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D-dopachrome tautomerase promotes IL-6 expression and inhibits adipogenesis in preadipocytes

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Abstract

We previously identified D-dopachrome tautomerase (DDT) as a novel adipokine whose mRNA levels in adipocytes are negatively correlated with obesity-related clinical parameters, and which acts on adipocytes to regulate lipid metabolism. Here we investigated functions of DDT on preadipocytes. Recombinant DDT (rDDT) enhanced both the expression and secretion of interleukin-6 (IL-6) in SGBS cells, a human preadipocyte cell line. Treatment with rDDT increased levels of phosphorylated ERK1/2, but not p38, in SGBS cells, and rDDT-induced IL-6 mRNA expression was attenuated by pretreatment with an ERK inhibitor, U0126. Knockdown of CD74, but not CD44, inhibited rDDT-induced IL-6 mRNA expression in SGBS cells. These results suggested that the rDDT-induced IL-6 expression in preadipocytes occurred through the CD74-ERK pathway. Furthermore, in SGBS cells subjected to adipogenic induction, rDDT decreased the amount of triacylglycerol, number of cells with oil droplets, and levels of mRNA encoding adipocyte marker proteins. Increased expression of CCAAT/enhancer binding protein families and peroxisome proliferation activated receptor γ2 during adipogenesis was inhibited in the cells treated with rDDT. These results suggested DDT to inhibit adipogenesis by suppressing the expression of genes encoding adipogenic regulators in preadipocytes.

Keywords

D-dopachrome tautomerase; Preadipocyte; Adipokine; ERK; IL-6; Adipogenesis

Abbreviations

AT, adipose tissue; SVF, stromal vascular fraction; DDT, D-dopachrome tautomerase; MIF, macrophage migration inhibitory factor; PKA, protein kinase A; AMPK, AMP-activated protein kinase; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IBMX, 3-isobutyl-1-methylxanthine; DAPI, 4',6'-diamidine-2-phenylindole dihydrochloride; MMP, matrix-metalloproteinase; PPAR, peroxisome proliferator-activated receptor; C/EBP, CCAAT/enhancer binding protein; STAT3, signal transducer and activator of transcription-3

1. Introduction

Adipose tissue (AT) is not only an energy storage organ, but also a source of various secreted functional factors, adipokines. Adipokines include proinflammatory factors such as resistin, tumor necrosis factor-α, interleukin-6 (IL-6), monocyte chemoattractant protein-1, and plasminogen activator inhibitor-1 and anti-inflammatory factors such as adiponectin and leptin [1]. AT is composed of mature adipocytes and a stromal vascular fraction (SVF) that includes preadipocytes, macrophages, and vascular cells. Obese AT is characterized by an increased AT mass and facilitates the infiltration of macrophages [2]. These macrophages interact with adipocytes or preadipocytes to induce secretion of inflammatory cytokines and free fatty acids [3], resulting in systemic insulin resistance which is a risk factor for type 2 diabetes, hypertension, and dyslipidemia [4].

D-dopachrome tautomerase (DDT) was identified as an enzyme converting D-dopachrome into 5,6-dihydroxyindole [5], but its physiological significance has not been elucidated due to an inactive substrate in mammals. DDT shares homology with macrophage migration inhibitory factor (MIF) in primary (33% identical) and tertiary structure [6]. MIF acts as a cytokine involved in the amplification of inflammatory and immune responses [7, 8]. Furthermore, MIF converts D-dopachrome into another tautomer, 5,6-dihydroxyindole-2-carboxylic acid [9]. Thus, the similarities with MIF suggest DDT to act as a cytokine with a certain function. Recently, DDT was reported to be detectable in human serum and to function as a MIF homolog

in non-small cell lung carcinomas and macrophages through CD74, a component of the MIF receptor complex [10, 11, 12].

Previously, we found DDT to be secreted from adipocytes by using a proteomic approach [13]. Levels of DDT mRNA in adipocytes were negatively associated with body mass index or fat areas and the expression of DDT was observed in mature adipocytes, but not in preadipocytes [13]. DDT acts on adipocytes in an autocrine/paracrine manner to regulate lipid metabolism through inhibition of protein kinase A (PKA) activity and/or activation of AMP-activated protein kinase (AMPK), and administration of a recombinant form of the protein in db/db mice improved glucose intolerance and serum levels of free fatty acids, suggesting that DDT acts as an adipokine with anti-obesity [13]. To clarify further the function of DDT in AT, we here investigated the actions of DDT on preadipocytes.

2. Materials and methods

2.1. Purification of recombinant human DDT (rDDT) and MIF (rMIF)

rDDT was obtained as described previously [13]. Human MIF cDNA was amplified by reverse transcription PCR (RT-PCR) using total RNA from THP-1 cells, a human monocyte cell line, and the recombinant protein was produced in the same manner. The concentration of endotoxin in these recombinant proteins was calculated to be below 1 EU/μg by using the ToxinSensor Endotoxin Detection System (Genscript, Piscataway, NJ). Tautomerase activity of rMIF was assessed using the L-dopachrome methyl ester as a substrate, measuring the changes in absorbance at 475 nm [9, 14]. Briefly, 720 μl of 10 mM sodium phosphate/1 mM EDTA, pH 6.0 was added to a 1 ml disposable cuvette, and then 48 μl of 10 mM L-dopa methyl ester (Sigma, St. Louis, MO) and 32 μl of sodium *m*-periodate (Sigma) were added to generate L-dopachrome methyl ester. The spontaneous decay of absorbance at 475 nm was monitored for 30 sec. Each 5 μg of rMIF and equal volume of PBS as a control was added to the cuvette and the accelerated decay of absorbance at 475 nm due to dopachrome tautomerization was monitored for 15 min.

