higher insulin sensitivity compared with the control group.

Discussion

Impaired adipocyte differentiation is associated with insulin resistance and type 2 diabetes in obesity-related diseases. As shown in Fig. 1, adipocytokines secreted from adipocytes, such as adiponectin and leptin, regulate insulin resistance. The ligand activity of PPARγ is important in adipocyte differentiation and plays a role in increasing with the expression of their gene. Consequently, promoting adipocyte differentiation improves insulin resistance. Furthermore, after the binding of insulin to its receptor, several cellular signaling pathways are activated. Specifically, the PI3K/Akt signaling pathway has been implicated in a variety of insulin-dependent cellular processes such

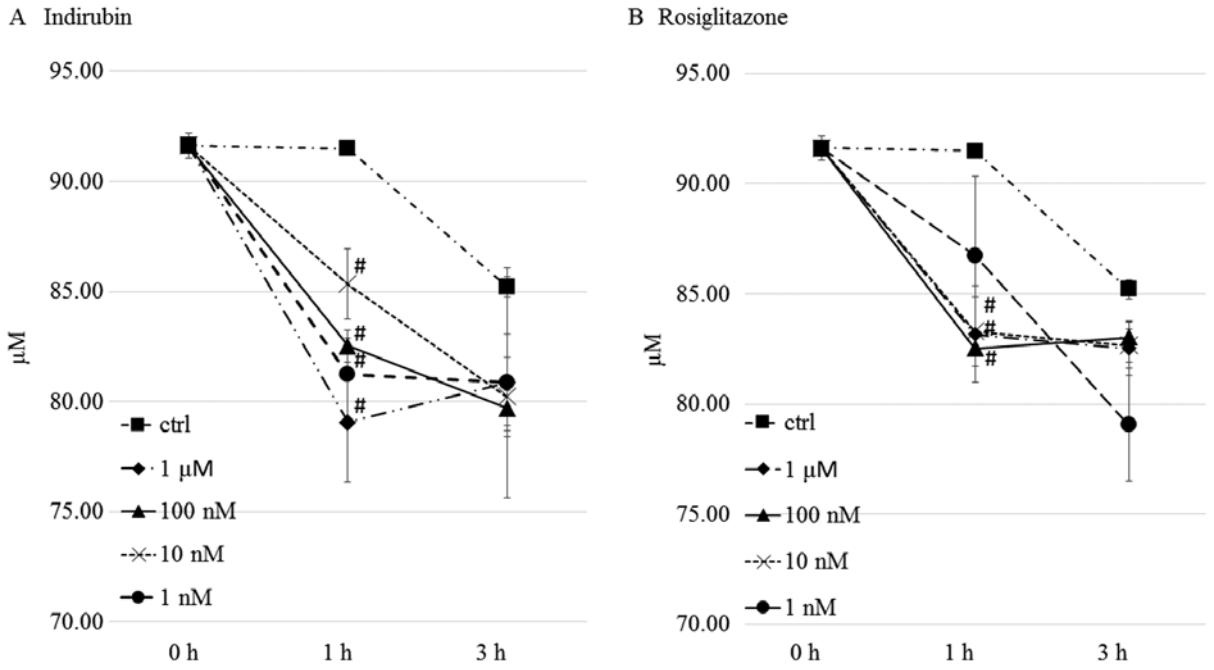

Figure 6. Effects of indirubin on glucose consumption in 3T3-L1 mature adipocytes. Fully differentiated 3T3-L1 adipocytes were incubated with samples for 72 h. The glucose concentrations in the medium were determined by the glucose oxidase method. (A) Effects of indirubin on glucose consumption. All indirubin treated groups indicated a significant difference. (B) Effects of rosiglitazone on glucose consumption. The 1 μM, 100 and 10 nM rosiglitazone treated groups indicated a significant difference. All values are presented as the mean ± SD. n=3. $^{\#}$P<0.001 vs. ctrl. ctrl, control.

