Supplementary Figure VI

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Liver-Specific Deletion of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Causes Hepatic Steatosis and Death

Shuichi Nagashima, Hiroaki Yagyu, Ken Ohashi, Fumiko Tazoe, Manabu Takahashi, Taichi Ohshiro, Tumenbayar Bayasgalan, Kenta Okada, Motohiro Sekiya, Jun-ichi Osuga and Shun Ishibashi

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Critical role of neutral cholesteryl ester hydrolase 1 in cholesteryl ester hydrolysis in murine macrophages[S]

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Abstract **Hydrolysis of intracellular cholesteryl ester (CE) is the rate-limiting step in the efflux of cholesterol from macrophage foam cells. In mouse peritoneal macrophages (MPMs), this process is thought to involve several enzymes: hormone-sensitive lipase (Lipe), carboxylesterase 3 (Ces3), neutral CE hydrolase 1 (Nceh1). However, there is some disagreement over the relative contributions of these enzymes. To solve this problem, we first compared the abilities of several compounds to inhibit the hydrolysis of CE in cells overexpressing Lipe, Ces3, or Nceh1. Cells overexpressing Ces3 had negligible neutral CE hydrolase activity. We next examined the effects of these inhibitors on the hydrolysis of CE and subsequent cholesterol trafficking in MPMs. CE accumulation was increased by a selective inhibitor of Nceh1, paraoxon, and two nonselective inhibitors of Nceh1, (+)-AS115 and (−)-AS115, but not by two Lipe-selective inhibitors, orlistat and 76-0079. Paraoxon inhibited cholesterol efflux to apoA-I or HDL, while 76-0079 did not. These results suggest that Nceh1 plays a dominant role over Lipe in the hydrolysis of CE and subsequent cholesterol efflux in MPMs.**—Sakai, K., M. Igarashi, D. Yamamuro, T. Ohshiro, S. Nagashima, M. Takahashi, B. Enkhtuvshin, M. Sekiya, H. Okazaki, J-i. Osuga, and S. Ishibashi. **Critical role of neutral cholesteryl ester hydrolase 1 in cholesteryl ester hydrolysis in murine macrophages.** ***J. Lipid Res.*** **2014.** 55: **2033–2040.**

Supplementary key words hormone-sensitive lipase • inhibitor • foam cells • efflux • paraoxon • lipoproteins • ATP binding cassette transporters

A prominent characteristic of atherosclerotic lesions is the presence of cholesteryl ester (CE)-laden macrophage foam cells. Foam cells develop in the vessel wall as a result of migration of circulating monocytes into the intima, where the monocytes differentiate into macrophages and take up excessive amounts of modified lipoproteins generated during prolonged retention in the arterial walls (1). Hydrolysis of intracellular CE is the rate-limiting step in the cholesterol efflux from macrophage foam cells (2). As the hydrolysis of CE takes place at neutral pH, the enzymes catalyzing it have been collectively called neutral CE hydrolases (NCEHs). Because this step is rate-limiting, particularly in macrophage foam cells (3, 4), it is important to clarify the mechanisms that mediate the hydrolysis of CE in foam cells.

To date, at least three enzymes have been proposed to serve as NCEHs in macrophages. One is hormone-sensitive lipase (LIPE) (5). Another is CE hydrolase (6), which is identical to human liver carboxylesterase 1 (CES1) (7). It is also identical to macrophage serine esterase 1 (8), also known as a human ortholog of triacylglycerol hydrolase (9). A third such enzyme is neutral cholesterol ester hydrolase 1 (NCEH1) (10), which is also known as KIAA1363 or arylacetamide deacetylase-like 1 (11).

Contradictory results, however, have been reported with regard to the relative contribution of each enzyme to the hydrolysis of CE in macrophages. Lipe is expressed in mouse peritoneal macrophages (MPMs), and its overexpression inhibits the accumulation of CE in macrophages derived from a human acute monocyte leukemia cell line, THP-1 (12). The reported contributions of Lipe to the hydrolysis of CE in MPMs have varied from negligible (13, 14) to intermediate (15) to substantial (16). A mouse ortholog of CES1, carboxylesterase 3 (Ces3), was barely detectable in MPMs (10) and had negligible NCEH activity (17, 18). In contrast, we found that Nceh1 was robustly

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Abbreviations: acLDL, acetylated LDL; Ad-Ces3, adenoviruses overexpressing Ces3; Ad-Lipe, adenoviruses overexpressing Lipe; Ad-Nceh1, adenoviruses overexpressing Nceh1; CE, cholesteryl ester; Ces1, carboxylesterase 1; Ces3, carboxylesterase 3; Lipe, hormone-sensitive lipase; MPM, mouse peritoneal macrophage; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NCEH, neutral cholesteryl ester hydrolase; Nceh1, neutral cholesteryl ester hydrolase 1; PNPB, *p*-nitrophenyl butyrate; SI, selectivity index.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of five figures.

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expressed in MPMs as well as in atherosclerotic lesions (10, 19). Its overexpression inhibited the accumulation of CE in THP-1 macrophages (10), and its knockdown or knockout significantly reduced NCEH activity of MPMs (10, 15). Recently, however, Buchebner et al. (16) have reported that the contribution of Nceh1 to the NCEH activity of MPMs was negligible based on the results obtained with a different line of *Nceh1* knockout mice.

To determine which enzyme is more relevant, we used a pharmacological approach, which can be more advantageous, because genetic modification might confound the results by potentially leading to not only unpredictable developmental changes but also compensatory regulation of other genes.

We selected six inhibitors, four of which have been reported to have inhibitory activity toward either Nceh1 or Lipe. Cravatt and his colleagues have previously reported that phosphatase activity of KIAA1363 (NCEH1) was inhibited by paraoxon (11) or AS115 (20, 21). We confirmed the inhibitory activity of AS115 on NCEH activity of NCHE1 (18), and 76-0079 was originally developed as a selective inhibitor of Lipe (18, 22). Benzil inhibits CES1 (23), and orlistat inhibits pancreatic lipase (24).

METHODS

Materials

ApoA-1 from human plasma, benzil (1,2-diphenylethane-1,2-dione), BSA fraction V (BSA), lecithin, leupeptin, orlistat, and *p*-nitrophenyl butyrate (PNPB) were purchased from Sigma-Aldrich (St. Louis, MO). Thioglycollate medium I was purchased from WAKO (Osaka, Japan). TRIzol was purchased from Invitrogen (Carlsbad, CA). (+)-AS115, (−)-AS115, and TO-901317 were purchased from Cayman Chemical (Ann Arbor, MI). Paraoxon was purchased from Santa Cruz Biotechnology (Dallas, TX). The 76-0079 (NNC 0076-0000-0079) was a gift from Novo Nordisk (Bagsvaerd, Denmark). K-604, an ACAT1 inhibitor, was provided by Kowa Pharmaceutical (Tokyo, Japan) (25). Cholesterol [1-^{14}C]oleate and [1-^{14}C]oleic acid were purchased from Perkin Elmer (Waltham, MA). [1,2,6,7-^{3}H(N)]Cholesteryl oleate was purchased from American Radiolabeled Chemicals (St. Louis, MO). Anti-murine GAPDH (#2118) was purchased from Cell Signaling Technology (Danvers, MA). The recombinant adenoviruses overexpressing LacZ (the Escherichia coli gene encoding β-galactosidase), Nceh1 (Ad-Nceh1), Ces3, (Ad-Ces3), or Lipe (Ad-Lipe) were described previously (10, 12, 17, 18).