2.2. Cell culture

SGBS cells, a human preadipocyte cell line with a high capacity for adipose differentiation [16], were kindly provided by Dr. Wabitsch. The cells were cultured on 6-well

plates in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 (1:1) (WAKO, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY), 33 μM biotin (Sigma), and 17 μM panthothenic acid (Sigma). DDT knockdown adipocytes were made by infection with an adenovirus expressing short hairpin RNA (shRNA) for the DDT gene as described previously [13]. After confluent SGBS cells were pre-incubated with serum-free DMEM/Ham's F12 (1:1) for 12 h, they were treated with rDDT or rMIF at 20 nM or the indicated concentrations for 12 h. To investigate the signal transduction pathway for DDT, SGBS cells were pretreated with 10 μM U0126 (Calbiochem, San Diego, CA) or dimethyl sulfoxide (DMSO; WAKO) for 30 min and then treated with 20 nM rDDT for 12 h.

2.3. RNA extraction and real-time RT-PCR

Total RNA from the cells was extracted with ISOGEN (Nippongene, Tokyo, Japan). Each cDNA was synthesized from total RNA using the Prime script™ RT Reagent Kit (TaKaRa, Shiga, Japan). Real-time RT-PCR was performed on an Applied Biosystems Prism 7300 Real Time PCR system (Applied Biosystems, Foster City, CA) using THUNDERBIRD™SYBR® qPCR Mix (TOYOBO, Tokyo, Japan) with each specific primer set (Table 1). The expression of each gene was normalized to that of glyceraldehyde-3-phosphate dehydrogenase. The data were analyzed using 7300 System Sequence Detection software (version 3.1; Applied Biosystems).

2.4. Western blotting

Each cell was washed with phosphate-buffered saline (PBS) and lysed in a mixture of sodium dodecyl sulfate (SDS) sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 125 mM Tris, pH 7.6) and lysis buffer (150 mM NaCl, 1 mM EDTA, 1% Triton-X, 50 mM Tris, pH 7.6) at a volume ratio of 1:4. The lysate was boiled at 95°C for 3 min, and centrifuged at 16,100 x *g* for 30 min. The supernatant was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to polyvinylidene difluoride membranes (Immobilon Transfer Membranes; Millipore, Bedford, MA). After incubation in blocking solution (Blocking One; Nakalai tesque, Kyoto, Japan), the membranes were incubated with a 1:1,000-diluted rabbit antibody against ERK1/2, phospho-ERK1/2, p38, or phospho-p38 (Cell signaling, Beverly, CA), or a 1:2,000-diluted mouse antibody against β-actin (Sigma). The membranes were incubated with an anti-rabbit or -mouse IgG-horseradish peroxidase-conjugated secondary antibody (1:40,000 or 1:80,000, respectively; GE Healthcare, Buckinghamshire, UK). Signal detection was performed using Immobilon Western Detection Reagent (Millipore) and exposure to X-ray film.

2.5. Enzyme-linked immunosorbent assay (ELISA)

SGBS cells were incubated with serum-free DMEM/Ham's F12 (1:1) with or without 20

nM rDDT for 24 h, and the cells and conditioned medium were collected. The conditioned medium and cell lysate in the lysis buffer were centrifuged at 16,100 x *g* for 20 min to remove debris. The concentration of IL-6 was measured by Quantikine Human IL-6 Immunoassay (R&D systems, Minneapolis, MN) according to the manufacturer's directions.

2.6. Transfection of small interfering RNA (siRNA)

SGBS cells were transfected with 1 μM CD74 siRNA (Invitrogen, Carlsbad, CA), 100 nM CD44 siRNA (Invitrogen), or negative control siRNA (Silencer® Negative Control #1 siRNA; Applied Biosystems) by electroporation using a Microporator MP-100 (Digital Bio, Seoul, South Korea). The CD74- and CD44-specific siRNA sequences were 5'-AUCCAUGACUGGCUUCUGAUCUUCC-3' and 5'-UAUUGAAAGCCUUGCAGAGGU-CAGC-3', respectively. Forty-eight hours after the transfection, the culture medium was changed to serum-free DMEM/Ham's F12 (1:1) containing 20 nM rDDT for 12 h.

2.7. Evaluation of adipogenesis

SGBS cells were preincubated with rDDT for 12 h and subjected to adipogenic induction as described by Wabitsch *et al.* [16] in the presence or absence of rDDT. Briefly, confluent SGBS cells were cultured in FBS-free differentiation medium: DMEM/Ham's F12 (1:1) supplemented with 33 μM biotin, 17 μM pantothenic acid, 500 μM

