1 **Caffeine-stimulated intestinal epithelial cells suppress lipid accumulation in adipocytes**

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11 **Running head**: Caffeine suppresses lipid accumulation

12 **1 Table**

13 **6 Figures**

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19 **Abbreviations used:** C/EBP, CCAAT/enhancer-binding protein; IL, Interleukin; PAI-1,

20 plasminogen activator inhibitor-1; PPAR, peroxisome proliferator-activated receptor; qRT-PCR,

21 quantitative real-time PCR

1 **Summary**

2 Caffeine is a methylxanthine derived from plant foods such as coffee beans and tea leaves, and
3 has multiple biological activities against physiological response and several diseases. Although
4 there are some reports about the direct effect of caffeine against anti-lipid accumulation *in vitro*,
5 the effect of caffeine on lipid accumulation in adipocytes through stimulating intestinal
6 epithelial cells is unknown. Since direct treatment with caffeine to 3T3-L1 cells did not affect
7 lipid accumulation, we determined whether caffeine-stimulated intestinal epithelial Caco-2
8 cells influence the lipid accumulation in 3T3-L1 adipocytes. Caco-2 cells were cultured on a
9 transwell insert with or without caffeine for 24 h. Subsequently, the basolateral component of
10 the Caco-2 cell culture on the transwell was collected and termed caffeine-conditioning medium
11 (CCM). When 3T3-L1 adipocytes were incubated with CCM, CCM decreased lipid
12 accumulation and suppressed gene expression of proliferator activated receptor (PPAR) γ and
13 CCAAT/enhancer binding protein (C/EBP) α in 3T3-L1 adipocytes. Furthermore, CCM
14 decreased the expression of C/EBPβ and C/EBPδ at the protein level, but not at the mRNA
15 level. We observed that a proteasome inhibitor, MG132, inhibited CCM-caused down-
16 expression of C/EBPβ and C/EBPδ proteins, and that CCM promoted the ubiquitination level
17 of C/EBPβ and C/EBPδ proteins. Protein microarray analysis showed caffeine suppresses the
18 secretion of inflammatory cytokines, interluikin-8 and plasminogen activator inhibitor-1 from
19 Caco-2 cells. These results suggest that caffeine indirectly suppresses lipid accumulation in
20 3T3-L1 adipocytes through decreasing secretion of inflammatory cytokines from Caco-2 cells.

21

22 ***Key Words*** caffeine, adipogenesis, 3T3-L1 adipocytes, Caco-2 cells,

1 **Introduction**

2

3 Obesity is a risk factor for multiple lifestyle diseases such as apoplexy, heart disease and
4 diabetes (*1, 2*). Adipocytes play a critical role in regulating lipid metabolism and energy balance,
5 which are associated with adiposity (*3*). An increase in the number of differentiated mature
6 adipocytes and excess accumulation of body fat leads to adiposity. Adipocytes accumulate
7 intracellular lipids during differentiation. As an *in vitro* model system for adipogenesis, murine
8 3T3-L1 cells, which were originally derived from mouse embryos, are often used.
9 Differentiation of 3T3-L1 cells is induced by exposure to the inducers, such as 3-isobutyl-1-
10 methylxanthine (IBMX), dexamethasone and insulin, and is responsible for the accumulation
11 of a large amount of intracellular lipid droplets during adipocyte differentiation. Adipogenesis,
12 the differentiation process for producing adipocytes, is a complex process that is regulated by
13 various transcription factors and their related genes. The expression levels of CCAAT/enhancer
14 binding protein (C/EBP) β and C/EBPδ, as transcriptional factors, are increased in the early
15 phase of adipocyte differentiation (*4*). Subsequently, these transcription factors up-regulate
16 proliferator activated receptor (PPAR) γ and C/EBPα, as the master regulators of adipocyte
17 differentiation (*5*). PPARγ and C/EBPα have been shown to regulate the expression of lipid and
18 glucose metabolism genes, such as fatty acid binding protein 4, CD36, adiponectin and glucose
19 transporter 4 (GLUT4) (*6, 7*).

20 As the *in vitro* model system for absorption and metabolism of intestinal epithelial cells,
21 many researchers have used human epithelial Caco-2 cells, which are derived from human
22 colon adenocarcinoma but this cell linage exhibits small intestinal epithelial cell-like
23 characteristics (*8, 9*). Moreover, Caco-2 cells secrete cytokines such as tumor necrosis factor-α
24 (TNF-α), interleukin (IL)-8 and monocyte chemotactic protein 1 (*10*). Cytokines are classified

as pro-inflammatory cytokines, such as IL-1, IL-8 and TNF-α, and anti-inflammatory cytokines, such as IL-4 and IL-10. IL-4 and granulocyte-colony stimulating factor (G-CSF) which are known to suppress adipocyte differentiation (*11, 12*). Thus, intestinal epithelial cell-derived cytokines might regulate adipogenesis and adipocyte differentiation.