Preparation of lipoproteins

After an overnight fast, blood was collected from normolipidemic volunteers to isolate plasma. LDL (d 1.019–1.063 g/ml) and HDL (d 1.063–1.21 g/ml) were isolated from the plasma by sequential density ultracentrifugation (26). LDL was acetylated by repeatedly adding acetic anhydride (27).

Mice

All mice [C57BL/6J (WT), *Nceh1* knockout (*Nceh1*$^{-/-}$) (15), and *Lipe* knockout (*Lipe*$^{-/-}$) (14, 15) mice] were maintained in a temperature-controlled (25°C) facility with a 12 h light/dark cycle and given free access to food and water. Mice were maintained and cared for according to the regulations of the Animal Care Committees of Jichi Medical University. All animals used in these studies were male.

Cells

HEK293A cells were cultured in DMEM containing 10% (v/v) FBS and antibiotics. MPMs were obtained 3 days after a 2 ml intraperitoneal injection of 5% (w/v) thioglycollate broth. MPMs were plated on 48- or 96-well plates and cultured in DMEM containing 10% (v/v) FBS and antibiotics for 2 h. Thereafter, cells were washed with PBS, and if not stated otherwise, the adherent macrophages were maintained in DMEM supplemented with 10% (v/v) FBS and antibiotics.

Western blot analyses

Cells were sonicated in buffer A (50 mM Tris-HCl, 250 mM sucrose, 1 mM EDTA, 2 μg/ml leupeptin, pH 7.0). Each lysate was separated on a 10% SDS-PAGE and transferred to a nitrocellulose membrane. For detection of the proteins, the membranes were incubated with each anti-murine Nceh1 (10), anti-murine Ces3 (17), anti-murine Lipe (10), or anti-murine GAPDH at a dilution of 1:500–4,000. Specifically bound immunoglobulins were detected in a second reaction with a horseradish peroxidase-labeled IgG conjugate and visualized by ECL detection (GE Healthcare) with Image Quant LAS 4000 Mini (GE Healthcare).

Enzymatic assays

Whole cell lysates were prepared from transfected HEK293A and used for the enzymatic assays. PNPB hydrolase activity was determined as described previously (10). NCEH activity was determined as described by Hajjar et al. (28), using a reaction mixture containing 6.14 μM cholesterol [1-^{14}C]oleate (48.8 μCi/μmol; 1 μCi = 37 kBq).

CE turnover assay

After incubation in DMEM containing 5 mg/ml lipoprotein deficient serum (LPDS) for 24 h, MPMs (1×10^6 cells/well) were incubated in DMEM containing 10 mM [1-^{14}C]oleic acid-albumin complex (58.2 μCi/μmol), 50 μg/ml acetylated LDL (acLDL), and 5 mg/ml BSA for 24 h. The cells were washed with PBS and incubated for 12 h with DMEM containing 5 mg/ml LPDS to allow hydrolyzing CE. Lipids were extracted and resolved by TLC. [^{14}C]CE was measured by liquid scintillation counter.

Intracellular neutral lipids stained with Oil Red O

After CE turnover assay, MPMs were washed with PBS, fixed with 4% paraformaldehyde for 30 min, and then stained with Oil Red O and hematoxylin eosin for microscopic analysis (IX70, Olympus).

Cholesterol efflux assay

Cholesterol efflux was determined as described previously (15, 29). Briefly, MPMs (1×10^6 cells/well) were loaded with [1,2,6,7-^{3}H(N)]cholesteryl oleate by incubating the cells with 50 μg/ml acLDL. After 24 h, cholesterol efflux was initiated by the addition of 100 μg/ml HDL or 25 μg/ml apoA-1 in the presence of K-604 and continued for 24 h. An aliquot of the medium was removed and centrifuged at 15,000 *g* for 2 min to remove cellular debris, and the radioactivity in the supernatant was measured with a liquid scintillation counter. The cells were lysed in 0.05% SDS buffer, and the radioactivity in an aliquot of the cell lysate was measured. The percent efflux was calculated as (media dpm)/(cell + media dpm) × 100.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay kit was purchased from Cayman

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Chemical (Ann Arbor, MI). Assay was performed following the manufacturer's protocol. Briefly, MPMs (5×10^4 cell/well) were incubated in DMEM containing 5 mg/ml BSA with each compound for 24 h. Four hours after the addition of Dye solution, Solubilization/Stop solution was added to the medium for measurement of absorbance using a spectrometer (E Max, Molecular Devices).

Quantitative real-time PCR

Total RNA was prepared from MPMs using TRIzol. Relative amounts of mRNA were calculated using a standard curve or the comparative cycle threshold method with the StepOnePlus Real-Time PCR instrument (Applied Biosystems) according to the manufacturer's protocol. Mouse β-actin (*Actb*) mRNA was used as the invariant control. Primer sequences were as follows: *Nceh1* forward, 5′-AGCCTGCAGTTTGAGCTTA-3′; *Nceh1* reverse, 5′-AGAGTCGGTATTTCTGGAGACG-3′; *Nceh1* probe, 5′-/56-FAM/AGGCTGGCA/ZEN/ACGTAGGTAAACTGTT/3IABkFQ/-3′; *Lipe* forward, 5′-CATATCCGCTCTCCAGTTGACC-3′; *Lipe* reverse, 5′-CCTATCTTCTCCATCGACTACTCC-3′; *Lipe* probe, 5′-/56-FAM/CGAGGCTCC/ZEN/CTTTCCCCGAG/3IABkFQ/-3′; *Abca1* forward, 5′-TGCCACTTTCCGAATAAAGC-3′; *Abca1* reverse, 5′-GGAGTTGGATAACGGAAGCA-3′; *Abca1* probe, 5′-ATGCCGTCTGCAGGAA-3′; *Abcg1* forward, 5′-TCGAATTCAAGGACCTTTCC-3′; *Abcg1* reverse, 5′-CCACTGTTGAATTTCCCAGA-3′; *Abcg1* probe, 5′-TGGTGGAAGAAGAAAG-3′; *Actb* forward, 5′-CGATGCCCTGAGGCTCTTT-3′; *Actb* reverse, 5′-TGGATGCCACAGGATTCCA-3′; *Actb* probe, 5′-CCAGCCTTCCTTCTT-3′.

Statistical analyses

Results are presented as the mean ± SD. Statistical differences between groups were analyzed by one-way ANOVA and the Dunnett's multiple comparisons test. All calculations were performed with Graph Pad Prism version 6.0 for Macintosh (MDF).

RESULTS

NCEH activity in the cells infected with Ad-Nceh1, Ad-Ces3, and Ad-Lipe

To confirm the ability of the overexpressed enzymes to hydrolyze CE, we infected HEK293A cells with recombinant adenoviruses to overexpress Nceh1, Ces3, or Lipe. Whole cell lysates were subjected to Western blot analyses and measurements of enzymatic activities (supplementary Fig. I). The Western blot analyses showed the expression of Nceh1 (45 and 50 kDa), Ces3 (60 kDa), and Lipe (80 kDa) (supplementary Fig. IA). Overexpression of all three enzymes caused substantial increases in PNPB hydrolase activity (Ad-Nceh1, 28.1- fold; Ad-Ces3, 26.5-fold; Ad-Lipe, 15.3-fold) (supplementary Fig. IB). NCEH activity was increased 24.9-fold by overexpression of Lipe and was increased 4.4-fold by overexpression of Nceh1, but it was not increased by overexpression of Ces3 (supplementary Fig. IC). Therefore, we used only Ad-Nceh1 and Ad-Lipe for further studies.