3-isobutyl-1-methylxanthine (IBMX; Sigma), 2 μM troglitazone (Sigma), 20 nM insulin (WAKO), 100 nM cortisol (WAKO), 0.2 nM triiodothyronine (WAKO), 25 nM dexamethasone (WAKO), and 10 μg/ml human transferrin (Calbiochem, Los Angels, CA). After 4 days, this medium was replaced with differentiation medium excluding IBMX, troglitazone, and dexamethasone, which was changed every 3 days. At 9 days after the induction, the cells were fixed with formaldehyde for 20 min. After being washed with 60% isopropanol, the cells were stained with Oil red O (Muto Pure Chemicals, Tokyo, Japan). The cells were washed with 60% isopropanol followed by distilled water, and observed under a microscope. After the cells were dried, Oil red O was eluted with 1 ml/well of isopropanol and the absorbance was measured at 500 nm by a spectrophotometer (Ultrospec 6300 pro; GE Healthcare). For the counting of cells with lipid droplets, fixed cells were also treated with 0.2% Triton-X100 for 15 min, washed with PBS, and subjected to 4',6'-diamidine-2-phenylindole dihydrochloride (DAPI) and Sudan III staining. The cells were observed under a fluorescence microscope (TE-2000; Nikon, Tokyo, Japan). The ratio of Sudan III-positive cells to DAPI-stained cells in 6 randomly selected low-power fields (x 100) was calculated.

2.8. Statistical analysis

Statistical analyses were performed using Student's t-test. Differences were considered to be significant when the *P*-value was less than 0.05.

3. Results

3.1. rDDT promotes IL-6 expression and secretion in preadipocytes

We previously reported that the expression of genes encoding enzymes related to lipid metabolism was increased in DDT knockdown adipocytes differentiated from SGBS cells [13]. Further analysis revealed a reduction of IL-6 mRNA levels in the cells (Fig. 1A). Because DDT knockdown adipocytes included undifferentiated preadipocytes and IL-6 was reported to be mainly secreted from preadipocytes rather than mature adipocytes [17, 18], we hypothesized that DDT acts on preadipocytes to induce IL-6 gene expression. To demonstrate this, we examined the effect of rDDT on IL-6 expression in SGBS preadipocytes. As expected, treatment with rDDT dose-dependently induced IL-6 gene expression in SGBS cells (Fig. 1B) and increased IL-6 protein levels both in the cells and in the conditioned medium (Fig. 1C). These results indicated that DDT acts on preadipocytes to promote IL-6 expression and secretion. To compare the effect of DDT with that of MIF, which is similar to DDT in tertiary structure, on SGBS cells, we prepared rMIF and confirmed its enzymatic activity (Fig. 1D). Treatment with rMIF did not influence IL-6 mRNA levels in SGBS cells (Fig. 1E). To confirm the biological activity of rMIF, effect of rMIF on mRNA expression of matrix metalloproteinase (MMP)-1 and MMP-3, which are induced by MIF in synovial fibroblasts [19], was investigated. rMIF up-regulated MMP-1 and MMP-3 gene expression in SGBS cells (Fig. 1F), suggesting that the recombinant retained the biological activity. In addition, MMP-1 and

MMP-3 gene expression in SGBS cells was also induced by rDDT (Fig. 1G). These results suggest that DDT partially, but not completely, acts as MIF's homolog in SGBS cells.

3.2. ERK signaling is involved in rDDT-induced IL-6 expression

We examined the involvement of MAPK signaling in rDDT-induced IL-6 expression in preadipocytes. Levels of phosphorylated ERK1/2, but not p38, were increased in SGBS cells after 30 min of rDDT treatment (Fig. 2A). In addition, IL-6 gene expression induced by rDDT was completely inhibited in the presence of U0126, an ERK inhibitor (Fig. 2B). These results indicated that DDT promotes IL-6 expression in SGBS cells through the ERK/MAPK pathway.

3.3. CD74, but not CD44, is involved in rDDT-induced IL-6 expression

Because the tertiary structure of DDT is similar to that of MIF [6], whose signal is mediated by a CD74/CD44 receptor complex [20, 21], we examined the involvement of these components in rDDT-induced IL-6 expression using SGBS cells transduced with siRNA against the CD74 or CD44 gene. CD74 and CD44 gene expression was confirmed to be inhibited in these cells (Fig. 3A, B). rDDT-induced IL-6 gene expression was attenuated in CD74 knockdown SGBS cells (Fig. 3C). Conversely, the knockdown of CD44 had no effect (Fig. 3D). These results suggested that CD74, but not CD44, is involved in the signaling for DDT-induced IL-6 expression in preadipocytes.

3.4. rDDT inhibits adipogenesis in preadipocytes

Next, we investigated the effects of rDDT on adipogenesis in preadipocytes. The amount of triacylglycerol in SGBS cells subjected to adipogenesis in the presence of rDDT was less than that in control cells (Fig. 4A) and rDDT dose-dependently decreased the number of cells with lipid droplets (Fig. 4B). Furthermore, mRNA levels of genes expressed in adipocytes, such as peroxisome proliferator-activated receptor (PPAR) γ2, CCAAT/enhancer binding protein (C/EBP) α, aP2, and adiponectin, were significantly lower in the cells treated with rDDT (Fig. 4C). These results clearly indicated that rDDT inhibited adipogenesis in SGBS cells.

3.5. rDDT inhibits induction of adipogenic regulators during adipogenesis

To reveal the molecular mechanism involved in the inhibition of adipogenesis by rDDT, mRNA levels of adipogenic regulators during adipogenesis were examined in rDDT-treated SGBS cells. The expression of C/EBPβ and C/EBPδ, involved in the early stages of adipogenesis, was inhibited in rDDT-treated cells (Fig. 5). Furthermore, mRNA levels of adipogenic regulators in the late stages, C/EBPα and PPARγ2, whose expression is induced by C/EBPδ and C/EBPβ, were lower at 6 days after adipogenic induction in rDDT-treated cells (Fig. 5). These results suggested that DDT impairs adipogenesis by inhibiting the induction of

adipogenic regulators during adipogenesis.