The regulation of adipocyte differentiation by food factors is a crucial therapeutic strategy for prevention and improvement of obesity and its related metabolic syndrome. However, little is known about the effect of food factors on adipocyte differentiation through the stimulation of intestinal epithelial cells. Caffeine (1,3,7-trimethylxanthine) is one of the methylxanthines and is included in coffee beans and tea leaves. Several studies have reported that caffeine increases lipolysis and energy expenditure (*13, 14*) and inhibits hypertrophy of mature adipocytes via suppression of glucose intake (*15*). Furthermore, it is reported that the intake of a diet containing caffeine reduced the weight of parametrical white adipose tissues in rats (*16*), and the weight of intraperitoneal adipose tissues in mice (*17*). To examined the effect of epithelial cells stimulated by caffeine on lipid accumulation in adipocytes, caffeine was added to the apical side of Caco-2 monolayers in a transwell, and 3T3-L1 cells were treated with the permeated basolateral medium, termed caffeine-conditioning medium (CCM).

Materials and Methods

Cell culture and treatment. Caco-2 cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing high glucose (4.5 g/L glucose) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. 3T3-L1 cells were cultured in Dulbecco modified Eagle's medium supplemented with 10% calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin. These cells were cultured in a CO_2 incubator with 5% CO_2/95%

1 air at 37°C. Adipocytes differentiation of 3T3-L1 cells was performed as described previously
2 (*18*). Briefly, 3T3-L1 cells to mature adipocytes were induced by treatment with 0.5 mmol/L
3 IBMX, 1 μmol/L dexamethasone and 10 μg/ml insulin in DMEM containing high glucose for
4 3 d after the cells reached confluence. Then, the cells were treated with 10 μg/ml insulin in
5 DMEM containing high glucose for an additional 3 d. Thereafter, they were maintained in
6 DMEM containing high glucose alone for an additional 2 d and used as mature 3T3-L1 cells.
7 An intestinal permeability system using Caco-2 cells and 3T3-L1 cells was constructed with
8 minor modification of the method described previously (*19*). Briefly, Caco-2 cells were seeded
9 on transwell inserts (polyethylene terephthalate track-etched membrane, 23.1-mm diameter and
10 0.4 μm pore size, BD Biosciences, Durham, NC) in a 6-well plate at a density of 8×10^5
11 cells/insert (4.2 cm^2/insert). Culture medium was replaced three times a week for 20-22 d. When
12 the transepithelial electrical resistance value of the monolayers, which was measured with a
13 Millicell-ERS instrument (Millipore Co., Bedford, MA) was 1985 ± 109 $\Omega \cdot cm^2$, the Caco-2
14 cells were used in experiments. Caffeine at 10 mmol/L was applied to the apical compartment
15 of Caco-2 cells on transwell inserts for 24 h. Then, CCM was collected from the basolateral
16 compartment, and 3T3-L1 cells were incubated in CCM. The culture system used is illustrated
17 in Fig.1.

Fig. 1

18
19 *Sudan II staining*. Post-confluent 3T3-L1 cells were cultured in CCM with 0.5 mmol/L
20 IBMX, 1 μmol/L dexamethasone and 10 μg/ml insulin for 3 d. Then, culture medium was
21 replaced with fresh medium and the cells were cultured for an additional 5 d. Determination of
22 lipid accumulation in 3T3-L1 cells was performed by Sudan II staining as described previously
23 (*18*). Lipid accumulation was also determined by direct application of caffeine to the cells.
24

1 *Quantitative real-time RT-PCR (qPCR).* Total RNA was extracted from 3T3-L1 cells and
2 Caco-2 cells using TRl-zol Reagent (Invitrogen, Carlsbad, CA), and cDNAs were synthesized
3 by reverse transcription using ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). qPCR was
4 performed on a real-time PCR system (TaKaRa PCR Thermal Cycler Dice, Takara Bio, Otsu,
5 Japan) using SYBR Premix Ex Taq II (Takara Bio). The primer sequences are described in Table
6 1. The relative gene expression levels were calculated by the comparative Ct method, using the
7 expression of the *Actb* or *ACTB* gene as an internal control.
8
9 *Western blotting.* Western blot analysis was performed as described previously (*20*). In
10 brief, 3T3-L1 cells were lysed in RIPA buffer, and the cell lysates were subjected to SDS-PAGE
11 and analyzed by western blotting using the following primary antibodies: rabbit polyclonal
12 antibodies [anti-PPARγ; H-100, anti-C/EBPβ; C-19, and anti-C/EBPδ; C-22 (Santa Cruz
13 Biotechnology, Santa Cruz, CA)] antibodies, and goat polyclonal anti-C/EBPα; N-19 (Santa
14 Cruz Biotechnology) antibody, and mouse monoclonal anti-β-actin (clone; C4; Santa Cruz
15 Biotechnology) antibody. The immunoreactive proteins were incubated with horseradish
16 peroxidase-conjugated secondary antibodies and reacted with Immunostar LD (Wako, Osaka,
17 Japan).
18
19 *Measurement of caffeine concentration.* Caffeine at 10 mmol/L was applied to Caco-2
20 monolayers on the transwell inserts in a 6-well plate for 24 h. The medium of the basolateral
21 side was collected. To extract caffeine, chloroform/2-propanol (85:15, v/v) was mixed with the
22 collected medium vigorously for 30 s. After centrifugation at 1,000 × g for 10 min, the aqueous
23 layer was collected, evaporated with a centrifugal concentrator and dissolved in 50% methanol.
24 Caffeine permeating through Caco-2 cells to the medium of the basolateral side was analyzed

