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3T3-L1 adipocytes possess anandamide- and epinephrine-responsive machinery for MDM2 distribution to the plasma membrane

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Abstract. The effects of biomolecules on peripheral tissues and their responsive machinery are not well understood. We examined MDM2 level in the plasma membrane (PM) and total MDM2 level of 3T3-L1 adipocytes treated with biomolecular anandamide, epinephrine, and other agents for 15 min. We also examined biomolecular responses in cells treated with mithramycin A, a binding inhibitor, or cells exposed to cooling and cell viability. Immunoblotting revealed that PM MDM2 level increased and total MDM2 level was not altered following treatment with anandamide, epinephrine, capsaicin, CL316243, and aluminum fluoride. PM MDM2 distribution caused by a biomolecular concentration was maintained by treatment with mithramycin A and exposure of cells to 28°C or 32°C but not to 18°C, and PM MDM2 levels after treatment with high concentrations of biomolecules were altered upon exposure to the inhibitor and mild hypothermia. These conditions did not decrease cell viability. Our findings indicate that 3T3-L1 adipocytes possess molecular machinery that responds differentially to anandamide and epinephrine under the inhibitor treatment and cool temperature conditions and that is sensitive to other agents (which mimic biomolecular responses); these machineries can induce subcellular alterations in molecular interactions. We provide information helpful for clarifying biomolecular responsive machinery present in 3T3-L1 adipocytes.

Key words: Anandamide — Epinephrine — 3T3-L1 Adipocytes — MDM2 — Plasma membrane

Introduction

Anandamide (AEA), an endogenous lipid in the endocannabinoid group, is produced in various tissue cells including blood cells (Vogeser et al. 2006) and macrophages treated with lipopolysaccharides (Liu et al. 2003) at 37°C or at room temperatures (22–25°C). The survival rate of platelets cultured at 37°C does not decrease in the presence of an AEA analog, and exposing platelets to a temperature of 22°C enables long-term cell survival and storage (Catani et al. 2010). AEA administration to animals induces decreased rectal temperatures (2–4°C) (Smith et al. 1994). Surgical patients with mild perioperative hypothermia, which leads to a decreased mean skin temperature to 32–33°C, and normal subjects with temperatures at 20–25°C exhibit increased plasma and urinary levels of adrenaline (Wilkerson et al. 1974; Frank et al. 1995). Mild hypothermia is useful in clinical therapy because it protects tissues such as brain from injury. However, the effects of biomolecules on peripheral tissue cells that can be exposed to cool temperatures are not well understood.

A β-adrenoceptor (β-AR) response involves MDM2 and its related molecule alterations and results in DNA damage (Hara et al. 2011). Cooling at 32°C alters MDM2 and its targeting molecule levels induced by DNA damage at 37°C and improves cell survival rate caused by the damage

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(Sakurai et al. 2005). Adipose cells are present in peripheral tissue locations and undergo adrenergic regulation. The cells are also implanted (Cho et al. 2006) and exposed to room temperatures in reconstructive surgery. Information that is helpful for clarifying molecular machinery related to adipose cell viability is insufficient, although some factors are modulated and adipose tissue survival is improved by treatment with a hormone (Hamed et al. 2010). 3T3-L1 (L1) adipocytes are model adipose cells and exhibit β-ARs that preferentially bind to epinephrine (EPI, adrenaline) (Lai et al. 1982). Exposing L1 adipocytes to 28°C and 18°C for 15 min and 32°C for 24 h does not decrease cell viability and induces responses related to MDM2 and its targeting molecule β-arrestin (Ohsaka and Nishino 2010, 2012). In L1 adipocytes cooled to 32°C for 24 h, EPI treatment for 15 min alters β-arrestin. However, the effects of EPI on MDM2 in adipocytes are unclear.

Exposing platelets to 22°C for more than min produces AEA analog-responsive phospho-Akt, an active kinase that modifies MDM2 (Mayo and Donner 2001), and maintains cell viability (Catani et al. 2010). In cortical neurons and C57BL/6J mouse adipocytes, treatment with a cannabinoid receptor type-1 (CB1) activator for 5 and 60 min induces MDM2 protein (Gowran et al. 2009) and activates an upstream kinase for MDM2 (Weber et al. 2005; Tedesco et al. 2010), respectively. AEA interacts with receptors, including CB1, TRPV1, and PPARγ (Ross 2003; Gasperi et al. 2007). Treating adipocytes with CB1 (Pagano et al. 2007; Bellocchio et al. 2008), TRPV1 (Lee et al. 2011), and PPARγ (Chung et al. 2006) activators and with EPI (Chernick et al. 1986; Wan et al. 2010) induces a number of responses including changes in molecular expression, transport, metabolism, and modification. In L1 adipocytes, treatment with AEA for 24 h alters the transport response stimulated by insulin *via* CB1 (Gasperi et al. 2007). However, the effects of AEA on adipocytes and its short-term treatment responses are poorly understood.

MDM2 responds to biomolecules including those for kinase-type receptor hormones (Malmlöf et al. 2007). This kinase-type hormone treatment for ≤15 min increases MDM2 level in the subcellular locus of cells (Mayo and Donner 2001; Ohsaka and Nishino 2010). However, whether MDM2 responds to other types of biomolecules for G protein-coupled receptors (GPCRs) in non-proliferative cells and what molecules are involved in MDM2 responses in these treated cells have not been fully elucidated, although MDM2 is known to be regulated by non-biomolecular activators including those for GPCRs of β-AR and CB1.

MDM2 forms complexes with receptor-signaling molecules such as β-arrestin, GPCR kinase 2 (GRK2) (Salcedo et al. 2006), and Gαs, a G protein (Tang et al. 2008), in a cell line and harbors the binding site for a complex with β-arrestin (Shenoy et al. 2001). MDM2 also ubiquitinates β-arrestin, GRK2, and β_2-AR in response to a non-selective β-AR activator. Treatment with the β-AR activator for 10 min and the CB1 activator for ≤15 min translocates β-arrestin or GRK2 to the plasma membrane (PM) (Li et al. 2006; Turu and Hunyady 2010). However, the subcellular loci for molecular interactions with MDM2 and for modification of MDM2-related molecules in response to the β-AR activator and whether the CB1 activator induces MDM2 interactions remain unclear. Treating L1 adipocytes with the β-AR activator for 5 min results in the formation of a β_2-AR complex with β-arrestin (Dalle et al. 2002). β-arrestin also interacts with other signaling molecules in L1 adipocytes, such as a receptor adaptor protein for TNF-α (Kawamata et al. 2007). The subcellular events that are induced in adipocytes treated with biomolecular agonists for β-AR and CB1 are not sufficiently clear, and the molecular interactions with MDM2 in these agonists-treated adipocytes have not been examined.