Selectivity of compounds against NCEH enzymes

We compared the inhibitory effects of each compound on NCEH enzymatic activities, which were expressed by overexpression of Nceh1 or Lipe in cell lysates. The IC_{50} values and selectivity index (SI) values are summarized in **Table 1**. (+)-AS115 and (−)-AS115 inhibited NCEH activities of both Nceh1 and Lipe (SI: 4.3 and 2.3, respectively). Paraoxon selectively inhibited NCEH activities of Nceh1 (IC_{50} values against Nceh1: 0.003 μM; SI: 400.0). On the other hand, orlistat and 76-0079 selectively inhibited NCEH activities of Lipe (SI: >51.0 and 115.0, respectively). Benzil selectively inhibited PNPB hydrolyzing activity of Ces3.

CE turnover in MPMs

To examine whether inhibition of the hydrolysis of CE by each compound affected cholesterol trafficking, we labeled MPMs with oleic acid and measured the amounts of cholesteryl oleate after exposure to each compound. Treatment with (+)-AS115 or (−)-AS115, which are non-selective inhibitors, and with paraoxon, which is an Nceh1-selective inhibitor, increased CE in MPMs from WT mice (**Fig. 1A–C**). Similar increase in CE was observed in MPMs from *Lipe*$^{-/-}$ mice. However, these CE-increasing effects were not observed in MPMs from *Nceh1*$^{-/-}$ mice. These results indicate that Nceh1 is critical for hydrolyzing CE and removal of cholesterol from the cell. On the other hand, treatment with orlistat or 76-0079, which are Lipe-selective inhibitors, did not significantly increase the CE contents in MPMs from any of three types of mice (Fig. 1D, E). Moreover, treatment with benzil, a Ces3-selective inhibitor, did not significantly increase the CE content either (Fig. 1F). These results indicate that neither Lipe nor Ces3 is significantly involved in the process.

To rule out the possibility that the compounds affect cholesterol trafficking via their cytotoxicity, we measured

TABLE 1. Inhibitory effect of compounds on the hydrolysis of PNPB or CE in cell lysates of HEK293A cells overexpressing Nceh1, Ces3, or Lipe

Compound	IC_{50} for PNPB Hydrolysis (μM)			IC_{50} for CE Hydrolysis (μM)		SI[a]	Selectivity
	Nceh1	Ces3	Lipe	Nceh1	Lipe		
(+)-AS115	1.2	0.5	0.6	0.3	0.07	4.3	Nceh1, Lipe
(−)-AS115	0.6	0.5	0.9	0.1	0.2	2.0	Nceh1, Lipe
Paraoxon	0.02	0.01	32.0	0.003	1.2	400.0	Nceh1
Orlistat	>20.4	>20.4	19.0	>20.4	0.4	>51.0	Lipe
76-0079	>19.5	>19.5	0.1	2.3	0.02	115.0	Lipe
Benzil	>47.6	0.5	>47.6	>47.6	>47.6	—	—

[a]SI = high IC_{50} (Nceh1 or Lipe)/low IC_{50} (Nceh1 or Lipe).

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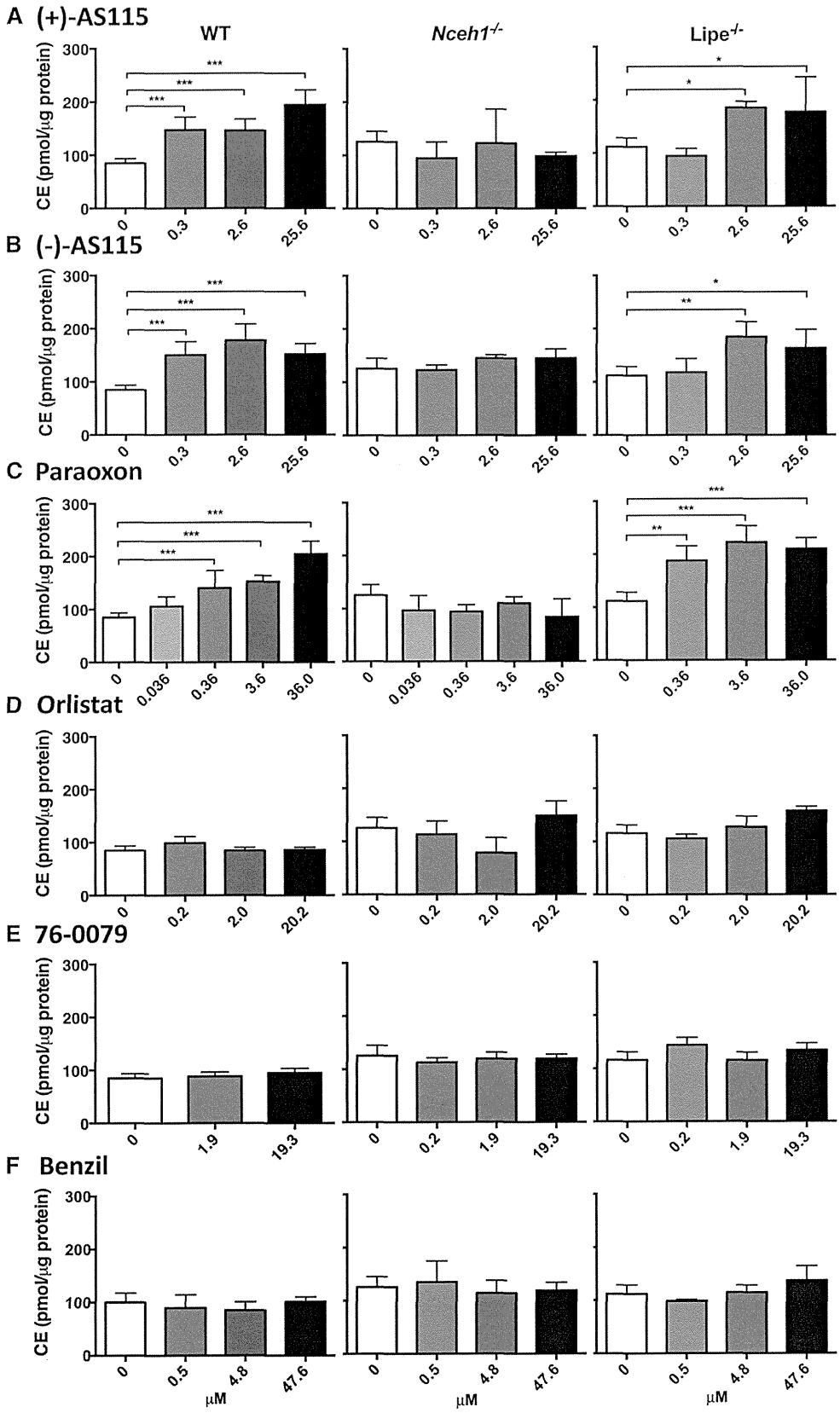

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MTT activities in the cells treated with the compounds. These compounds did not show cytotoxicity against MPMs in an MTT assay (supplementary Fig. II). To examine whether the compounds affect the expression of each enzyme, we performed RT-PCR analysis of *Nceh1* and *Lipe*. Paraoxon, orlistat, and 76-0079 did not affect the expression of *Nceh1* and *Lipe* (supplementary Fig. III). On the other hand, (+)-AS115 decreased the expression of *Lipe*, and (−)-AS115 decreased the expression of *Nceh1* and *Lipe* at 25.6 μM. However, the effects were not significant at the lower concentrations (0.3 and 2.6 μM). Therefore, (+)-AS115 and (−)-AS115 inhibited both the hydrolase activity and the expression of *Nceh1* and *Lipe* (supplementary Fig. III). Conceivably, the inhibition of the expression of *Nceh1* and *Lipe* did not mediate the CE-increasing effects of AS115s at the lower concentrations.