by a HPLC system consisting of Shimadzu liquid chromatograph model CBM-20A (Kyoto, Japan) equipped with an autosampler using a Cadenza CL-C18 column (250 × 4.6 mm inner diameter, 3 μm particle diameter; Imtakt, Kyoto, Japan) on a flow rate at 0.5 mL/min, column temperature at 40°C and UV detection at 275 nm. The mobile phase consisted of isocratic solvent (0.05% acetic acid/acetonitrile; 88:12, v/v).

Secretory cytokine analysis. Caffeine at 10 mmol/L was added on the apical side of Caco-2 cells for 24 h. Then, the medium from the basolateral side was subjected to a Proteome Profiler Human Cytokine Array Panel A Array Kit (R&D systems, Minneapolis, MN) according to the manufacturer's instructions.

Statistical analysis. All data are expressed as the means ± SE of triplicate independent determinations for each experiment except the cytokine array. Statistical significance was analyzed by Student's *t*-test, or one- or two-way analyses of variance with Turkey's post hoc testing. Statistical analysis was performed with JMP statistical software version 11.2.0 (SAS Institute, Cary, NC), and statistically significant differences were set at $p < 0.05$.

Results

We examined the effect of intestinal epithelial cells stimulated by caffeine (10 mmol/L) on lipid accumulation in 3T3-L1 adipocytes. Treatment with CCM miniaturized lipid droplets (Fig. 2A) and significantly decreased lipid accumulation in 3T3-L1 cells (Fig. 2B). Ingested caffeine was metabolized by cytochrome P450 (*21*). We hypothesized that caffeine metabolites miniaturized lipid droplets. We determined the concentration of caffeine and its metabolites in

Fig. 2

CCM by HPLC analysis. The peaks of caffeine and its metabolites (theobromine, theophylline, 1-methylxanthine and 1,7-dimethyl uric acid) were well resolved (Fig. 2C, top panel). CCM included the caffeine (28 μmol/L) (Fig. 2C, middle panel), whereas caffeine was not detected in nontreated medium (Fig. 2C, bottom panel). On the other hand, no caffeine metabolites were detected (data not shown). We also examined the effect of caffeine on lipid accumulation after direct application to adipocytes. As shown in Fig. 2D, caffeine itself did not affect lipid accmulation by 50 μmol/L in 3T3-L1 cells. These results indicate that caffeine decreases lipid accumulation in 3T3-L1 cells indirectly through the stimulation of Caco-2 cells.

We next investigated the changes in the expression levels of adipocyte-specific differentiation markers in 3T3-L1 cells after treatment with CCM. CCM significantly decreased the mRNA levels of PPARγ and C/EBPα (Fig. 3A). In addition, CCM also decreased the gene expression of adiponectin (Fig. 3A). As the expression levels of adipocyte differentiation markers during early stages, CCM decreased the protein levels of C/EBPβ and C/EBPδ (Fig. 3B), whereas it did not affect their mRNA levels (Fig. 3C).

Fig. 3

To determine whether CCM promotes degradation of C/EBPβ and C/EBPδ proteins, 3T3-L1 cells were co-incubated with CCM and the proteasome inhibitor MG132. As shown in Fig. 4A, MG132 increased their protein levels and canceled the CCM-induced decrease of C/EBPβ and C/EBPδ proteins. Furthermore, result from the immunoprecipitation assay showed that the CCM increases complex formation between ubiquitin and C/EBPβ, and C/EBPδ (Fig. 4B). These results indicate that CCM induces degradation of C/EBPβ and C/EBPδ protein through the ubiquitin-proteasome pathway.