GRK2 interacts with Gαq/11, another G protein, in L1 adipocytes (Usui et al. 2004b). MDM2 interactions with GRK2 induced by treatment with the β-AR activator for 5 and 10 min are impaired by the expression of β-arrestin (Salcedo et al. 2006). Mithramycin A is a transcription factor-binding inhibitor that decreases MDM2 expression (Bond et al. 2004). Treating L1 adipocytes with mithramycin A for 24 h alters molecular expression including decreased MDM2 and increased β-arrestin expression (Ohsaka and Nishino 2010, 2012) and also alters receptor responses including those induced by a PPARγ agonist (Chung et al. 2006). Molecular machinery in adipocytes for MDM2 interaction responses is unknown.

Treating L1 adipocytes with TNF-α impairs a receptor response for MDM2 (Medina et al. 2005), and treatment with capsaicin, a TRPV1 agonist, suppresses a response required for TNF-α production induced in a macrophage-conditioned medium in L1 adipocytes (Kang et al. 2007). Responses similar to those of C57BL/6J mouse and human adipocytes (Pagano et al. 2007) to the CB1 activator or of L1 adipocytes to EPI are induced in such adipocytes by ≤15 min of treatment with capsaicin (Zhang et al. 2007), CL316243 (Kumar et al. 2007), a specific agonist for β_3-AR that is highly expressed among β-ARs in L1 adipocytes (Monjo et al. 2005), and with aluminum fluoride (Ohsaka and Nishino 2012), a receptor-signaling (G protein signaling) activator. Molecular targeting experiments designed on the basis of these similarities are helpful for elucidating the signaling machinery responsive to biomolecules.

To investigate the responses of peripheral tissue cells to biomolecules for GPCRs and molecular machinery present in cells, we treated L1 adipocytes for 15 min with the biomolecules AEA and EPI, with AEA and EPI after treatment with mithramycin A, and with capsaicin, CL316243, and aluminum fluoride, and we examined MDM2 level in the plasma membrane and total MDM2 level of L1 adipocytes. We also

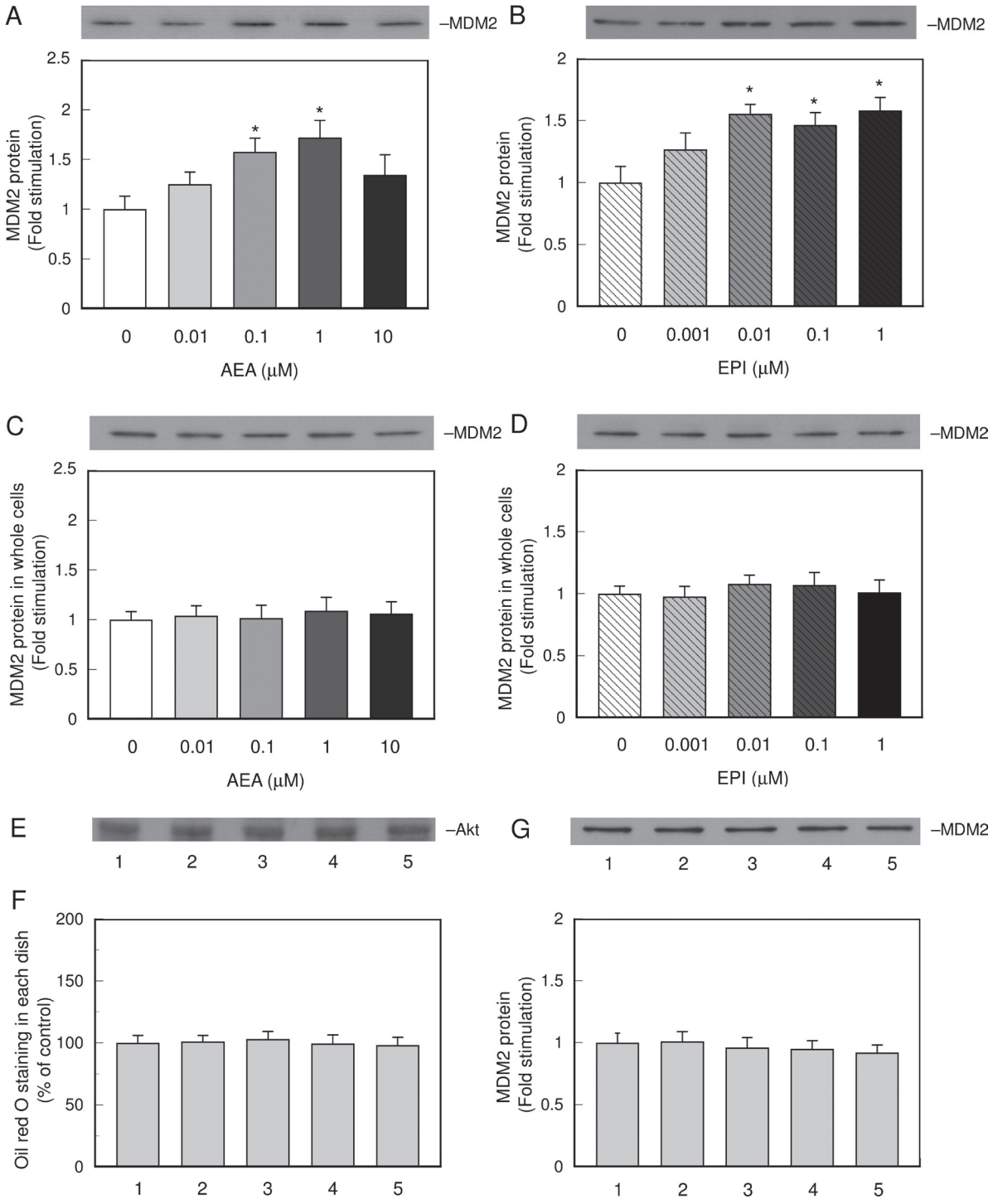
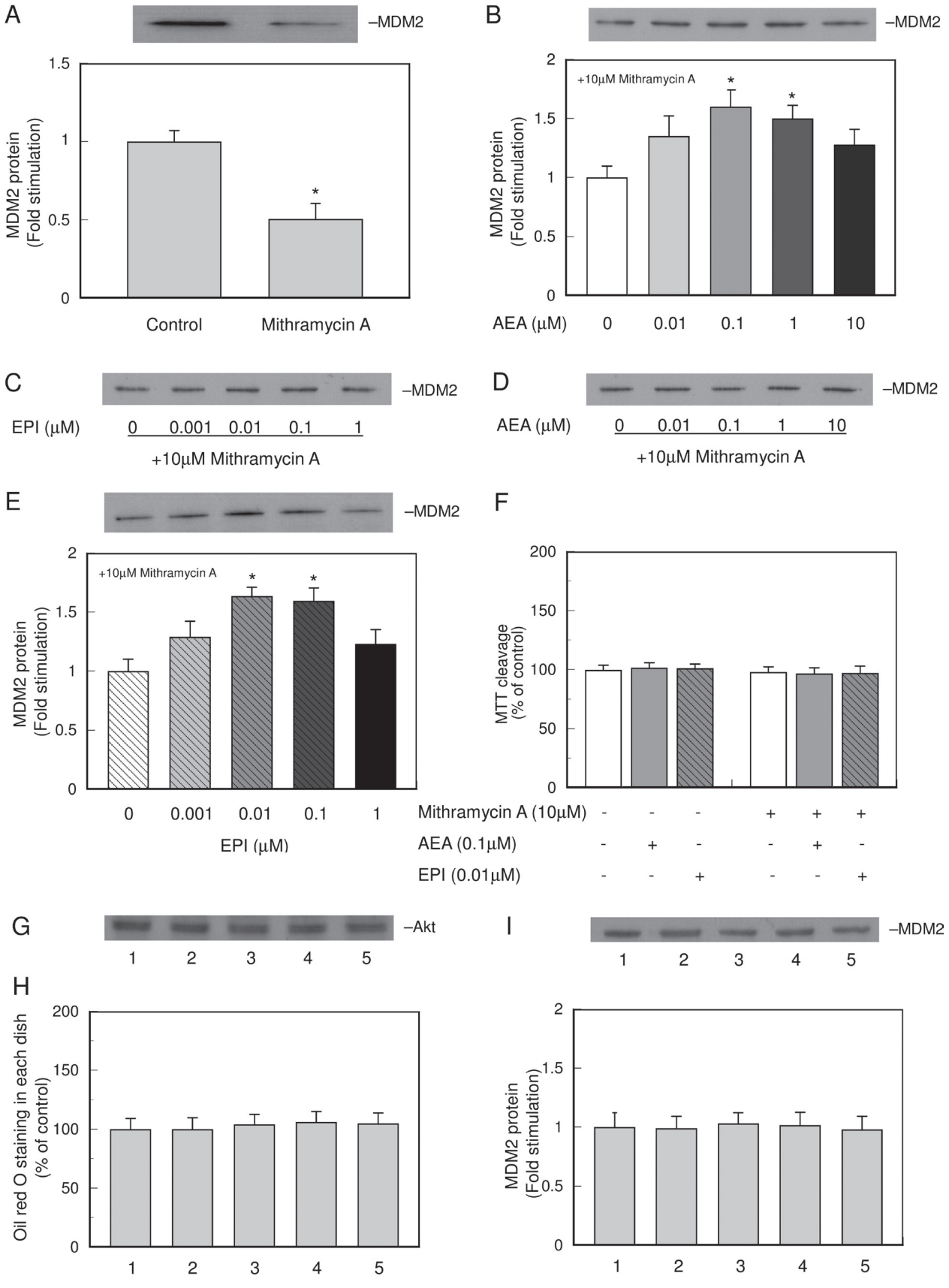