Lipid droplet accumulation in MPMs

After loading the cells with CE by incubation with acLDL, intracellular neutral lipid droplets were stained with Oil Red O. Treatment with (+)-AS115, (−)-AS115, or paraoxon increased lipid droplet accumulation compared with the control (**Fig. 2A–D**). On the other hand, neither orlistat, 76-0079, nor benzil caused significant lipid droplet accumulation (Fig. 2E–G). These results suggest that selective inhibition of Nceh1, but not Lipe or Ces3, increased CE accumulation in MPMs.

Cholesterol efflux in MPMs

To directly investigate whether the inhibition of CE hydrolysis decreases the release of free cholesterol from the cell, we measured cholesterol efflux in MPMs treated with paraoxon, an Nceh1-selective inhibitor, and 76-0079, a Lipe-selective inhibitor in the presence of K-604 to inhibit de novo esterification of cholesterol (**Fig. 3**). When HDL or apoA-1 was used as a cholesterol acceptor, only treatment with paraoxon decreased cholesterol efflux from MPMs of WT mice (Fig. 3A, B). Similar decrease was observed in MPMs from *Lipe*$^{-/-}$ mice. However, these effects of paraoxon were not observed in MPMs from *Nceh1*$^{-/-}$ mice (Fig. 3A, B). To examine whether the changes in cholesterol efflux are associated with changes in the expression of *Abca1* and *Abcg1*, we measured the expressions of these genes by RT-PCR. While TO-901317, a liver X receptor agonist, increased the expressions of *Abca1* and *Abcg1*, neither paraoxon nor 76-0079 affected them significantly (supplementary Fig. IVA, B). K-604 (19.7 μM), an ACAT1 inhibitor, did not inhibit Nceh1 and Lipe in NCEH assay (supplementary Fig. V). These results indicate that Nceh1 is primarily involved in CE hydrolysis and that it is the rate-limiting step in the cholesterol efflux from MPMs.

DISCUSSION

Based on their selectivities on Nceh1 or Lipe, the six inhibitors were classified into four groups: (1) nonselective inhibitors [(+)-AS115 and (−)-AS115], (2) Nceh1-selective inhibitor (paraoxon), (3) Lipe-selective inhibitors (orlistat and 76-0079), and (4) inhibitor of PNPB-hydrolyzing activity of Ces3 (Benzil). Treatment with paraoxon, an Nceh1-selective inhibitor, increased CE accumulation, as shown by the accumulation of neutral lipid droplets, in MPMs (Figs. 1C, 2D). Moreover, paraoxon decreased cholesterol efflux from MPMs without changing the expression of *Abca1* or *Abcg1* (supplementary Fig. IVA, B). Similar effects were observed in MPMs from *Lipe*$^{-/-}$ mice. In contrast, these effects were not detectable in MPMs from *Nceh1*$^{-/-}$ mice. These results indicate that Nceh1 substantially contributes to the NCEH and subsequent cholesterol efflux in MPMs, which is in good agreement with our previous reports (15, 18). On the other hand, orlistat and 76-0079 (Lipe-selective inhibitors) did not significantly increase the CE contents (Fig. 1D, E) or lipid droplets in MPMs (Fig. 2E, F). It is possible that orlistat is not transported to the intracellular sites where Lipe is localized. In contrast, 76-0079 has been widely used for cell-based experiments showing its efficacy (18, 22). Thus, we conclude that Lipe does not contribute to the NCEH activity in MPMs. These observations are very consistent with the results that AS115s (nonselective inhibitors) increased CE accumulation as much as paraoxon did. Based on these results, we can conclude that Nceh1 plays a dominant role over Lipe in the hydrolysis of CE in MPMs.

In contrast to these current and previous observations that Nceh1 significantly contributes to CE hydrolysis in MPMs, Buchebner et al. (16) proposed that Lipe, but not Nceh1, is essential for the hydrolysis of CE in MPMs. It is unclear why they reached opposite conclusions even though they used a similar strategy: use of MPMs obtained from genetically modified mice. We assume that the completeness of the deficiency of Nceh1/KIAA1363 might be different between the mice used in the two labs because of the use of different targeting vectors. Furthermore, sensitivity of the assay to measure NCEH activity might have complicated the results.

The negligible contribution of Lipe to the hydrolysis of CE in MPMs is very consistent with the results of two earlier studies on MPMs from *Lipe*$^{-/-}$ mice (13, 14). Buchebner et al., however, reported that NCEH activity was almost abrogated in MPMs from *Lipe*$^{-/-}$ mice. We do not know the reason for this discrepancy. Because Lipe deficiency leads to developmental changes in several tissues such as testis and adipose tissue (14), it is possible that Lipe deficiency also causes developmental changes in the macrophage lineage in an age-dependent manner. If Lipe

Fig. 1. Effect of inhibitors against Nceh1 and/or Lipe on CE turnover in MPMs from WT, *Nceh1*$^{-/-}$, or *Lipe*$^{-/-}$ mice. MPMs were incubated with [1-^{14}C]oleic acid in the presence of acLDL. After 24 h, MPMs were changed to new medium and incubated up to 12 h with each inhibitor. TLC was used to separate CE from the cellular lipids. A: (+)-AS115. B: (−)-AS115. C: Paraoxon. D: Orlistat. E: 76-0079. F: Benzil. Data are presented as the means ± SD of 3–10 measurements. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, each concentration of inhibitor versus control (determined by ANOVA followed by the Dunnett's multiple comparisons test for A–F).

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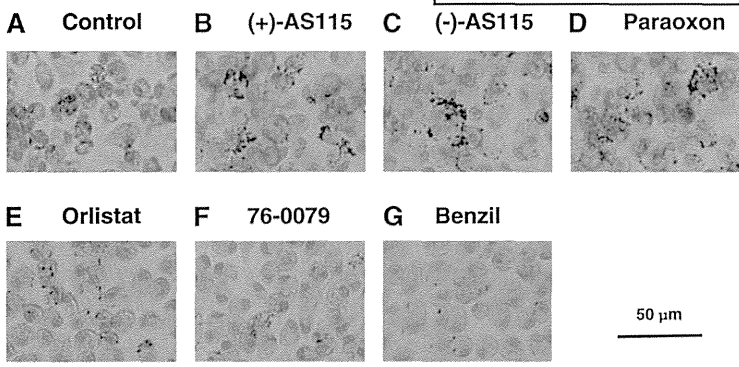

Fig. 2. Effect of inhibitors against Nceh1 and/or Lipe on neutral lipid droplet accumulation in MPMs from WT mice. Intracellular neutral lipids were stained with Oil Red O. A: Control (methanol). B: (+)-AS115, 25.6 μM. C: (−)-AS115, 25.6 μM. D: Paraoxon, 36.0 μM. E: Orlistat, 20.2 μM. F: 76-0079, 19.3 μM. G: Benzil, 47.6 μM.

deficiency somehow decreases the NCEH activity of Nceh1 in macrophages under certain conditions, this may explain the contradiction stated previously.