Fig. 4

To investigate the effect of caffeine treatment on cytokine secretion from Caco-2 cells, we analyzed cytokine levels in CCM using protein array. Caco-2 cells secreted some cytokines, and the secretion level of interleukin-8 and plasminogen activator inhibitor (PAI)-1 was

1 increased more by 24 h-incubation than that by 2 h-incubation (Fig. 5A). Caffeine treatment

Fig. 5

2 increases secretion levels of G-CSF (1.55-fold) from Caco-2 cells (Fig. 5B). In contrast,
3 caffeine decreased secretion of some cytokines from Caco-2. In particular, caffeine
4 downregulated secretion of PAI-1 (0.79-fold) and IL-8 (0.57-fold) levels from Caco-2 cells.
5 Furthermore, we determined whether caffeine regulates the mRNA expression levels of PAI-1
6 and IL-8 in Caco-2 cells. Caffeine suppressed the gene expression of *SERPINE1*, encoding PAI-
7 1 and *IL8* (Fig. 6). These results indicate that caffeine increases secretion of anti-inflammatory
8 cytokine G-CSF and decreases secretion of pro-inflammatory cytokines PAI-1 and IL-8.
9

10 **Discussion**

11

12 Obesity is the main cause of metabolic syndrome. Many studies have documented that
13 adipocyte differentiation and lipid accumulation are associated with the development of obesity.
14 Certain dietary compounds derived from plants are considered to be a prescription for anti-
15 obesity by suppression of adipocyte differentiation and regulation of lipid metabolism in culture
16 cells and animal experiments (*22-24*). However, there is little research that shows the effect of
17 dietary compounds on adipogenesis through stimulation of intestinal epithelial cells. This study
18 reports that caffeine-stimulated medium from Caco-2 cells suppresses adipogenesis in 3T3-L1
19 cells.

20 Our results show the indirect effect of caffeine against anti-lipid accumulation through
21 intestinal epithelial cells (Fig. 2). Some studies have reported the effect of caffeine against anti-
22 lipid accumulation *in vitro* and *in vivo*. Caffeine decreases lipid accumulation by suppression
23 of insulin-stimulated glucose uptake in 3T3-L1 adipocytes (*15*). Furthermore, a high
24 concentration of caffeine suppresses the protein expression of C/EBPβ through regulating

1 mitotic clonal expansion in 3T3-L1 adipocytes. On the other hand, a combination of caffeine
2 and other food components exhibits anti-obesity effects *in vitro* and *in vivo* (*25, 26*), indicating
3 that caffeine supports the anti-obesity effect of other food components. Taken together, these
4 results demonstrate that caffeine has direct and indirect effects against lipid accumulation
5 Caffeine decreased the expression of pro-inflammatory cytokines IL-8 and PAI-1 in Caco-2
6 cells (Fig. 4B). Pro-inflammatory cytokines from other tissues promote lipid accumulation in
7 adipocytes. PAI-1 belongs to a family of serine protease inhibitors, and an increased plasma
8 PAI-1 level has been associated with insulin resistance (*27*). Knockout of PAI-1 suppresses
9 weight gain in mice (*28*), and some pharmacological inhibitors of PAI-1 improve high fat diet-
10 induced obesity in mice (*29, 30*), indicating that inhibition of PAI-1 reduces adipogenesis.
11 Although IL-8 increases the leptin secretion in human adipocytes (*31*), the effect of IL-8 derived
12 from Caco-2 on fat accumulation in adipocytes is unclear. Further studies are needed to analyze
13 the effect of Caco-2-derived IL-8 on adipogenesis. On the other hand, our results also showed
14 caffeine increased the secretion level of anti-inflammatory cytokine G-CSF (Fig. 4B). G-CSF
15 belongs to the class-1 cytokine superfamily, and the cytokines in this superfamily play an
16 important role in energy homeostasis and exert anti-obesity effects (*32, 33*). Intraperitoneal
17 administration of G-CSF decreased body weight and increased energy expenditure in diabetic
18 model rats (*12*). These results indicate that caffeine-stimulated medium suppresses lipid
19 accumulation through decreased secretion levels of PAI-1 and IL-8 from Caco-2 cells.
20 The secretion of IL-8 and PAI-1 is induced by inflammatory cytokines, oxidative stress and
21 heavy metals. Some studies have reported that food factors suppress inflammation and reduce
22 the secretion of pro-inflammatory cytokines in intestinal epithelial cells. Soy isoflavone reduces
23 the secretion of TNF-α-induced IL-8, but not of hydrogen peroxide-induced IL-8 (*34*).
24 Histamine inhibits TNF-α- and oxidative stress-induced IL-8 secretion in Caco-2 cells (*35*).