Figure 1. Effects of anandamide (AEA) and epinephrine (EPI) on MDM2 level in the plasma membrane of L1 adipocytes. L1 adipocytes were treated with the indicated concentrations of AEA (A and C) and EPI (B and D) for 15 min at 37°C in KRH buffer containing 1% BSA. Plasma membrane fractions (A, B, and G) and whole cell lysates (C, D, and E) were obtained from treated or untreated cells, and MDM2 or Akt levels were determined using immunoblot analysis with an MDM2 (A–D and G) or Akt (E) antibody. Immunoblot lanes (E, 1–5) and columns (F, 1–5) show the expression of Akt and amount of oil red O dye staining, respectively, in each dish of cells prior to treatment with agents. Data are expressed as percentages of values of cells treated for 15 min without agents (F). Immunoblot lanes (G, 1–5) show the MDM2 level in the plasma membrane of cells treated for 15 min without agents. Values are presented as means ± SD of 3 or 4 experiments (A–D, F, and G). Blotted lanes represent typical results (3 experiments, E). * $p < 0.05$ *vs.* cells not treated with AEA (A) or EPI (B).


A
–MDM2
MDM2 protein (Fold stimulation)
1.5
1
0.5
0
*
Control
Mithramycin A
B
–MDM2
+10μM Mithramycin A
MDM2 protein (Fold stimulation)
2
1.5
1
0.5
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*
*
AEA (μM) 0 0.01 0.1 1 10
C
–MDM2
EPI (μM) 0 0.001 0.01 0.1 1
+10μM Mithramycin A
D
–MDM2
AEA (μM) 0 0.01 0.1 1 10
+10μM Mithramycin A
E
–MDM2
+10μM Mithramycin A
MDM2 protein (Fold stimulation)
2
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EPI (μM)
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MTT cleavage (% of control)
200
150
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Mithramycin A (10μM) - - - + + +
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EPI (0.01μM) - - + - - +
G
–Akt
1 2 3 4 5
I
–MDM2
1 2 3 4 5
H
Oil red O staining in each dish (% of control)
200
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100
50
0
1 2 3 4 5
MDM2 protein (Fold stimulation)
2
1.5
1
0.5
0
1 2 3 4 5

examined responses to the biomolecules in cells exposed to 32°C, 28°C, and 18°C and the viability of treated cells.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and phosphate-buffered saline (PBS) were purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). AEA (*N*-arachidonylethanolamide), capsaicin, CL316243, EPI, mithramycin A (product numbers: A0580, M2028, C5976, E4250, and M6891, respectively), and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Aprotinin was obtained from Roche Diagnostics (Indianapolis, IN, USA). Anti-MDM2 (C-18) rabbit and Akt antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and EMD Bioscience Inc. (Madison, WI, USA), respectively. Aluminum chloride and sodium fluoride (which were used to prepare aluminum fluoride), calcium chloride, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), ethylenediaminetetraacetic acid (EDTA), fatty acid-free bovine serum albumin (BSA), 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), magnesium sulfate, oil red O, phenylmethylsulfonyl fluoride (PMSF), polyacrylamide, potassium chloride, sodium chloride, and sucrose were purchased from Nacalai Tesque (Kyoto, Japan).

Culture of 3T3-L1 adipocytes

3T3-L1 (L1) cells were plated at a density of 3.5×10^4 cells/cm^2 in DMEM supplemented with heat-inactivated 10% fetal calf serum (FCS) (HyClone, South Logan, UT, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C and were differentiated into adipocytes as described previously (Ohsaka and Nishino 2010). L1 adipocytes at 7–9 days after the onset of differentiation were used for all experiments. Before exposure to agents, differentiated L1 adipocytes were examined by staining triacylglycerol droplets in the cells with oil red O and spectrophotometrically measuring the absorbance of dye present in lipids at 510 nm as described previously (Ramírez-Zacarías et al. 1992). We also examined the cellular differentiation-inducible protein level of Akt (Sakaue et al. 2004).

Treatment with agents and exposure to cooling

L1 adipocytes were washed twice using PBS, and culture medium containing serum was replaced with Krebs-Ringer HEPES (KRH) buffer (2.5 mM $CaCl_2$, 10 mM HEPES (pH 7.4), 4.7 mM KCl, 1.25 mM $MgSO_4$, and 118 mM NaCl) containing 1% BSA. L1 adipocytes were then treated with or without AEA, EPI, capsaicin, CL316243, or aluminum fluoride for 15 min at 37°C. In addition, L1 adipocytes were treated in KRH buffer containing 1% BSA with or without AEA or EPI for 15 min at 37°C following treatment in serum-containing medium containing mithramycin A for 24 h at 37°C or were treated in KRH buffer containing 1% BSA with or without AEA or EPI for 15 min at a temperature of 28°C or 18°C. Furthermore, L1 adipocytes were treated in KRH buffer containing 1% BSA in the presence and absence of AEA or EPI for 15 min at 32°C after the cells cultured in serum-containing medium had been exposed to cooling at 32°C for 24 h.