4. Discussion

DDT is secreted from adipocytes and acts in a paracrine or autocrine manner to regulate lipid metabolism in adipocytes [13]. In the present study, we demonstrated that DDT also acts on preadipocytes to promote the expression of IL-6 and to inhibit adipogenesis. DDT is similar to MIF in tertiary structure [6], and both proteins exhibit tautomerase activity using D-dopachrome as a substrate [5, 9]. Recently, DDT was reported to be a functional homolog of MIF because it binds to CD74, a component of the MIF receptor complex, leading to the activation of ERK and its downstream proinflammatory pathways [11, 12]. Indeed, MIF and DDT had the same effects on MMP-1 or MMP-3 mRNA expression in SGBS cells in the present study. However, DDT, but not MIF, increased IL-6 expression in SGBS cells. Furthermore, expression of DDT was observed in adipocytes, but not in preadipocytes, and increased during adipogenesis [13], whereas MIF is expressed in both cell types and its mRNA levels do not increase with differentiation [22]. These results suggest that the function of DDT in AT is not completely the same as that of MIF. MIF activates the MAPK cascade (the ERK pathway) through a CD74/CD44 receptor complex and is involved in cell proliferation, differentiation, survival, and inflammatory responses in various cell types [20, 21, 23]. It is considered that CD74 and CD44 are involved in MIF binding and intracellular signal

transduction, respectively [20]. In the present study, knockdown of CD74, but not CD44, attenuated rDDT-induced IL-6 gene expression in preadipocytes, suggesting that the DDT receptor complex may consist of CD74 and component(s) other than CD44 in preadipocytes. Alternatively, there is a possibility that CD74 solely mediates DDT to stimulate IL-6 expression in preadipocytes. The difference in the actions of DDT and MIF on preadipocytes may be derived from different components of the receptor complex. Further experiments are necessary to identify the DDT receptor or the complex in preadipocytes.

IL-6 is a pleiotropic inflammatory cytokine involved in immune and inflammatory responses and associated with hematopoiesis and carcinogenesis [24]. On the other hand, transient IL-6 up-regulation is reported to contribute improvement of insulin sensitivity [25]. For example, enhancement of insulin signaling in liver by adiponectin is mediated by IL-6 secretion from AT macrophages [26] and IL-6 released from skeletal muscle during exercise enhances insulin secretion from pancreatic islets directly or indirectly through glucagon-like peptide-1 [27, 28]. We previously showed that the administration of rDDT in db/db mice improved their glucose intolerance [13]. Therefore, we hypothesized that rDDT-induced IL-6 in preadipocytes is involved in improvement of glucose intolerance in db/db mice treated with rDDT, however, we have not demonstrated the hypothesis yet. Further experiments need to clarify the significance of rDDT-induced IL-6 secretion from preadipocytes in glucose tolerance.

DDT was revealed to have an inhibitory effect on adipogenesis. Although IL-6 secreted from obese AT is reported to inhibit adipogenesis [29], adipogenesis inhibited by rDDT may mainly depend on mechanisms other than induction of IL-6 in preadipocytes. IL-6 is reported to inhibit the expression of C/EBPα, but not C/EBPβ, C/EBPδ, and PPARγ, during adipogenesis in 3T3-L1 [29]; however, the gene expression of these four adipogenic regulators was inhibited in rDDT-treated SGBS cells. For example, phosphorylation of ERK induced by Pref1, a factor inhibiting adipogenesis, is reported to up-regulate the expression of Sox-9, which directly binds to the promoter region of C/EBPβ and C/EBPδ to suppress their transcription [30]. As well as Pref1, DDT may inhibit adipogenesis by activating the ERK/MAPK pathway in preadipocytes. However, we could not exclude the involvement of other factors in the inhibition of adipogenesis by DDT.

In conclusion, DDT secreted from adipocytes acts on preadipocytes to promote IL-6 expression and to inhibit adipogenesis by suppressing the induction of genes encoding adipogenic regulators.

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Figure Legends

Fig. 1. rDDT induces IL-6 expression in SGBS cells. (A) IL-6 mRNA levels in adipocytes differentiated from SGBS cells expressing shNC and shDDT were measured by real-time RT-PCR. The levels are shown relative to those in cells expressing shNC. (B) IL-6 mRNA levels were measured in SGBS cells treated with each concentration of rDDT. The levels are shown relative to those in untreated cells. (C) IL-6 protein levels in the cell lysate (a left graph) and conditioned medium (a right graph) from untreated cells (rDDT(-)) or rDDT-treated cells (rDDT(+)) were measured by ELISA. IL-6 protein levels in the lysate were normalized to total cellular protein levels and are shown relative to those in the untreated cell lysate. (D) Enzymatic activity of rMIF was measured by decrease in absorbance at 475 nm of L-dopachrome methyl ester. The data are shown relative to absorbance of controls. Data are the mean ± S.E. (n=3). (E) IL-6 mRNA levels in SGBS cells treated with rMIF at the indicated concentration were measured by real-time RT-PCR. The levels are shown relative to those in untreated cells. (F, G) MMP-1 and MMP-3 mRNA levels in SGBS cells treated with rMIF (F) or rDDT (G) were measured by real-time RT-PCR. The levels are shown relative to those in untreated cells. Each graph is representative of at least 3 independent experiments. Data are the mean ± S.E. (n=3). *P<0.05.