The compounds used in the present study were not strictly specific. Paraoxon and/or AS115, the Nceh1-inhibiting compounds, also inhibit PNPB-hydrolyzing activity of Ces3 (Table 1). Furthermore, it is well known that organophosphorus toxicants, to which paraoxon belongs, target at least 50 serine hydrolases and receptors including acetylcholinesterase, butyrylcholinesterase, chymotrypsin, arylformamidase, and fatty acid amide hydrolase (30). However, overexpression of Ces3 did not show a significant NCEH activity (supplementary Fig. IC) (17). Moreover, benzil, a Ces3-selective inhibitor, did not inhibit the hydrolysis of CE in MPMs (Figs. 1F, 2G). Therefore, it is unlikely that paraoxon or AS115 increased CE accumulation by specifically inhibiting Ces3.

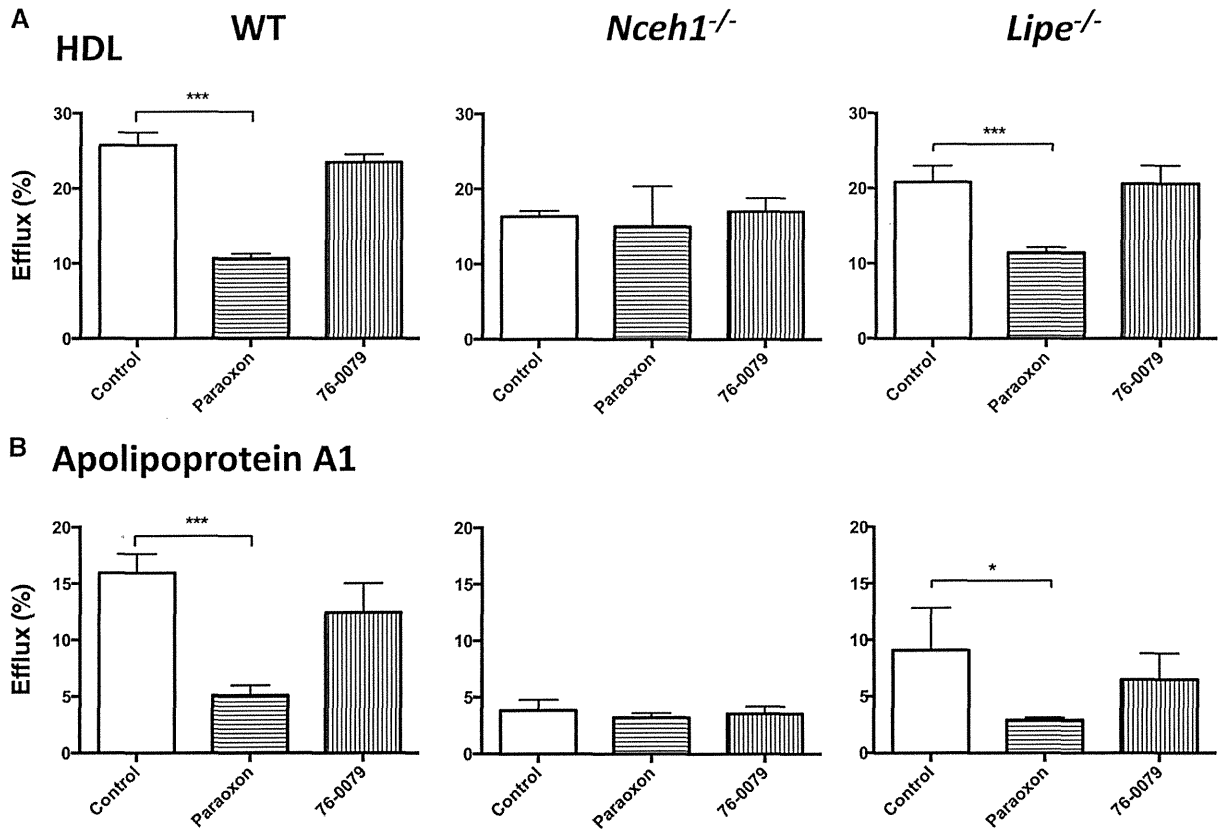

Fig. 3. Effect of inhibitors against Nceh1 or Lipe on cholesterol efflux in MPMs from WT, *Nceh1*$^{-/-}$, or *Lipe*$^{-/-}$ mice. MPMs were loaded with [1,2,6,7-^{3}H(N)]cholesteryl oleate by incubating the cells with acLDL. After 24 h, cholesterol efflux was initiated by the addition of HDL or apoA-1 in the presence of K-604 with each inhibitor and continued for 24 h. The percent efflux was calculated as (media dpm)/(cell + media dpm) × 100. Cholesterol efflux was examined with paraoxon (36.0 μM), 76-0079 (19.3 μM), or methanol (control) in the presence of HDL (A) or apoA-1 (B). Data are presented as the means ± SD of three to four measurements. * $P < 0.05$, *** $P < 0.001$, each inhibitor vs control (determined by ANOVA followed by the Dunnett's multiple comparisons test for A).

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Recently, Marcel and his colleagues (31, 32) proposed a novel and intriguing pathway for CE hydrolysis: autophagy. In one of the key experiments, they used chloroquine to disrupt the lysosomal pathway. Treatment with chloroquine increased cellular CE as much as treatment with paraoxon did. These results were interpreted as evidence of the involvement of autophagy in the hydrolysis of CE. However, a high concentration of chloroquine can be cytotoxic. Indeed, we found that incubation of MPMs with 30 and 100 μM chloroquine for 24 h decreased MTT activity by 60% and 90%, respectively (unpublished observations). The resulting dying cells might be taken up by neighboring macrophages by efferocytosis, where cellular CE is directly targeted to lysosomes via fusion with phagosomes. This pathway involves lysosomes, but certainly not autophagy. Another caveat concerning the use of chloroquine is its potential effect on ataxia telangiectasia mutated (ATM). Schneider et al. (33) reported that treatment with low-dose chloroquine attenuated atherosclerosis in apoE knockout mice by suppressing c-Jun N-terminal kinase activity, which suppresses LPL activity via activating ATM. In a pioneering paper addressing the lysosomal pathway for CE hydrolysis, Avart and his colleagues (34) showed that chloroquine inhibited the hydrolysis of CE only when CE is in anisotropic inclusions. Further studies are needed to correctly interpret the antiatherosclerotic effect of chloroquine.

In conclusion, we show pharmacological evidence that Nceh1 has a critical role in the hydrolysis of CE in MPMs. These findings should provide the basis for understanding the pathophysiology of atherosclerosis and can be exploited to develop new therapeutic approaches.