Preparation of plasma membrane fractions and immunoblot analysis

The L1 adipocytes treated with or without the indicated agents under various conditions or cells before treatment with agents were washed with PBS, homogenized, and sonicated in a buffer containing 30 U/ml aprotinin, 1 mM EDTA, 20 mM HEPES (pH 7.4), 1 mM PMSF, and 0.25 mM sucrose.

◂ **Figure 2.** Effects of anandamide (AEA) and epinephrine (EPI) on MDM2 level in the plasma membrane of L1 adipocytes treated with mithramycin A. L1 adipocytes were treated in the presence or absence of 10 μM of mithramycin A for 24 h at 37°C in DMEM containing serum (A) or were treated with mithramycin A for 24 h at 37°C in serum-containing DMEM and further incubated with the indicated concentrations of AEA (B and D) and EPI (E and C) for 15 min at 37°C in KRH buffer containing 1% BSA. Plasma membrane fractions (A, B, E, and I) and whole cell lysates (C, D, and G) were obtained from treated or untreated cells, and MDM2 or Akt levels were determined using immunoblot analysis with an MDM2 (A–E and I) or Akt (G) antibody. L1 adipocytes treated as described above (in 2B–E) or treated as described in the legend to Figure 1A–D were incubated in a culture medium containing MTT to determine the amount of MTT formazan formed by MTT cleavage. Data are expressed as percentages of values obtained from untreated cells (0 h) (F). Immunoblot lanes (G, 1–5) and columns (H, 1–5) show the expression of Akt and amount of oil red O dye staining, respectively, in each dish of cells prior to AEA and EPI treatment. Data are expressed as percentages of values of cells treated for 15 min without agents (H). Immunoblot lanes (I, 1–5) show the MDM2 level in the plasma membrane of cells treated for 15 min without agents after mithramycin A treatment. Values are presented as means ± SD of 3 or 4 experiments (A, B, E, F, H, and I). Blotted lanes represent typical results (3 experiments; C, D, and G). * $p < 0.05$ *vs.* cells not treated with mithramycin A (A) or not treated with AEA (B) or EPI (E) after mithramycin A treatment.

The homogenates were centrifuged at 2000 × *g* for 10 min at 4°C to remove the cell nucleus and fat, and plasma membrane fractions were prepared as described previously (Heller-Harrison et al. 1995). Protein content was measured using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL). Proteins (10 μg) were separated using 10% SDS–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The proteins blotted on the membrane were incubated for 1.5 h with the indicated antibody (1:500–1000), and the immune complexes were detected and quantified as described previously (Ohsaka and Nishino 2010). The relative intensities of the blots were normalized according to the ratio of protein to DNA in aliquots (DNA determined using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany)) and expressed as values for each cell.

Measurement of cell viability

Treated L1 adipocytes were washed with PBS and incubated with 5 mg/ml MTT for 2 h at 37°C in culture medium, and the amount of MTT formazan produced by MTT cleavage was determined using a spectrophotometric assay as described previously (Hansen et al. 1989).

Statistical analysis

Comparisons among multiple groups were performed by using analysis of variance (ANOVA) with Scheffe's test, and differences between two groups were evaluated by using unpaired Student's *t*-test. Statistical significance was defined as $p < 0.05$.

Results

Increases in the level of MDM2 in the plasma membrane of L1 adipocytes treated with AEA and EPI

We examined MDM2 level in the plasma membrane of L1 adipocytes treated with AEA and EPI for 15 min at 37°C. MDM2 level was significantly increased in the plasma membrane of cells treated with 0.1 and 1 μM of AEA (Fig. 1A) and with 0.01–1 μM of EPI (Fig. 1B) for 15 min at 37°C but was not increased after treatment with 0.01 and 10 μM of AEA (Fig. 1A) or with 0.001 μM of EPI (Fig. 1B) for 15 min at 37°C. These treatments did not alter the total MDM2 level in cells (Fig. 1C and D).

PM MDM2 levels induced by AEA and EPI in L1 adipocytes treated with mithramycin A

We treated L1 adipocytes with mithramycin A for 24 h and then incubated the cells with AEA and EPI for 15 min at 37°C. Treatment with 10 μM of mithramycin A for 24 h decreased the basal level of PM MDM2 in L1 adipocytes (Fig. 2A). In cells treated with 10 μM of mithramycin A, the PM MDM2 level was not increased after treatment with 1 μM of EPI (Fig. 2E) for 15 min and was increased by treatment with 0.1 and 1 μM of AEA (Fig. 2B) and with 0.01 and 0.1 μM of EPI (Fig. 2E). No change was observed in the total MDM2 level in these treated cells (Fig. 2C and D, some data not shown). Incubation of cells without AEA and EPI for 15 min did not alter the PM MDM2 level decreased by mithramycin A (data not shown). We also examined the amount of MTT formazan formed by MTT cleavage in treated cells to evaluate cell viability. Treatment conditions did not decrease the viability of L1 adipocytes (Fig. 2F, some data not shown).

PM MDM2 levels induced by other agents in L1 adipocytes

We examined MDM2 level in the plasma membrane of L1 adipocytes treated with capsaicin, CL316243, and aluminum fluoride for 15 min at 37°C. PM MDM2 level was increased in L1 adipocytes treated with capsaicin (0.01–100 μM, Fig. 3A), CL316243 (0.001–0.1 μM, Fig. 3B), and aluminum fluoride (0.08–5 mM, Fig. 3E) for 15 min at 37°C without alteration in the total cellular MDM2 level (Fig. 3C, D and F; some data not shown); 0.1 nM and 1 μM of CL316243 did not significantly alter PM MDM2 or total MDM2 level in cells (Fig. 3B and D).

AEA- and EPI-induced changes in PM MDM2 levels under low-temperature conditions

We examined cellular responses to AEA and EPI in L1 adipocytes exposed to temperatures of 32°C, 28°C, and 18°C. In cells incubated at 28°C for 15 min, treatment with 0.1, 1, and 10 μM of AEA (Fig. 4A) and with 0.01, 0.1, and 1 μM of EPI (Fig. 4B) significantly increased PM MDM2 level, but these responses were not induced in cells incubated at 18°C for 15 min (Fig. 4E and F). Similar responses of increased PM MDM2 levels were observed in cells incubated at 32°C for 15 min following exposure to 32°C for 24 h (Fig. 5A). Treatment conditions at temperatures of 32°C, 28°C, and 18°C did not alter the total cellular MDM2 level (Figs. 4C, D, G, and H and 5B) and did not induce a decrease in cell viability (Fig. 5C, some data not shown). In our experiments, prior to treatments with agents, L1 adipocytes plated on each dish did not exhibit different levels of differentiation-responsive molecules (Figs. 1E and F, 2G and H, 3G and H, and 5D and E; some data not shown).