1 Orally administered caffeine decreases pro-inflammatory cytokines (TNF-α and IL-17F) and
2 increases anti-inflammatory cytokines IL-10 in mouse colons (*36*). Furthermore, caffeine
3 attenuates acute colitis in a dextran sodium sulfate-induced model of accumulated oxidative
4 stress (*36*). These results suggest that caffeine decreases gene expression of PAI-1 and IL-8
5 through suppression of inflammation or oxidative stress in Caco-2 cells.
6 CCM induced degradation of C/EBPβ and C/EBPδ protein (Fig. 3). Knockout of C/EBPβ
7 suppresses fat accumulation in mice fed a high-fat diet (*37*). Thus, suppression of C/EBPβ
8 expression is an attractive target for diet-induced obesity. Food factors suppress the gene
9 expression of C/EBPβ through regulation of mitotic clonal expansion in 3T3-L1 adipocytes and
10 zebrafish (*38, 39*). In addition, direct treatment with caffeine also suppresses mitotic clonal
11 expansion in 3T3-L1 adipocytes (*40*). However, little is known about food factors which
12 promote the degradation of C/EBPβ protein. C/EBPβ protein is degraded by the proteasome
13 pathway through increasing its ubiquitination (*41*). Our results showed that CCM promoted
14 ubiquitination of C/EBPβ (Fig. 3B). Thus, CCM might induce the expression of ubiquitin ligase,
15 but not regulate mitotic clonal expansion in 3T3-L1 adipocytes.
16 In this study, Caco-2 cells were treated with caffeine at 10 mmol/L. Caffeine is included in
17 various kinds of food and drink, such as coffee and green tea. The concentrations of caffeine
18 contained in coffee are highly variable and are influenced by the extraction method. In the case
19 of espresso, the caffeine content (25 mL) ranged from 111.21 to 257.57 mg (=from 11.5 to 26.5
20 mmol/L) (*42*). Moreover, Thomas et al. report that the median value of caffeine content among
21 20 commercial espresso coffees is 140 mg/43 mL (=17. 1 mmol/mL) (*43*). Taken together, we
22 consider that when people drank a cup of espresso, epithelial cells in the gastrointestinal tract
23 are exposed to caffeine at about 10 mmol/L and suggest that caffeine at 10 mmol/L is a
24 physiological concentration for Caco-2 cells.

1 In conclusion, this study shows that caffeine decreases lipid accumulation through regulation
2 of cytokine secretion from intestinal epithelial cells. It is important to understand the regulation
3 of crosstalk between the intestinal cells and other cells by dietary components, in addition to
4 regulation of their absorption in intestinal cells.

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2

1 **Table 1.** Primer sequences

Gene	5'-3' Primer sequence	Reference
Actb	F; GGTCATCACTATTGGCAACG R; TCCATACCCAAGAAGGAAGG	44
Adipoq	F; GAACTTGTGCAGGTTGGATG R; TGCATCTCCTTTCTCTCCCT	44
Cebpa	F; GGAACTTGAAGCACAATCGATC R; TGGTTTAGCATAGACGTGCACA	20
Cebpb	F; GGGGTTGTTGATGTTTTTGG R; CGAAACGGAAAAGGTTCTCA	20
Cebpd	F; GATCTGCACGGCCTGTTGTA R; CTCCACTGCCCACCTGTCA	20
Pparg	F; ACGTGCAGCTACTGCATGTGA R; AGAAGGAACACGTTGTCAGCG	20
ACTB	F; GGACTTCGAGCAAGAGATGG R; AGCACTGTGTTGGCGTACAG	45
IL8	F; AGAGTGATTGAGAGTGGACC R; ACTTCTCCACAACCCTCTG	46
SERPINE1	F; TGCTGGTGAATGCCCTCTACT R; CGGTCATTCCCAGGTTCTCTA	47

2

1 **Legends to Figures**

2

3 Fig. 1. Design of cell culture and treatment. Caffeine at 10 mmol/L was added to the apical
4 side of Caco-2 monolayers and incubated for 24 h. Caffeine-conditioning medium (CCM) was
5 collected from basolateral side and applied to 3T3-L1 cells.

6

7 Fig. 2. Effect of CCM on lipid accumulation in 3T3-L1 cells. (A) Staining for lipid droplets
8 in 3T3-L1 cells by Sudan II after incubation with CCM or caffeine-nontreated medium (Veh)
9 for 3 d. (B) Measurement of the dye of Sudan II staining in the cells treated with CCM or Veh.
10 Error bars represent the mean ± SE (n = 3). * p<0.05 vs. Veh. (C) Detection of caffeine and
11 measurement of caffeine concentration in medium using HPLC analysis. *Top panel* shows each
12 peak of caffeine and its metabolites. 1; 1-methylxanthine, 2; theobromine, 3; 1,7-dimethyl uric
13 acid, 4; theophylline, 5; caffeine. *Middle* and *bottom panels* show HPLC chart in CCM and
14 caffeine-nontreated medium, respectively. Arrow point show peak of caffeine. (D)
15 Measurement of lipid accumulation in 3T3-L1 cells treated with various concentrations of
16 caffeine directly. Error bars represent the mean ± SE (n = 3). Statistical significance was
17 analyzed by one-way analyses of variance with Turkey's post hoc testing. N.S.; not significant.
18 All data are representative of three independent experiments.