Fig. 2. rDDT-induced IL-6 expression in preadipocytes is mediated by ERK activation. (A)

Phosphorylation of ERK1/2 and p38 in rDDT-treated SGBS cells at the indicated time points was assessed by Western blotting. β-actin was used as internal control. (B) IL-6 mRNA levels in SGBS cells treated with or without rDDT in the presence of U0126 or DMSO were measured by real-time RT-PCR. The levels in rDDT-treated cells (black bars) are shown relative to those in untreated cells (white bars). Each graph is representative of at least 3 independent experiments. Data are the mean ± S.E. (n=3). *P<0.05.

Fig. 3. rDDT-induced IL-6 expression in preadipocytes is mediated by CD74. (A, B) mRNA levels of CD74 (A) and CD44 (B) in SGBS cells transfected with siRNA against CD74 (siCD74) and CD44 (siCD44) were measured by real-time RT-PCR, respectively. The levels are shown relative to those in cells transfected with nontargeting siRNA (siNC). (C, D) IL-6 mRNA levels in rDDT-treated SGBS cells transfected with siCD74 (C) or siCD44 (D) were measured by real-time RT-PCR. The levels in rDDT-treated cells (black bars) are shown relative to those in untreated cells (white bars). Each graph is representative of at least 3 independent experiments. Data are the mean ± S.E. (n=3). *P<0.05.

Fig. 4. rDDT inhibits adipogenesis in SGBS cells. Confluent SGBS cells were pre-incubated with serum-free medium supplemented with or without rDDT (20 nM or the indicated concentration), and then induced to differentiate into adipocytes in the presence or absence of

rDDT. At 9 days after the adipogenic induction, the degree of differentiation into adipocytes was evaluated by Oil red O staining (A), Sudan III staining (B), and mRNA levels of adipogenic markers (C). (A) The cells stained by Oil red O were observed under a microscope at 400 x magnification (left images), and absorbance of eluted Oil red O was measured (right graph). (B) The cells were double-stained by DAPI and Sudan III and observed under a microscope at 400 x magnification (left images). The right graph represents the average percentage of Sudan III- positive cells among DAPI-stained cells per microscopic low-power field (x 100). (C) mRNA levels of adipocyte-specific genes, PPARγ2, C/EBPα, adiponectin, and aP2, were measured by real-time RT-PCR. The levels in rDDT-treated cells (rDDT(+)) are shown relative to those in untreated cells (rDDT(-)). Each graph is representative of at least 3 independent experiments. Data are the mean ± S.E. (n=3). *P<0.05.

Fig. 5. rDDT inhibits induction of adipogenic regulators during adipogenesis. The experiment was performed in the same manner as Fig. 3. At 0, 2, 4, and 6 day(s) after the adipogenic induction, mRNA levels of C/EBPβ, C/EBPδ, C/EBPα, and PPARγ2 in the cells treated with rDDT (dashed lines) and in control cells (solid lines) were measured by real-time RT-PCR. The levels are shown relative to those in untreated cells at day 0. Each graph is representative of at least 3 independent experiments. Data are the mean ± S.E. (n=3). *P<0.05.

Table 1

Sequences of primers used for real-time RT-PCR

Gene	Forward (5'-3')	Reverse (5'-3')
adiponectin	GTGATGGCAGAGATGGCAC	ACACTGAATGCTGAGCGGTA
aP2	CCTGGTACATGTGCAGAAAT	AGAGTTCAATGCGAACTTCA
CD44	GGCGCAGATCGATTTGAATA	TTCTCCATCTGGGCCATTGT
CD74	TAGACAGATCCCCGTTCCTG	TGGAAAACATTGGCTCTTCC
C/EBPα	AAGAAGTCGGTGGACAAGAACAG	GCAGGCGGTCATTGTCACT
C/EBPβ	CTGGAGACGCAGCACAAG	ACAGCTGCTCCACCTTCTTC
C/EBPδ	GGTGCCCGCTGCAGTTT	CTCGCAGTTTAGTGGTGGTAAGTC
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
IL-6	TACCCCCAGGAGAAGATTCC	TTTTCTGCCAGTGCCTCTTT
MMP-1	ATGCTGAAACCCTGAAGGTG	CTGCTTGACCCTCAGAGACC
MMP-3	GCAGTTTGCTCAGCCTATCC	GAGTGTCGGAGTCCAGCTTC
PPARγ2	AGCAAACCCCTATTCCATGCT	ATCAGTGAAGGAATCGCTTTCTG