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Absence of Nceh1 augments 25-hydroxycholesterol-induced ER stress and apoptosis in macrophages[S]

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Abstract **An excess of cholesterol and/or oxysterols induces apoptosis in macrophages, contributing to the development of advanced atherosclerotic lesions. In foam cells, these sterols are stored in esterified forms, which are hydrolyzed by two enzymes: neutral cholesterol ester hydrolase 1 (*Nceh1*) and hormone-sensitive lipase (*Lipe*). A deficiency in either enzyme leads to accelerated growth of atherosclerotic lesions in mice. However, it is poorly understood how the esterification and hydrolysis of sterols are linked to apoptosis. Remarkably, *Nceh1*-deficient thioglycollate-elicited peritoneal macrophages (TGEMs), but not *Lipe*-deficient TGEMs, were more susceptible to apoptosis induced by oxysterols, particularly 25-hydroxycholesterol (25-HC), and incubation with 25-HC caused massive accumulation of 25-HC ester in the endoplasmic reticulum (ER) due to its defective hydrolysis, thereby activating ER stress signaling such as induction of CCAAT/enhancer-binding protein-homologous protein (CHOP). These changes were nearly reversed by inhibition of ACAT1. In conclusion, deficiency of *Nceh1* augments 25-HC-induced ER stress and subsequent apoptosis in TGEMs. In addition to reducing the cholesteryl ester content of foam cells, Nceh1 may protect against the pro-apoptotic effect of oxysterols and modulate the development of atherosclerosis.**—Sekiya, M., D. Yamamuro, T. Ohshiro, A. Honda, M. Takahashi, M. Kumagai, K. Sakai, S. Nagashima, H. Tomoda, M. Igarashi, H. Okazaki, H. Yagyu, J-i. Osuga, and S. Ishibashi. **Absence of Nceh1 augments 25-hydroxycholesterol-induced ER stress and apoptosis in macrophages.** ***J. Lipid Res.*** **2014. 55: 2082–2092.**

Supplementary key words KIAA1363 • arylacetamide deacetylase-like 1 • atherosclerosis • foam cells • acyl-CoA:cholesterol acyltransferase • lipase/hormone-sensitive lipase • oxysterols • reverse cholesterol transport • lipid droplets • apoptosis • neutral cholesterol ester hydrolase 1 • endoplasmic reticulum

Atherosclerotic cardiovascular diseases are the leading cause of mortality in industrialized countries, despite advances in the management of coronary risk factors. Heart attacks arise from the thrombotic occlusion of coronary arteries following the rupture of plaques. Characteristic of these rupture-prone plaques is their lipid-rich nature due to the presence of cholesteryl ester (CE)-laden macrophage foam cells (1).

The hydrolysis of intracellular CE, the initial step of reverse cholesterol transport, is catalyzed by multiple enzymes: neutral cholesterol ester hydrolase 1 (NCEH1) (2), also known as KIAA1363 or arylacetamide deacetylase-like 1 (AADACL1) (3), hormone-sensitive lipase (LIPE) (4), and possibly carboxylesterase 1 (CES1) (5, 6). NCEH1 is a microsomal protein tethered to the endoplasmic reticulum (ER) membrane by its N terminus with the rest of the protein containing the catalytic domain residing in the ER

Abbreviations: acLDL, acetyl-LDL; Bip, immunoglobulin heavy chain-binding protein; CE, cholesteryl ester; CHOP, CCAAT/enhancer-binding protein-homologous protein; ER, endoplasmic reticulum; 25-HC, 25-hydroxycholesterol; 27-HC, 27-hydroxycholesterol; *Hmgcs1*, 3-hydroxy-3-methylglutaryl-CoA synthase 1; 7-KC, 7-ketocholesterol; LD, lipid droplet; LIPE, hormone-sensitive lipase; LPDS, lipoprotein deficient serum; LXR, liver X receptor; NCEH1, neutral cholesterol ester hydrolase 1; PPPA, pyripyropene A; Srebp, sterol regulatory element binding protein; TEM, transmission electron microscopy; TGEM, thioglycollate-elicited peritoneal macrophage; TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; Xbp-1, X-box-binding protein 1.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of three figures.

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lumen (7) and is robustly expressed in macrophages (2). In addition to CE, NCEH1 may catalyze the hydrolysis of 2-acetyl monoalkylglycerol (8) and TG (2). We have recently reported that disruption of *Nceh1* promotes the formation of foam cells and accelerates the development of atherosclerosis in mice lacking either *Apoe* or LDL receptor (*Ldlr*) (9). In humans, NCEH1 plays a more critical role in cholesterol removal from monocyte-derived macrophages (10). Ultimately, however, accumulating evidence has suggested that reverse cholesterol transport is only one of a number of diverse functions of macrophages in atherogenesis (11). Macrophage apoptosis is another important feature of atherosclerosis (12, 13). Interestingly, the metabolism of cholesterol and its metabolites is closely involved in the apoptosis of macrophages. For example, oxysterols such as 25-hydroxycholesterol (25-HC) and 7-ketocholesterol (7-KC) are major bioactive molecules that initiate the apoptosis of macrophages exposed to oxidized LDL (14). Sinensky and his colleagues reported that increased Ca^{2+} influx (15) and subsequent activation of cytosolic phospholipase A2 (cPLA2) (16), as well as increased proteasomal degradation of Akt (17), underlie the oxysterol-induced apoptosis in CHO-K1 and P388D1 cells. They further showed that ACAT mediates the 7-KC-induced apoptosis in P388D1 cells and mouse peritoneal macrophages (18). In contrast to this pro-apoptotic role, Rothblat and Tabas reported that ACAT can serve an anti-apoptotic role by showing inhibition of ACAT causes apoptosis of macrophages after exposure to acetyl-LDL (acLDL) (19, 20). Tabas and colleagues further proposed that ER stress pathways mediate apoptotic signaling in this process (21). Thus, the role of ACAT1 in apoptosis may depend on the sort of sterol, which probably explains the conflicting results concerning the effects of ACAT1's inhibition on atherosclerosis (22, 23). Because NCEH1 counteracts ACAT activity, it is possible that macrophages lacking Nceh1 are more susceptible to apoptosis, particularly in response to various sterols. These considerations have prompted us to examine the anti-apoptotic role of Nceh1 in the apoptosis of macrophages.

Herein, we demonstrate that *Nceh1*-deficient macrophages are highly susceptible to apoptosis induced by 25-HC, and the underlying mechanism may involve the activation of ER stress signaling due to accumulation of 25-HC ester in the ER.

MATERIALS AND METHODS

Materials

27-Hydroxycholesterol (27-HC) was purchased from Research Plus (Bayonne, NJ); all other oxysterols and MG-132 were purchased from Sigma (St. Louis, MO). Ca^{2+}-free DMEM medium was purchased from Gibco (Carlsbad, CA). K-604 (24) and CS-505 (25) were provided by Kowa Pharmaceutical, Daiichi Sankyo and Kyoto Pharmaceutical Industries, respectively. Pyripyropene A (PPPA) was purified from a culture broth of the fungus, *Aspergillus fumigatus* FO-1289 (26, 27). Cholesterol [1-^{14}C]oleate and [1-^{14}C]oleic acid were purchased from Perkin Elmer (Waltham, MA). 25-HC oleate was synthesized from 25-HC and oleic acid with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and 4-dimethylaminopyrindine in dichloromethane. After reaction, 25-HC oleate was purified by preparative TLC on silica gel (10:1 hexane:ethyl acetate). Chemical structure was determined by the NMR analysis and MS. 25-HC [1-^{14}C]oleate was synthesized from 25-HC and [1-^{14}C]oleic acid.

Lipoproteins

acLDL and lipoprotein deficient serum (LPDS) were prepared as described previously (28).

Animals

Mice lacking *Nceh1* (*Nceh1*$^{-/-}$), *Lipe* (*Lipe*$^{-/-}$) or both (*Nceh1*$^{-/-}$; *Lipe*$^{-/-}$) were generated as described previously (9, 29). Mice used in this study were crossed onto the C57BL/6J background for more than five generations. All experimental procedures and handling of animals were conducted according to our institutional guidelines.