19

20 Fig. 3. Expression pattern of transcriptional factors and lipid metabolism gene in 3T3-L1
21 cells during adipocyte differentiation. (A) mRNA expression of PPARγ (*Pparg*), C/EBPα
22 (*Cebpa*) and Adiponectin (*Adipoq*) in 3T3-L1 cells after incubation with CCM for 3 d. * p<0.05
23 vs. vehicle. All data are representative of three independent experiments. (B) Western blotting
24 analysis of C/EBPβ and C/EBPδ in 3T3-L1 cells after incubation with CCM for 2 h. β-Actin

1 was used as a loading control (left panel). Intensities of each band were quantified by ImageJ
2 1.44, and the ratio of C/EBPβ and C/EBPδ levels was normalized to the β-actin level (middle
3 and right panels). Error bars represent the mean ± SE (n = 3). (C) qPCR analysis of C/EBPβ
4 (*Cebpb*) and C/EBPδ (*Cebpd*) in 3T3-L1 cells after incubation with CCM for 2 h. Relative
5 levels are expressed as means ± SE (n = 3). * p<0.05 vs. vehicle. All data are representative of
6 three independent experiments.
7
8 Fig. 4. Involvement of ubiquitin-proteasome in caffeine-conditioning medium-induced
9 reduction of C/EBPs protein. (A) Protein analysis of C/EBPβ and C/EBPδ in 3T3-L1 cells after
10 incubation with CCM in the presence of MG132, a proteasome inhibitor (top panel). Intensities
11 of each band were quantified by ImageJ 1.44, and the ratio of C/EBPβ and C/EBPδ levels was
12 normalized to the β-actin level (lower panels). Error bars represent the mean ± SE (n = 3).
13 Significant differences (p<0.05) are indicated by different letters. All data are representative of
14 three independent experiments. (B) Immunoprecipitation assay (IP) using C/EBPβ or C/EBPδ
15 IgG in 3T3-L1 cells after incubation with CCM. Immunoprecipitated proteins were analyzed
16 by western blotting against C/EBPβ, C/EBPδ and ubiquitin antibodies. All data are
17 representative of three independent experiments
18
19 Fig. 5. Suppression effect of caffeine on the secretion of pro-inflammatory cytokines from
20 Caco-2 cells. (A) CCM obtained from 2 h- or 24 h-incubation was reacted with the proteome
21 profiler array according to the manufacturer's instructions. Binding of protein to specific
22 antibody was visualized (top panel). The different exposure spots of each membrane were
23 analyzed, and intensities of each spot were quantified by ImageJ 1.44. The ratio of spots was
24 normalized to the spot in 2 h-incubation (lower panels). (B) Caffeine (10 mmol/L)-treated

1 medium or caffeine-nontreated medium (vehicle) was reacted with the proteome profiler array

2 according to the manufacturer's instructions. Binding of protein to specific antibody was

3 visualized (top panel). The ratio of spots was normalized to the spot in vehicle group (lower

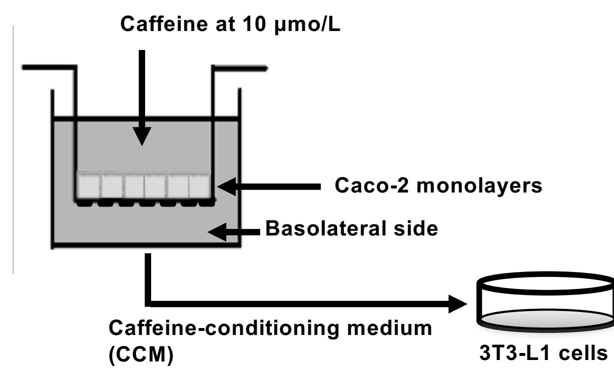
4 panels).

5 Fig. 6. qPCR analysis of IL-8 (*Il8*) and PAI-1 (*SERPINE1*) in Caco-2 cells after incubation

6 with caffeine (10 mmol/L) for 24 h. Relative levels are expressed as means ± SE (n = 3). *

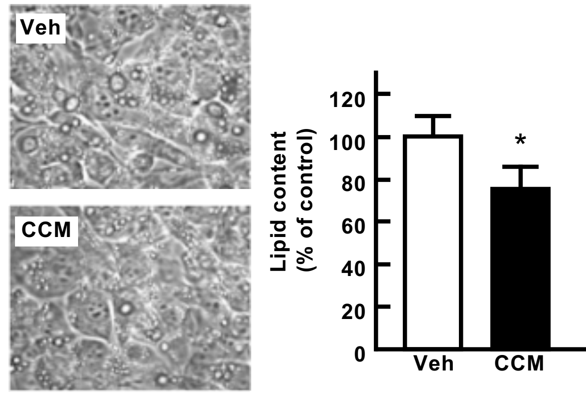
7 p<0.05 vs. vehicle. All data are representative of three independent experiments.