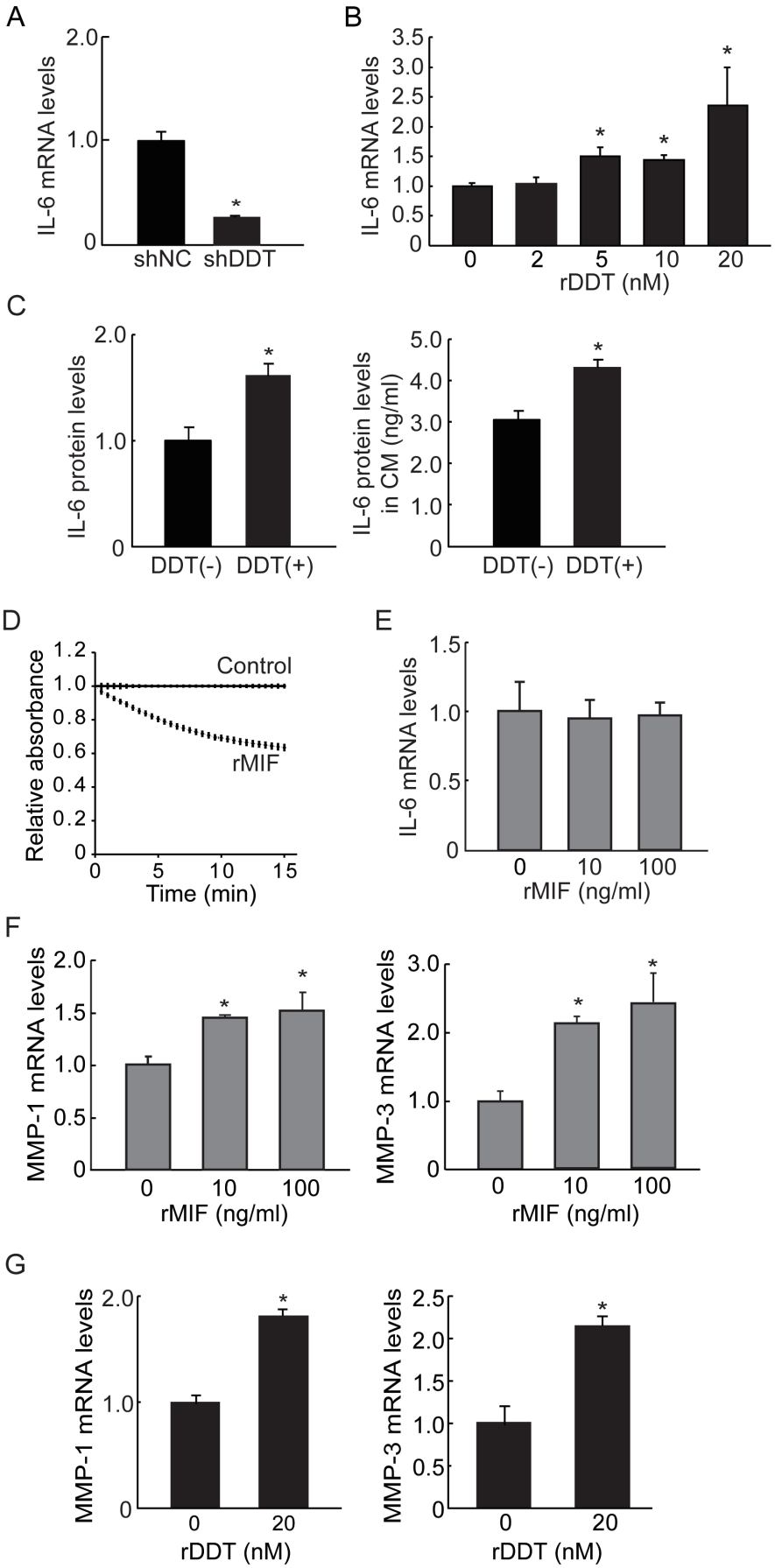
A
IL-6 mRNA levels
2.0
1.0
0
shNC
shDDT
B
IL-6 mRNA levels
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0
2
5
10
20
rDDT (nM)
C
IL-6 protein levels
2.0
1.0
0
DDT(-)
DDT(+)
IL-6 protein levels in CM (ng/ml)
5.0
4.0
3.0
2.0
1.0
0
DDT(-)
DDT(+)
D
Relative absorbance
1.2
1.0
0.8
0.6
0.4
0.2
0
Control
rMIF
0
5
10
15
Time (min)
E
IL-6 mRNA levels
1.5
1.0
0.5
0
0
10
100
rMIF (ng/ml)
F
MMP-1 mRNA levels
2.0
1.5
1.0
0.5
0
0
10
100
rMIF (ng/ml)
MMP-3 mRNA levels
3.0
2.0
1.0
0
0
10
100
rMIF (ng/ml)
G
MMP-1 mRNA levels
2.0
1.0
0
0
20
rDDT (nM)
MMP-3 mRNA levels
2.5
2.0
1.5
1.0
0.5
0
0
20
rDDT (nM)

Fig.1

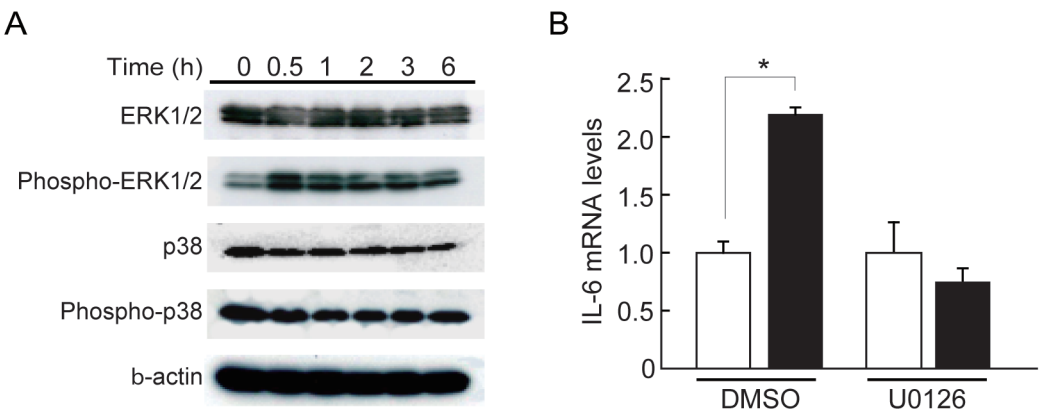
A
Time (h) 0 0.5 1 2 3 6
ERK1/2
Phospho-ERK1/2
p38
Phospho-p38
b-actin
B
IL-6 mRNA levels
2.5
2.0
1.5
1.0
0.5
0
*
DMSO
U0126