Peritoneal macrophages

Thioglycollate-elicited peritoneal macrophages (TGEMs) were obtained from 8-week-old mice as described (9) and incubated in DMEM containing 10% FCS or 10% LPDS.

Transmission electron microscopy

Cells were fixed in modified Karnovsky's phosphate-buffered (0.1 M) glutaraldehyde (2.5%)-paraformaldehyde (4%) mixture at room temperature for 12 h, postfixed for 2 h in 2% osmium tetroxide in 0.1 M phosphate buffer, dehydrated in ethanol and propylene oxide, and embedded in Epon. The sections were cut and then counterstained with uranyl acetate and lead citrate for transmission electron microscopy (TEM).

Detection of DNA ladder

DNA (0.5 μg), which was extracted from the cells, was end-labeled with [α-^{32}P]dCTP by Klenow and subjected to electrophoresis in a 1.5% agarose gel and then transferred to nylon membranes, as described previously (30). Mouse thymocyte apoptotic DNA was used as a control (31).

TUNEL

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was performed by using a kit (Takara Biomedicals, Tokyo). At least 1,500 cells from five random fields were counted in each individual sample, and the percentage of apoptotic cells was calculated as (TUNEL-positive cells)/(TUNEL-positive cells + surviving TUNEL-negative cells).

Subcellular fractionation

Cells were sonicated in buffer A [20 mM Tris-HCl (pH 7.0), 250 mM sucrose with protease inhibitors], ultracentrifuged at 100,000 *g* for 45 min at 4°C, microsomal pellet was resuspended and re-ultracentrifuged to enhance purity to give a supernatant fraction (cysotol) and a microsomal pellet (22, 23).

TLC

Lipid was extracted from the cytosolic (100 μg of protein) and microsomal fraction (50 μg of protein), and was separated by TLC with toluene-ethyl acetate (67:33) as the solvent. Visualization was done with 10% sulfuric acid.

Measurements of oxysterols

Concentrations of oxysterols in subcellular fractions were measured using LC-MS/MS as described (32). After the addition of

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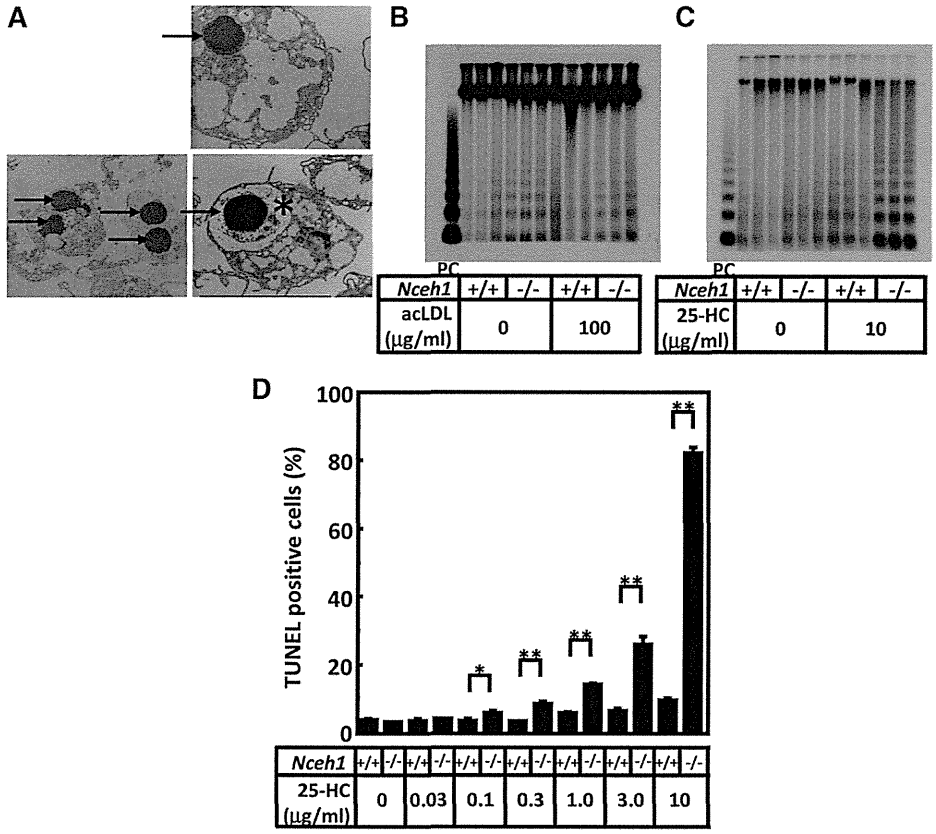

Fig. 1. Nceh1-deficient TGEMs are prone to apoptosis. A: TEM observation. TGEMs were incubated in DMEM containing 10% FCS. Nuclear condensation (indicated by arrows), a characteristic feature of apoptotic cells, was observed predominantly in *Nceh1*-deficient TGEMs (representative images are shown). *The apoptotic macrophage engulfed by another macrophage. B, C: DNA ladder. DNA (0.5 μg) from macrophages was loaded in each lane. PC denotes positive control DNA (0.5 μg) from dexamethasone-treated thymocytes. Three wells of cells were incubated either in DMEM containing 10% FCS with or without acLDL (100 μg/ml) for 24 h (B) or in DMEM containing 10% LPDS with vehicle or 25-HC (10 μg/ml) for 24 h (C). D: Four wells of cells were incubated with increasingly higher concentrations of 25-HC for 24 h. The apoptotic cells were detected by TUNEL. Data are expressed as the mean ± SEM. $*P < 0.05$; $**P < 0.01$.

deuterated internal standards and butylated hydroxytoluene, each fraction was either hydrolyzed with 1 N ethanolic KOH and derivatized into picolinyl esters, or directly converted into picolinyl esters.

Northern blot analysis

Northern blot analyses were performed as described (9).

Analysis of Xbp-1 mRNA splicing

Total RNA was reverse transcribed and amplified using a sense primer (5′-AAACAGAGTAGCAGCGCAGACTGC-3′) and an antisense primer (5′-GGATCTCTAAAACTAGAGGCTTGGTG-3′). This fragment was further digested by *PstI* as described previously (33).

Quantitative real-time PCR

Two micrograms of total RNA were reverse-transcribed using the ThermoScript RT-PCR system (Invitrogen). Quantitative real-time PCR was performed using SYBR Green dye (Applied Biosystems, Foster City, CA) in an ABI Prism 7900 PCR instrument (Applied Biosystems). The relative abundance of each transcript was calculated from a standard curve of cycle thresholds for serial dilutions of a cDNA sample and normalized to *Rplp0* or *Atcb*. Primer sequences for *Abca1* and 3-hydroxy-3-methylglutaryl-CoA synthase 1 (*Hmgcs1*) were described previously (9). Other primer sequences were as follows: immunoglobulin heavy chain-binding protein (*Bip*) (sense 5′-TCATCGGACGCACTTGGAA-3′, antisense 5′-CAACCACCTTGAATGGCAAGA-3′), CCAAT/enhancer-binding protein-homologous protein (*Chop*) (sense 5′-GTCCCTAGCTTGGCTGACAGA-3′, antisense 5′-TGGAGAGCGAGGGCTTTG-3′), *Nceh1* (sense 5′-AGCCTGCAGTTTGAGCTTA-3′, antisense 5′-AGAGTCGGTATTTCTGGAGACG-3′), *Acat1* (sense 5′-GGAAGTTGGGTGCCACTTCG-3′, antisense 5′-GGTGCTCTCAGATCTTTGG-3′), *Rplp0* (sense 5′-GAAGACAGGGCGACCTGGAA-3′, antisense 5′-TTGTGGCTCCCACAATGAAGC-3′), and *Atcb* (sense 5′-CGATGCCCTGAGGCTCTTT-3′, antisense 5′-TGGATGCCACAGGATTCCA-3′).