Discussion

In this study, we found that the MDM2 level is increased in the plasma membrane of L1 adipocytes treated with

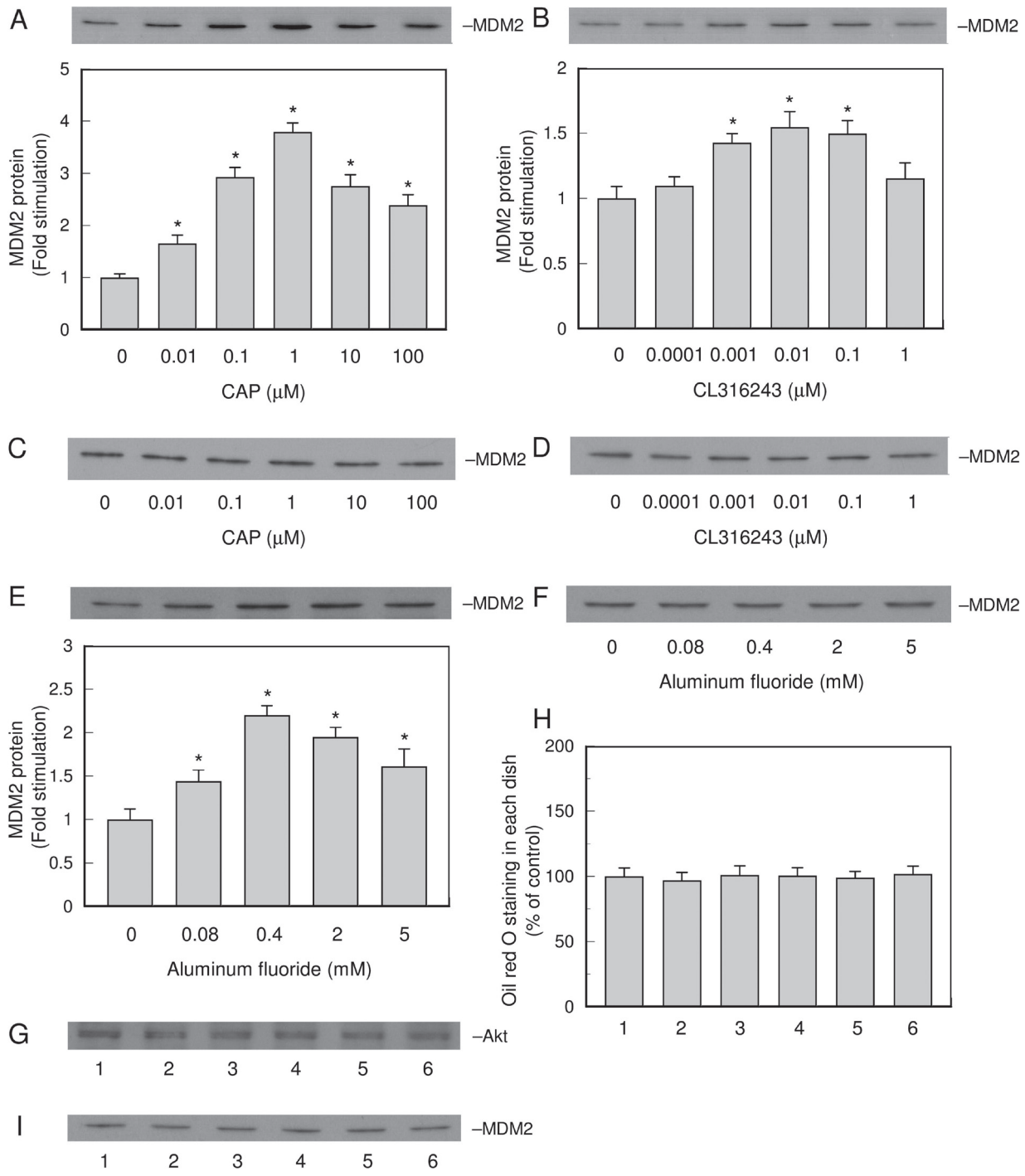
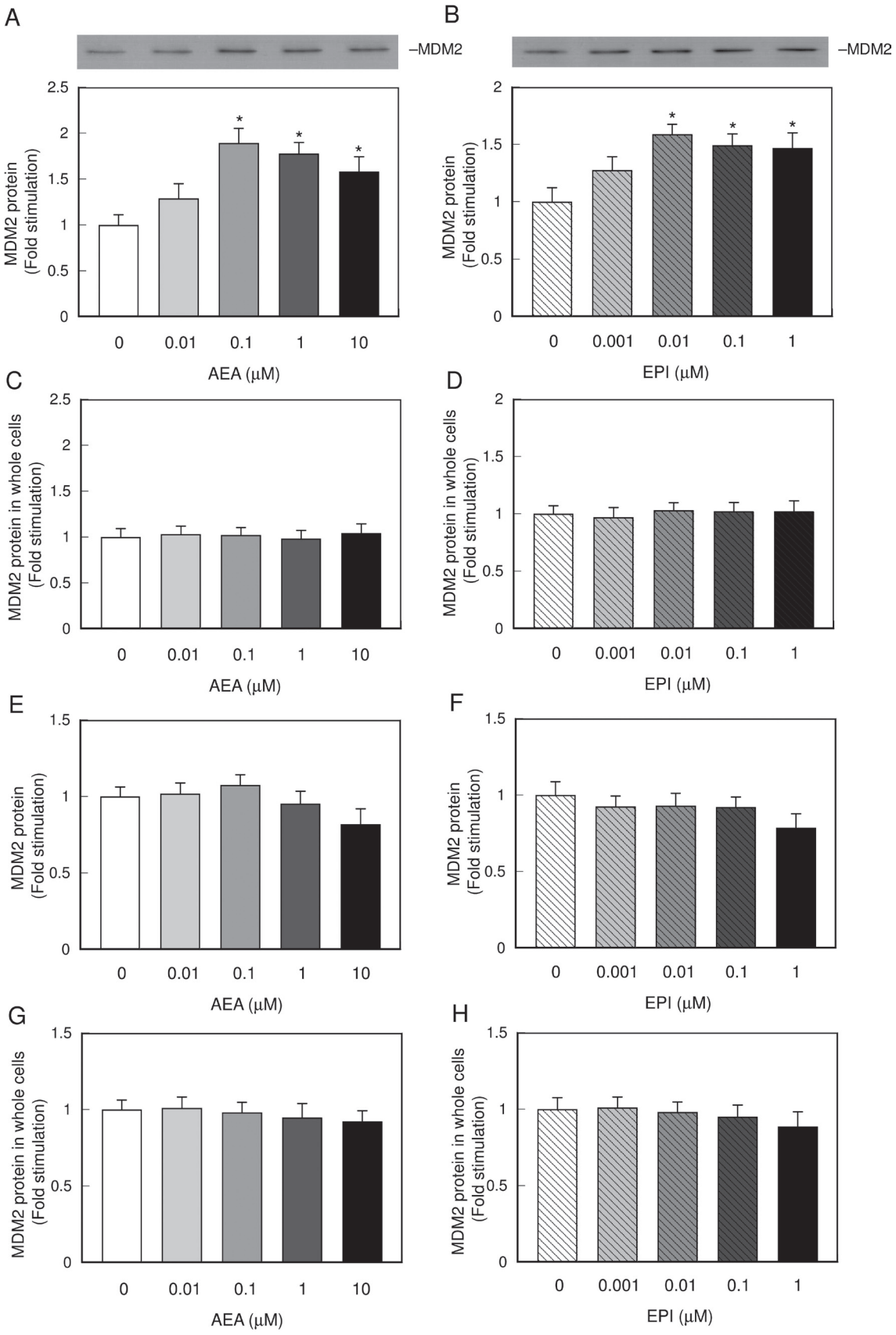