Fig.2

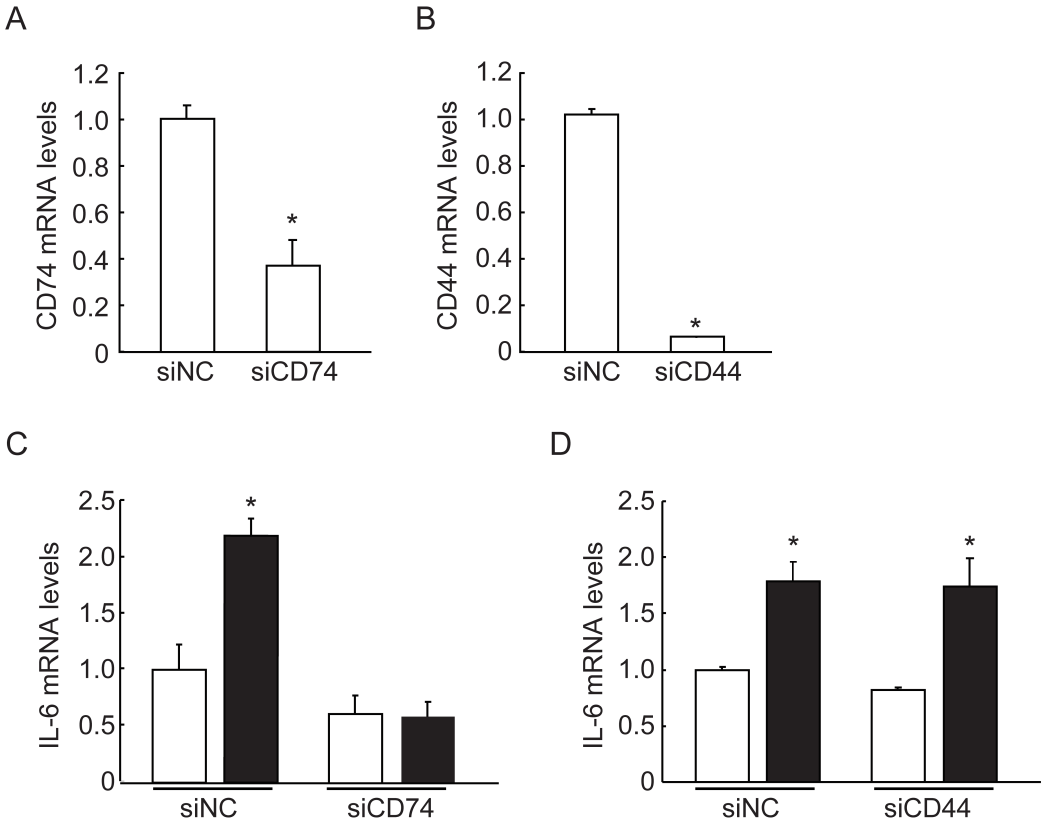
A
B
C
D
CD74 mRNA levels
CD44 mRNA levels
IL-6 mRNA levels
IL-6 mRNA levels
1.2
1.0
0.8
0.6
0.4
0.2
0
2.5
2.0
1.5
1.0
0.5
0
*
siNC
siCD74
siCD44