8



Takakazu MITANI, Figure 1

A



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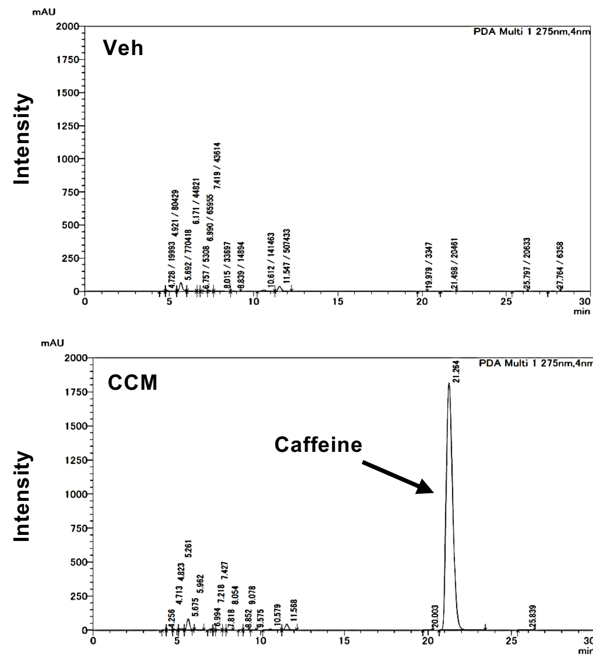

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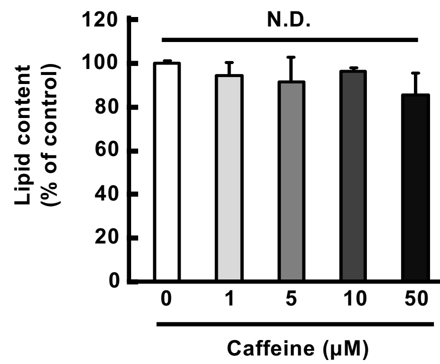
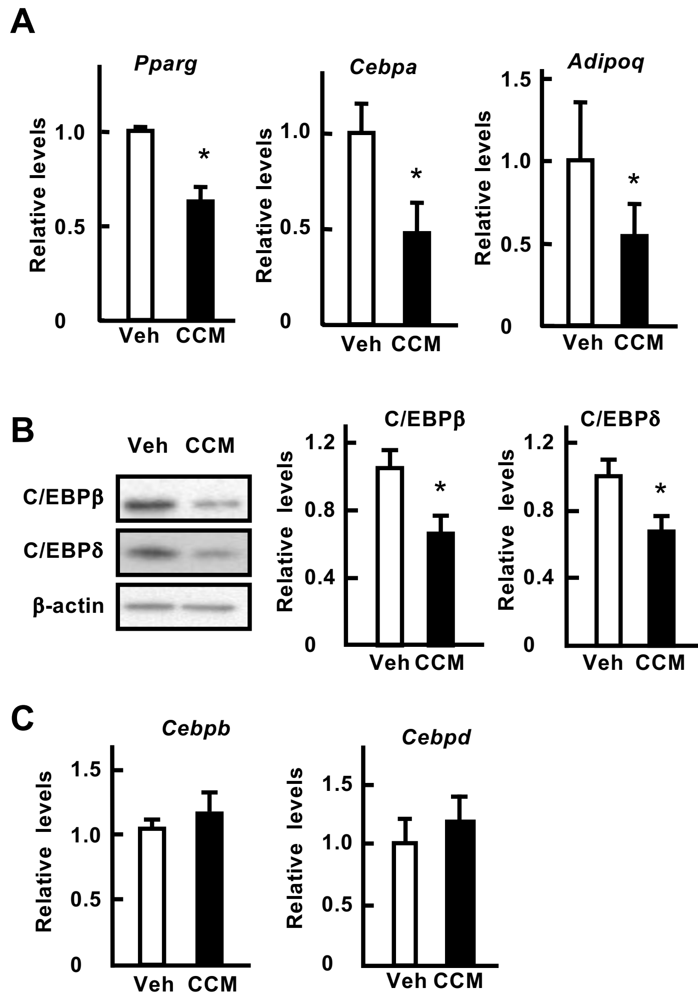