Figure 3. Effects of capsaicin (CAP), CL316243, and aluminum fluoride on MDM2 level in the plasma membrane of L1 adipocytes. L1 adipocytes were treated with the indicated concentrations of CAP (A and C), CL316243 (B and D), and aluminum fluoride (E and F) for 15 min at 37°C in KRH buffer containing 1% BSA. Plasma membrane fractions (A, B, E, and I) and whole cell lysates (C, D, F, and G) were obtained from treated or untreated cells, and MDM2 or Akt levels were determined using immunoblot analysis with an MDM2 (A–F and I) or Akt (G) antibody. Immunoblot lanes (G, 1–6) and columns (H, 1–6) show the expression of Akt and amount of oil red O dye staining, respectively, in each dish of cells prior to treatment with agents. Data are expressed as percentages of values of cells treated for 15 min without agents (H). Immunoblot lanes (I, 1–6) show the MDM2 level in the plasma membrane of cells treated for 15 min without agents. Values are presented as means ± SD of 3 or 4 experiments (A, B, E, and H). Blotted lanes represent typical results (3 experiments; C, D, F, G, and I). * $p < 0.05$ *vs.* cells not treated with CAP (A), CL316243 (B), or aluminum fluoride (E).


A
–MDM2
MDM2 protein (Fold stimulation)
AEA (μM)
0 0.01 0.1 1 10
B
–MDM2
MDM2 protein (Fold stimulation)
EPI (μM)
0 0.001 0.01 0.1 1
C
MDM2 protein in whole cells (Fold stimulation)
AEA (μM)
D
MDM2 protein in whole cells (Fold stimulation)
EPI (μM)
E
MDM2 protein (Fold stimulation)
AEA (μM)
F
MDM2 protein (Fold stimulation)
EPI (μM)
G
MDM2 protein in whole cells (Fold stimulation)
AEA (μM)
H
MDM2 protein in whole cells (Fold stimulation)
EPI (μM)

AEA and EPI for 15 min. Treatment of L1 adipocytes with insulin, a kinase-type receptor hormone, for 15 or 30 min induces MDM2-related responses (Medina et al. 2005; Ohsaka and Nishino 2010) including increased PM MDM2 levels. Incubation of L1 adipocytes at 23°C and 19°C for 15 min induces or does not induce a response to insulin for 15 min (Elmendorf et al. 1999; van Dam et al. 2005). In addition, treatment of L1 adipocytes with mithramycin A for 24 h induces responses for alterations in molecular expression similar to those induced by AR agonists, which bind to α- and β-ARs, (Ohsaka et al. 2010; Ohsaka and Nishino 2012). Insulin-stimulated glucose transport in L1 adipocytes is augmented by 24-h AEA treatment (Gasperi et al. 2007) and is decreased by 10-min EPI treatment (Mulder et al. 2005). On the other hand, responses of adipocytes to agonists such as insulin-induced plasma membrane glucose transporter 4 (GLUT4) and phospho-Akt are similarly induced following treatment with CB1 (Pagano et al. 2007) and β_3-AR (Zmuda-Trzebiatowska et al. 2007) agonists. Adipocyte machinery for responses to biomolecular hormones including AEA and EPI is not fully understood. In the present study, we also found that the MDM2 levels induced by AEA and EPI are partially altered upon exposure of L1 adipocytes to mithramycin A and mild hypothermia and that L1 adipocytes respond to treatment with other agents.

Treatment of L1 adipocytes with mithramycin A for 24 h alters β-arrestin expression and inhibits a receptor response to endothelin-1 (ET-1, which induces its receptor complex formation with β-arrestin (Imamura et al. 2001)) (Chai et al. 2009). This treatment condition decreased the basal level of PM MDM2 distribution and impaired the response of L1 adipocytes to EPI for 15 min at the high concentration of 1 μM. Responses of L1 adipocytes to low concentrations of AEA (0.1 and 1 μM) and EPI (0.01 and 0.1 μM) were retained by treatment with mithramycin A irrespective of alterations in molecular expression. Altered molecular expression, such as that for β-arrestin, induced by mithramycin A appears not to be involved in the molecular machinery response to low concentrations of AEA and EPI but to affect the machinery that is responsive to a high EPI concentration.

Similar to previous studies showing that L1 adipocytes respond differently to insulin for 15 min in cool temperatures, AEA and EPI treatment for 15 min induced PM MDM2 in L1 adipocytes incubated at 28°C and 32°C but not at 18°C. Interestingly, the PM MDM2 level observed after treatment with a high concentration of AEA (10 μM) was increased by exposure to 28°C and 32°C. L1 adipocytes contain molecular machinery that responds differentially to AEA and EPI under cool temperature conditions.

Inhibition studies using known antagonists that are specific for receptor subtypes showed that the effects of AEA and EPI on insulin responses are not completely restored by the antagonists (Mulder et al. 2005; Gasperi et al. 2007). β-arrestin is required for the kinase responses of Src binding and ERK activation induced by β_2-AR signaling but not those induced by β_3-AR signaling (Cao et al. 2000). β_3-AR is reportedly less prone to desensitization (which is induced by receptor sequestration/internalization, etc.) (Langin et al. 1995). In cells expressing wild-type β-arrestin or a β-arrestin mutant (which mimics dephosphorylation), decreased binding of labeled β-AR ligand to the cell-surface receptor is induced by treatment with a non-selective β-AR activator (Lin et al. 1997). Treatment of L1 adipocytes with mithramycin A increases β-arrestin expression and decreases phospho-β-arrestin expression, while exposure to cooling at 32°C for 24 h increases phospho-β-arrestin and β-arrestin expression in L1 adipocytes (Ohsaka and Nishino 2012). Differential responses of TRPV1 are observed when the same concentration of AEA is used at 37°C and 22°C (Ross 2003), and phosphatase inhibitor treatment results in recovery of the TRPV1 response desensitized by AEA (Lizanecz et al. 2006). Decreased phosphatase activity can be induced at 20–25°C (Oehler-Jänne et al. 2008). In addition, differential transport of AEA into cells and altered subcellular distribution of α_{2C}-AR are observed as a result of exposure to mild hypothermia (Hillard et al. 1997; Filipeanu et al. 2011). The altered response to the high concentrations of AEA and EPI seems to be due to mithramycin A- and mild hypothermia-altered molecular machinery involved in the response to high concentrations of biomolecules.