Fig. 3

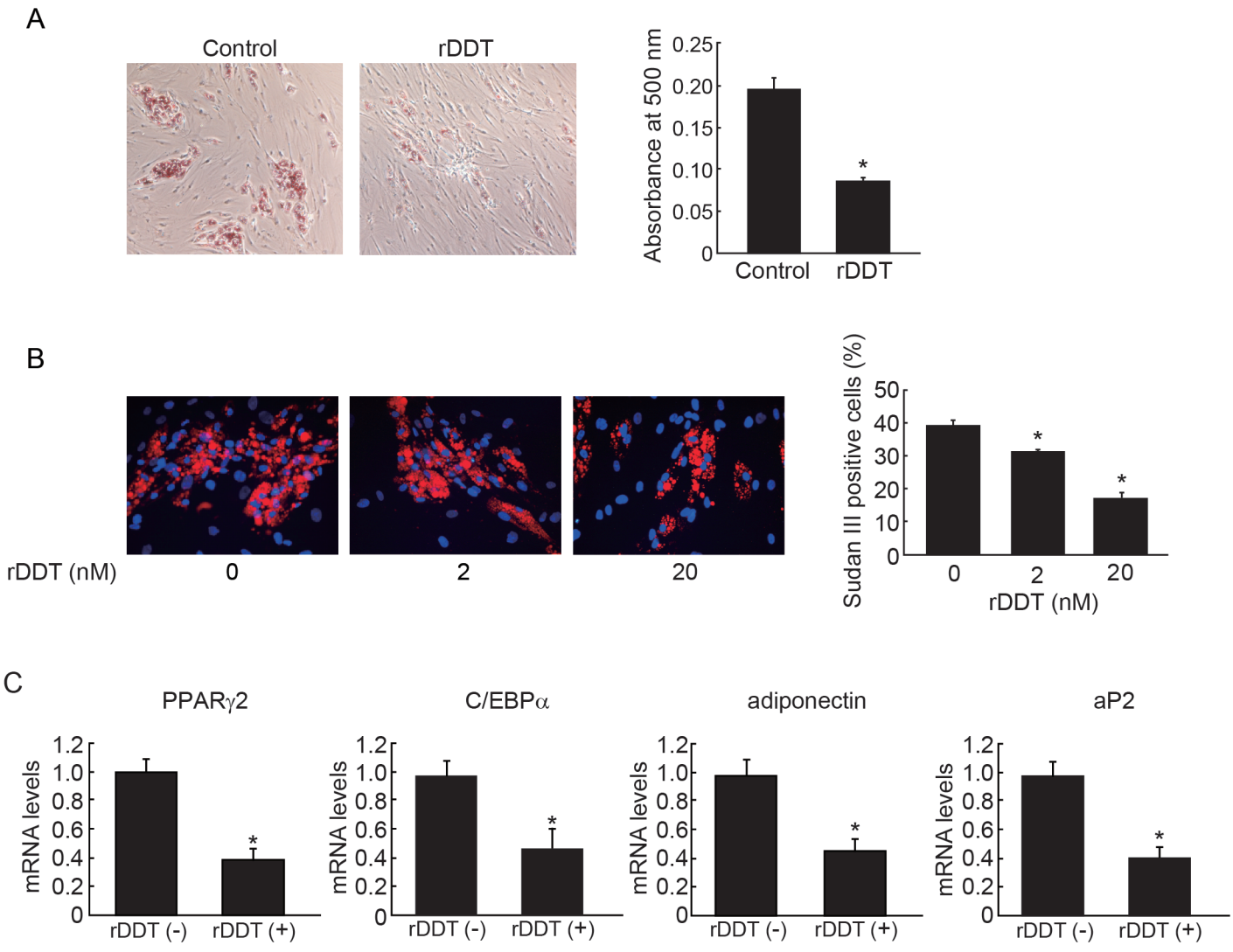
A
Control
rDDT
Absorbance at 500 nm
0.25
0.20
0.15
0.10
0.05
0
*
Control
rDDT
B
rDDT (nM)
0
2
20
Sudan III positive cells (%)
50
40
30
20
10
0
*
*
0
2
20
rDDT (nM)
C
PPARγ2
C/EBPα
adiponectin
aP2
mRNA levels
1.2
1.0
0.8
0.6
0.4
0.2
0
*
rDDT (-)
rDDT (+)

Fig. 4

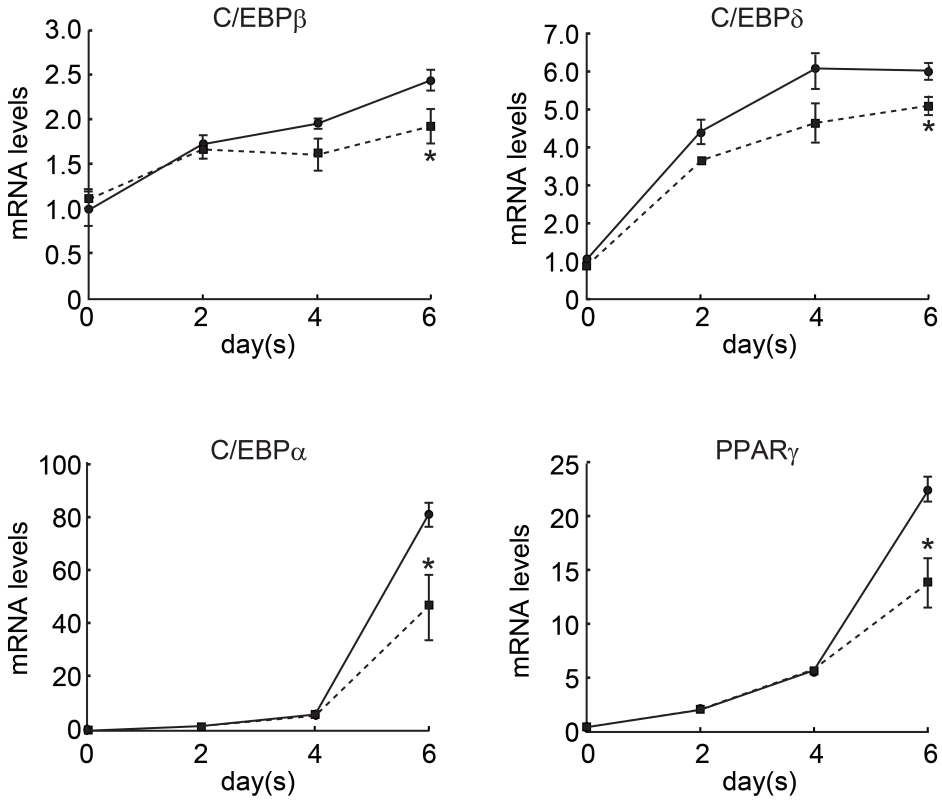
C/EBPβ
mRNA levels
day(s)
C/EBPδ
mRNA levels
day(s)
C/EBPα
mRNA levels
day(s)
PPARγ
mRNA levels
day(s)

Fig. 5