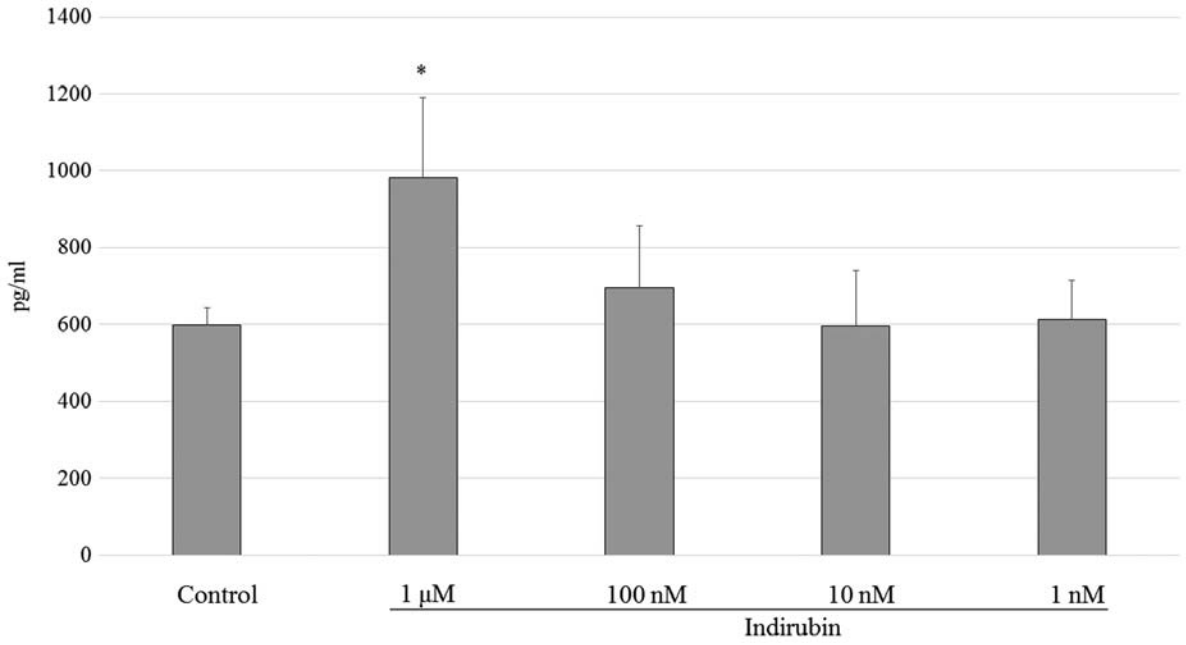

Figure 7. Effects of indirubin on estrogen biosynthesis in 3T3-L1 mature adipocytes. After culturing for a further 5 days, the estrogen concentration in the culture medium was quantified. All values are presented as the mean ± SD. n=3. *P<0.05 vs. control.

as cell survival, glucose transport and adipocyte differentiation. Insulin stimulates cellular differentiation, however, in the present study, cellular differentiation and activation of the PPARγ signaling pathway were also observed in indirubin-treated pre-adipocytes in the absence of insulin. Therefore, indirubin may promote the differentiation of pre-adipocytes into mature cells via the activation of PPARγ regardless of insulin stimulation, followed by the restoration of insulin sensitivity.

The present study revealed that whereas a low concentration of indirubin induced a strong PPARγ ligand activity, this effect was inhibited at high concentrations. Therefore, indirubin has a biphasic effect on PPARγ ligand activity and the activation of PPARγ may be involved in multiple mechanisms. It was reported that PPAR ligands activate the ERK pathway via PPARγ in a dose-dependent manner (12). On the other hand, it is also reported that PPARγ-specific agonists activate

AMP-activated kinase (AMPK) in a PPARγ dose-independent manner (13). Thus, the biphasic effect of indirubin may be due to both its PPARγ-dependent and independent mechanisms, suggesting that the optimal concentration should be evaluated *in vivo* in future experiments.