Subcutaneous fat grafts whose survival rate was improved by treatment with a hormone induce phospho-Akt (Hamed et al. 2010). An active form of AMPK, which changes fat metabolism and results in apoptosis (Dagon et al. 2006), is induced in L1 adipocytes treated with the non-selective β-AR activator for 15 min (Yin et al. 2003) and is also induced by treatment with mithramycin A for 24 h at 37°C and expo-

◂ **Figure 4.** Effects of anandamide (AEA) and epinephrine (EPI) on MDM2 level in the plasma membrane of L1 adipocytes exposed to cooling at 28°C and 18°C. L1 adipocytes were treated with the indicated concentrations of AEA (A, C, E and G) and EPI (B, D, F, and H) for 15 min at 28°C (A–D) or 18°C (E–H) in KRH buffer containing 1% BSA. Plasma membrane fractions (A, B, E, and F) and whole cell lysates (C, D, G, and H) were obtained from treated cells, and MDM2 levels were determined using immunoblot analysis with an MDM2 antibody (A–H). Values are presented as means ± SD of 3 or 4 experiments (A–H). Blotted lanes represent typical results. * $p < 0.05$ *vs.* cells not treated with AEA (A) or EPI (B).

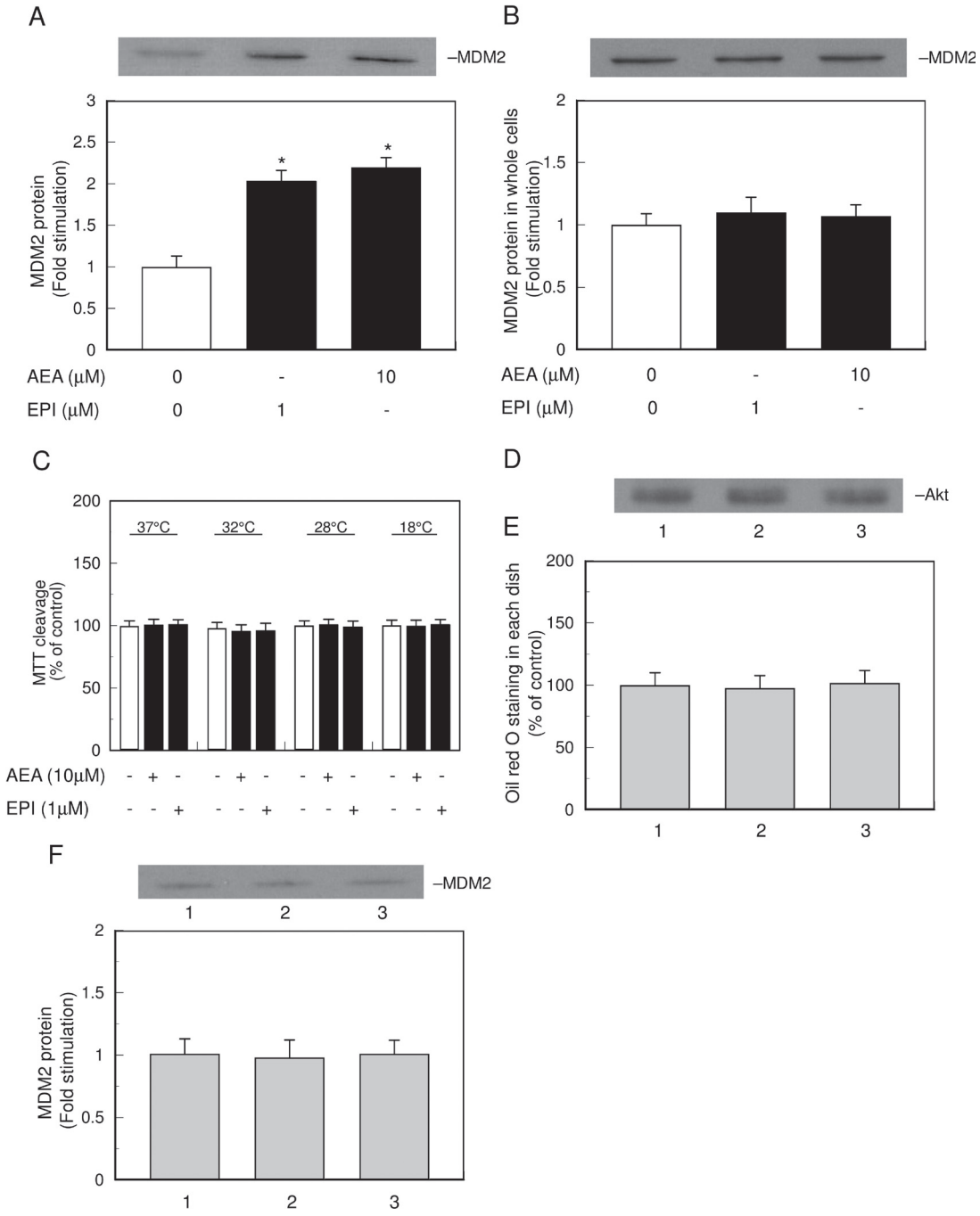


Figure 5. Effects of anandamide (AEA) and epinephrine (EPI) on MDM2 level in the plasma membrane of L1 adipocytes cooled at 32°C. L1 adipocytes were exposed to cooling at 32°C for 24 h in serum-containing medium and further incubated with the indicated concentrations of AEA and EPI for 15 min in KRH buffer containing 1% BSA (A and B). Plasma membrane fractions (A and F) and whole cell lysates (B and D) were obtained from treated or untreated cells, and MDM2 or Akt levels were determined using immunoblot analysis with an MDM2 (A, B, and F) or Akt (D) antibody. MTT cleavage in the L1 adipocytes treated as described above (in 5A and B) or treated as described in the legend to Figure 4 was measured as mentioned previously (in Figure 2F). Data are expressed as percentages of values obtained from untreated cells (0 h) (C). Immunoblot lanes (D, 1–3) and columns (E, 1–3) show the expression of Akt and amount of oil red O dye staining, respectively, in each dish of cells prior to AEA and EPI treatment. Data are expressed as percentages of values of cells treated for 15 min without agents (E). Immunoblot lanes (F, 1–3) show the MDM2 level in the plasma membrane of cells treated for 15 min without agents after exposure to cooling at 32°C. Values are presented as means ± SD of 3 or 4 experiments (A–C, E, and F). Blotted lanes represent typical results (3 experiments; D). *$p < 0.05$ *vs.* cells not treated with AEA and EPI after exposure to cooling (A).

sure to 32°C for 24 h in L1 adipocytes (Ohsaka et al. 2010). Exposure of L1 adipocytes to cooling alone for 15 min at 28°C and 18°C increases the PM MDM2 level (Ohsaka and Nishino 2010). Under the condition of cool temperatures, induction of PM MDM2 by AEA and EPI seems to occur through a signaling machinery-insensitive or signaling machinery-sensitive mechanism to cooling at 28°C and 32°C and not to occur at 18°C due to altered biomolecular machinery caused by cooling. Treatment with AEA and EPI is thought to induce PM MDM2 through a signaling machinery insensitive or sensitive to mithramycin A and mild hypothermia. Treatment conditions used in this study did not reduce cell viability. Our results of responses to AEA and EPI may be helpful for clarifying signaling machineries related to fat cell viability.