Takakazu MITANI, Figure 2



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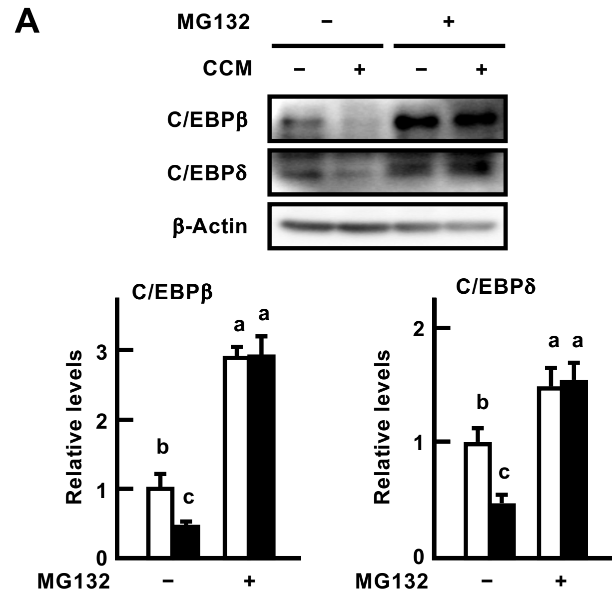
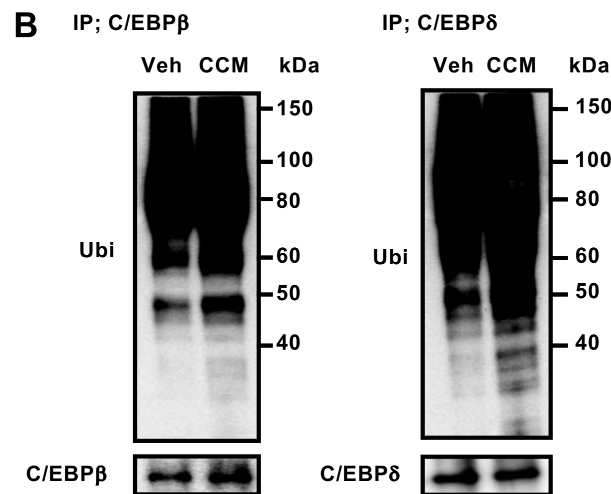
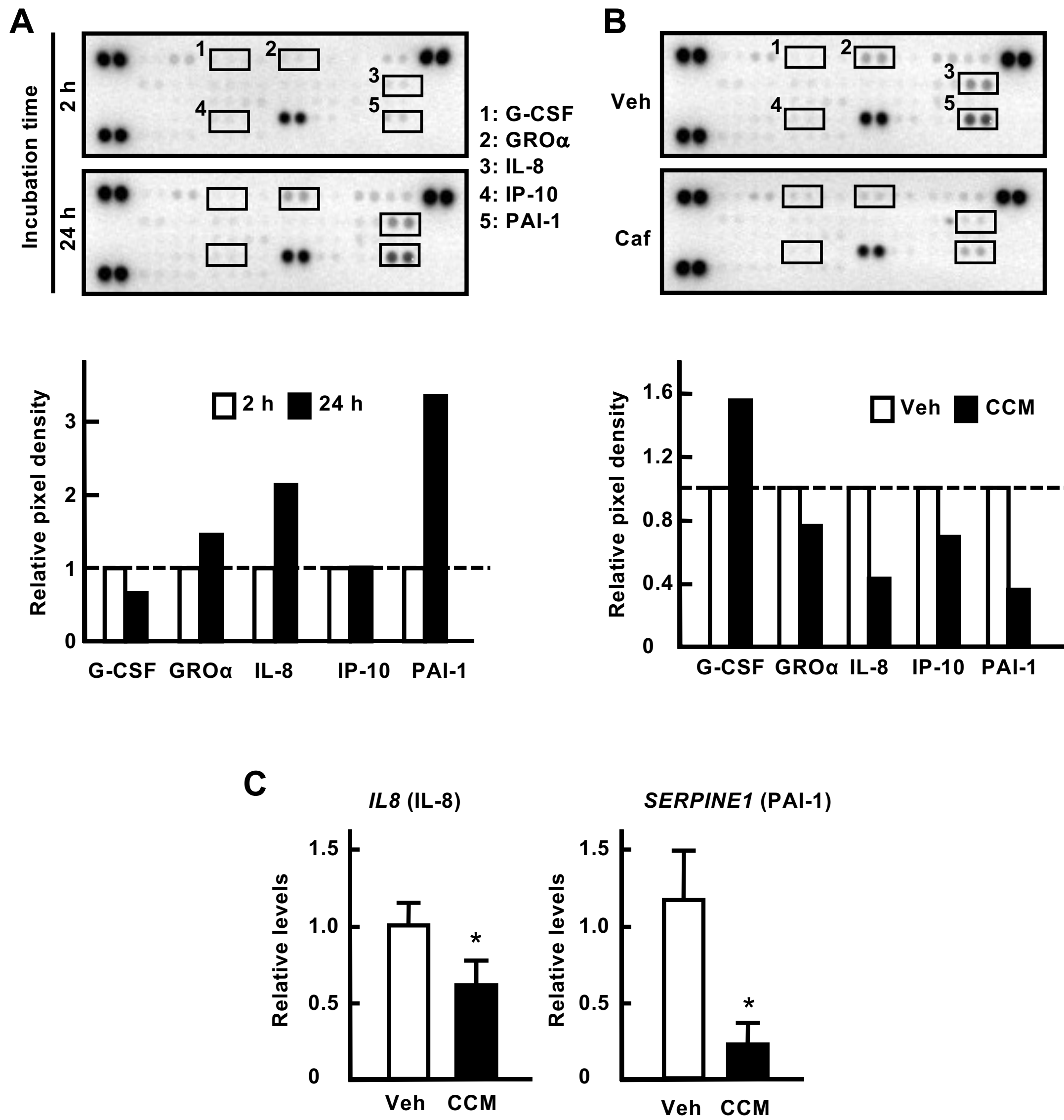




Takakazu MITANI, Figure 4



Takakazu MITANI, Figure 5