The development of insulin resistance is characterized by the impairment of glucose uptake mediated by GLUT4 in adipocytes (14). The result showed that indirubin increased insulin-stimulated glucose uptake in mature adipocytes. PPARγ ligands are reported to increase glucose transport in adipocytes by regulating the expression of several target genes directly involved in glucose metabolism (15). Therefore, indirubin may promote adipocyte glucose utilization through the upregulation of GLUT4.

In the process of differentiation of pre-adipocytes into mature adipocytes, morphological and biochemical characteristics are determined by hormone-sensitive metabolic processes. Besides promoting glucose metabolism, adipocytes also acquire the ability to perform lipolysis and lipogenesis and to secrete adipokines (3,16). Perilipin, which is a type of adipophilin, is located on the surface of triglycerides (TG) droplets in mature adipocytes. In the present study, small LD was observed in mature cells, similarly to pre-adipocytes. Dispersion of LD is considered to affect the localization of perilipin and hormone-sensitive lipase, which is one of the main regulators of lipid metabolism. The smaller LD exhibit higher lipolytic activity compared with the larger LD (17). Lipolytic activity affects the surface-volume ratio of LD, which results in more efficient degradation of stored TG by several lipid metabolic enzymes and perilipin. These findings suggested that treatment of cells with indirubin significantly enhanced adipocyte differentiation and lipolysis. Therefore the intracellular distribution of TG and the generation of micro LDs may enhance lipid metabolism and improve insulin resistance.

Estrogen is produced in mature adipocytes and its deficiency leads to numerous metabolic disturbances, including insulin resistance (18). The present study revealed that indirubin promoted estrogen biosynthesis in 3T3-L1 cells, suggesting that it may directly and/or indirectly regulate the activity of aromatase and generate estradiol via PPARγ. The mitogen-activated protein kinase (MAPK) and AMPK are regulated by different metabolic pathways, such as PPARγ and C/EBPα, which are required to maintain adipocyte function (19,20). Therefore, indirubin may result in the enhancement of aromatase activity by directly and/or indirectly acting on the mitogen-activated protein kinase (MAPK) or PI3K/Akt signaling pathways. Thus, indirubin may serve as a novel modulator of estrogen biosynthesis that can act locally in adipose tissues.

The PPARγ and PI3K/Akt signaling pathway regulate adipocyte differentiation and lipid metabolism. It was reported that hyperglycemia in 3T3-L1 adipocytes was ameliorated by the upregulation of PPARγ and PI3K/Akt and promoting fat accumulation (21). On the other hand, it was revealed that water-extracted plum (Prunus salicina L. cv. Soldam) attenuates adipogenesis in murine 3T3-L1 adipocyte cells through the PI3K/Akt signaling pathway (22). These studies suggested that the activities of anti-adipogenic and/or lipolysis via enhancing cell differentiation without cytotoxicity were enhanced. The results of the present study revealed that indirubin, an extract from *Polygonum tinctorium*, exhibited PPARγ ligand-binding ability and enhanced adipocyte differentiation by increasing its transcriptional activity. In mature adipocytes, indirubin was involved in the promotion of glucose uptake, reduction of TG accumulation and lipid size and enhancement of estrogen biosynthesis. Therefore, indirubin may serve as a novel agent in the treatment of diabetes mellitus by improving insulin resistance.

Finally, there are some limitations to this study. These assays were performed assuming that insulin resistance in patients with type 2 diabetes was improved by the use of thiazolidinedione. In other words, this study attempted to elucidate the mechanism in which indirubin improves insulin resistance in the presence of insulin. In addition, inhibition of PPARγ by GW9662 has a critical effect on the survival of mature adipocytes. Therefore, it should be demonstrated whether indirubin improves insulin resistance without insulin after proving its detailed mechanism in the presence of insulin.

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Availability of data and materials

All data generated or analyzed during the current study are included in this published article.

Authors' contributions

TK and KS conceived and designed the study. TK wrote the manuscript and KS revised the manuscript. TK, KK and TM performed and analyzed the experiments. TK, KS, KK and TM interpreted experiment data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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