The effects of AEA and EPI on the transport response to insulin in L1 adipocytes are altered after treatment with GPCR antagonists for CB1 (Gasperi et al. 2007) and β-ARs including β_1-, β_2-, and β_3-AR (Mulder et al. 2005); however, these responses are not altered after treatment with other antagonists for PPARγ, TRPV1 (Gasperi et al. 2007), or α-AR (Monjo et al. 2005), although L1 adipocytes express these receptors, and AEA, capsaicin, and EPI can interact with them. L1 adipocytes also have membrane transport activity for AEA and amide hydrolase activity for fatty acid (Gasperi et al. 2007). Treatment with arachidonic acid, a metabolite for AEA, for <30 min produces cAMP in L1 adipocytes (Long and Pekala 1996). Aluminum fluoride modulates receptor signaling systems such as the adenylyl cyclase system via G protein activation. Treatment with CL316243 for 5 and 15 min activates cAMP-dependent kinase and ERK in L1 adipocytes (Robidoux et al. 2006). Induction of PM MDM2 caused by AEA and EPI was mimicked by treatment with other agents that modulate receptors, G proteins, adenylyl cyclase systems, and protein kinases. Some of the agents AEA, EPI, capsaicin, CL316243, and aluminum fluoride may induce PM MDM2 through a common signaling molecule(s). The effects of agents that mimic the AEA- and EPI-induced responses provide possible clues for elucidating the signaling mechanism underlying these biomolecular responses. Inhibitory experiments targeting molecules that are responsive to capsaicin, CL316243, and aluminum fluoride are needed to reveal the machinery of biomolecular responses.

Induction of PM MDM2 by agents used in this study differed between cells treated with the same concentration of 0.01 or 10 μM of AEA and capsaicin and of 0.001 or 1 μM of EPI and CL316243, while such differences were not observed at other concentrations. The observed differences seem to be attributable to the affinity of the agent to the receptors and/or the ability of the agent to transduce signals and subsequently influence PM MDM2 induction.

Treatment of L1 adipocytes with AEA, EPI, and other agents induced PM MDM2 and did not alter the total MDM2 level. This indicates that intracellular MDM2 is decreased and increased in the plasma membrane by translocation and that MDM2 is involved in molecular interactions induced in the plasma membrane. Treatment with the agents used in this study translocates MDM2 to the plasma membrane and induces subcellular alterations in molecular interactions.

GRK2 binds to β-AR-containing vesicles in the presence of G protein subunits $G_{\beta\gamma}$ and phosphoinositides (Pitcher et al. 1995). Treatment of L1 adipocytes with insulin for 1–15 min increases the complex between GRK2 and Gαq/11 and phosphorylates this G protein (Usui et al. 2004b). An active form of Gαq/11 and an anti-Gαq/11 antibody partially alter some responses of L1 adipocytes to insulin, including responses for plasma membrane GLUT4 and phospho-Akt induction, (Imamura et al. 1999). PM MDM2 is induced in insulin-treated L1 adipocytes, and such induction of PM MDM2 was similarly observed after treatment of L1 adipocytes with the G protein activator and GPCR agonists used in this study. Treatment of a tumor cell line with IGF-I results in translocation of MDM2 from the cytoplasm into the nucleus (inducing MDM2 interactions in the nucleus) (Mayo and Donner 2001). In L1 adipocytes (differentiated and non-proliferative cells), MDM2 is responsive to G protein signaling activation and has the ability to interact with other molecules in the plasma membrane.

MDM2 harbors the binding site for complexes not only with β-arrestin but also insulin receptor substrate 1 (IRS-1) (Usui et al. 2004a). IRS-1 is a target molecule for insulin and other biomolecules such as IGF and TNF-α in L1 adipocytes (Rui et al. 2001). Receptors including the insulin and IGF receptors form complexes with G_β, β-arrestin, or Src in adipocytes untreated or treated with agonists (Dalle et al. 2001, 2002). GRK2 and β-arrestin antibodies or siRNAs modulate L1 adipocyte responses including those for GLUT4 translocation and lipolysis induced by treatment with insulin, ET-1, IGF-I, and TNF-α (Dalle et al. 2001; Imamura et al. 2001; Usui et al. 2004b; Kawamata et al. 2007). MDM2 also induces molecular ubiquitination, including that of the IGF receptor and β-AR *in vitro* (Shenoy et al. 2001; Girnita et al. 2003), through its ubiquitin ligase activity. MDM2 may interact with molecules present in complexes in the plasma membrane or those formed in the plasma membrane of treated cells and alter the ubiquitin content of molecules in the plasma membrane. Moreover, MDM2 may interact with MDM2-targeting molecules responsive to other biomolecules and might modulate other biomolecules-responsive signaling in fat metabolism. Further studies are needed to determine the interactive response induced by subcellular alterations in molecular interactions and the responsive significance in treated L1 adipocytes.

Treatment of adipocytes with signaling activators, including CB1 and β_3-AR activators, alters molecular expression and complex formation in the plasma membrane

(Pagano et al. 2007; Ahmad et al. 2009). Treatment of L1 adipocytes with insulin for 10–20 min induces Akt expression and phospho-Akt production in the plasma membrane (Sweeney et al. 2004; Ahmad et al. 2009) and translocates GLUT4 (Elmendorf et al. 1999), phosphoinositides-binding molecules (van Dam et al. 2005), and MDM2 to the plasma membrane. Such distribution to the subcellular locus is thought to be induced by alterations in the expressions of plasma membrane molecules and/or alterations in the interactive activity of subcellular molecules. Modified MDM2 is induced in insulin-treated L1 adipocytes and observed in the plasma membrane of L1 adipocytes treated with aluminum fluoride for 15 min (see discussion in Ohsaka and Nishino 2010). L1 adipocytes harbor molecular machinery to activate kinases that can modify MDM2, including Akt. Such kinases can be activated by treatment with an AEA analog, EPI, capsaicin (Zhuang et al. 2004; Amantini et al. 2007), CL316243, and aluminum fluoride (Bogoyevitch et al. 1995) for ≤30 min. The plasma membrane fractions obtained from treated L1 adipocytes in this study are useful for clarifying the molecules that interact with MDM2 in the subcellular locus. Moreover, these fractions are useful for elucidating the subcellular events that are induced and machinery that is regulated in treated L1 adipocytes. Our results advance the understanding of the responses to biomolecular GPCR signaling in adipocytes that are located in peripheral tissues and understanding of molecular machinery present in adipocytes.

L1 adipocytes possess molecular machinery that responds differentially to AEA and EPI under mithramycin A treatment and cool temperature conditions and that is sensitive to capsaicin, CL316243, and aluminum fluoride; these molecular machineries can induce subcellular alterations in molecular interactions. We provide information that is helpful for clarifying biomolecular responsive machinery present in L1 adipocytes.

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