Western blot analyses

TGEMs were homogenized in buffer A [50 mM Tris-HCl, 250 mM sucrose, 1 mM EDTA, 2 μg/ml leupeptin (pH 7.0)]. Ten micrograms of proteins of whole lysates were separated by SDS-PAGE on the NuPAGE 10% Bis-Tris gel and transferred to a nitrocellulose membrane. For detection of the proteins, the membranes were incubated with each anti-murine Akt (Abcam) or anti-murine GAPDH at a dilution of 1:1,000 in Hikari A solution (Nacalai Tesque). Specifically bound immunoglobulins were detected in a second reaction with a horseradish peroxidase-labeled IgG conjugate and visualized by ECL detection (GE Healthcare) with Image Quant LAS 4000 Mini (GE Healthcare).

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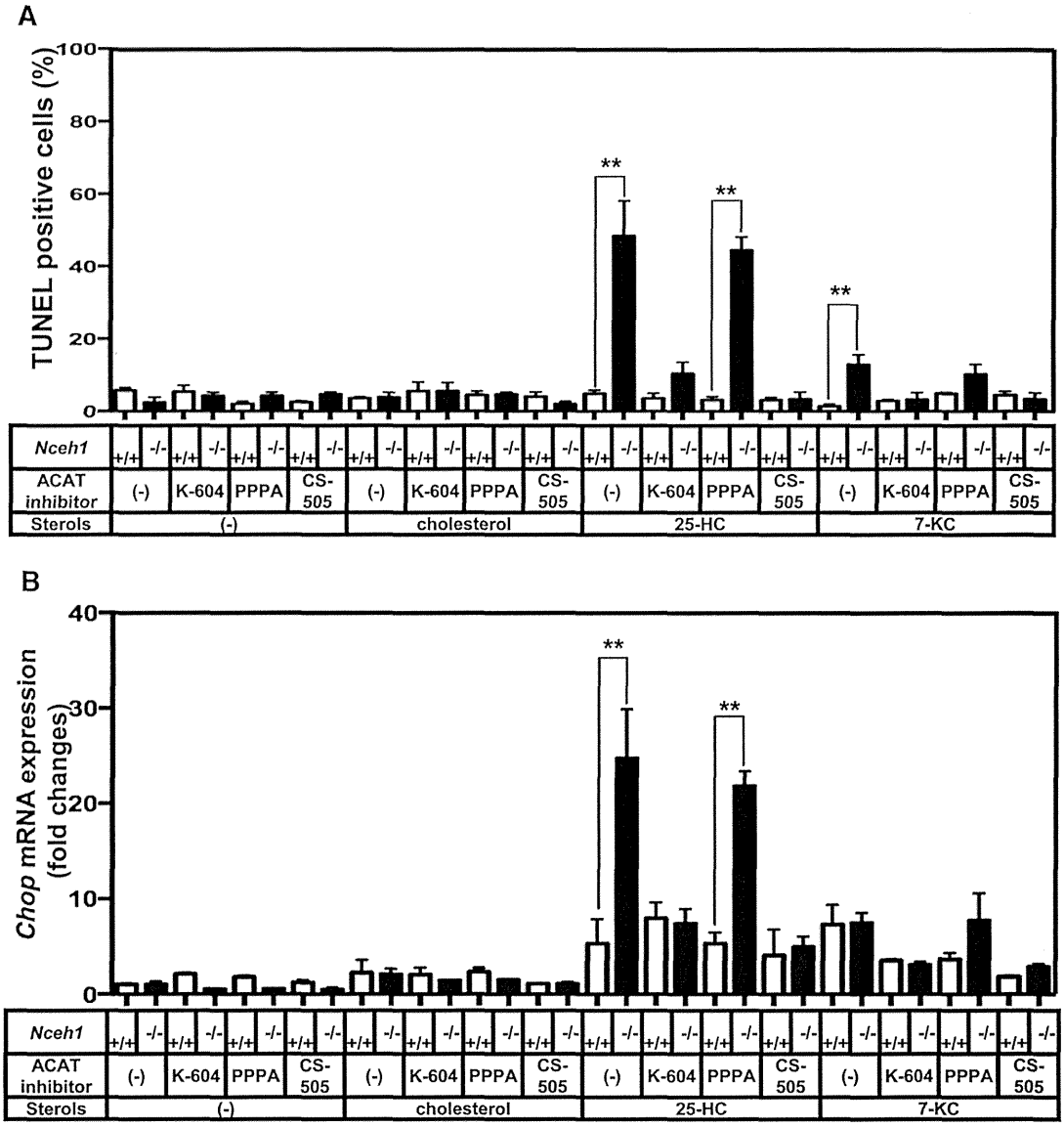

Fig. 2. Inhibition of ACAT1 suppresses the augmentation of 25-HC-induced apoptosis in *Nceh1*-deficient TGEMs. Four wells of TGEMs were incubated in DMEM containing 10% LPDS with vehicle or 25-HC (10 μg/ml) in the presence or absence of the ACAT inhibitors: K-604, PPPA, and CS-505 (10 μM) for 24 h. A: The apoptotic cells were detected by TUNEL. B: Expression of *Chop* was measured by RT-PCR. Data are expressed as the mean ± SEM. $^{**}P < 0.01$; NS, a nonsignificant difference.

Statistics

Statistical differences between groups were analyzed by one-way ANOVA and the post hoc Tukey-Kramer test or two-tailed Student's *t*-test, unless otherwise stated.

RESULTS

Nceh1 deficiency increases the susceptibility of macrophages to apoptosis

While attempting to examine the intracellular structures of the *Nceh1*$^{-/-}$ TGEMs using TEM, we noticed a small number of apoptotic cells featuring condensed nuclei in *Nceh1*$^{-/-}$ TGEMs (**Fig. 1A**). Interestingly, while cellular cholesterol loading with acLDL did not alter the frequency of apoptotic nuclei (Fig. 1B), treatment of Nceh1-deficient TGEMs with 25-HC (Fig. 1C, D; **Fig. 2A**), 7-KC (Fig. 2A), and 27-HC (data not shown) augmented apoptosis. The Nceh1-dependent augmentation of apoptosis was not observed for other compounds including lipopolysaccharide, tunicamycin, staurosporin, 5α6α-epoxycholesterol, 5β6β-epoxycholesterol, 7β-hydroxycholesterol, and 24-hydroxycholesterol (data not shown). The effects were far more pronounced for 25-HC than for 7-HC and 27-HC, and detectable even at a concentration of 0.1 μg/ml (0.26 μM) (Fig. 1D), which is close to a physiological concentration of oxysterols (0.01–0.1 μM in plasma) (34).

The augmentation of the 25-HC-induced apoptosis, which was clearly observed in *Nceh1*$^{-/-}$ TGEMs, was not detectable in nonelicited macrophages; treatment with 25-HC significantly increased the number of apoptotic cells as well as the expression of *Chop*, even in nonelicited WT

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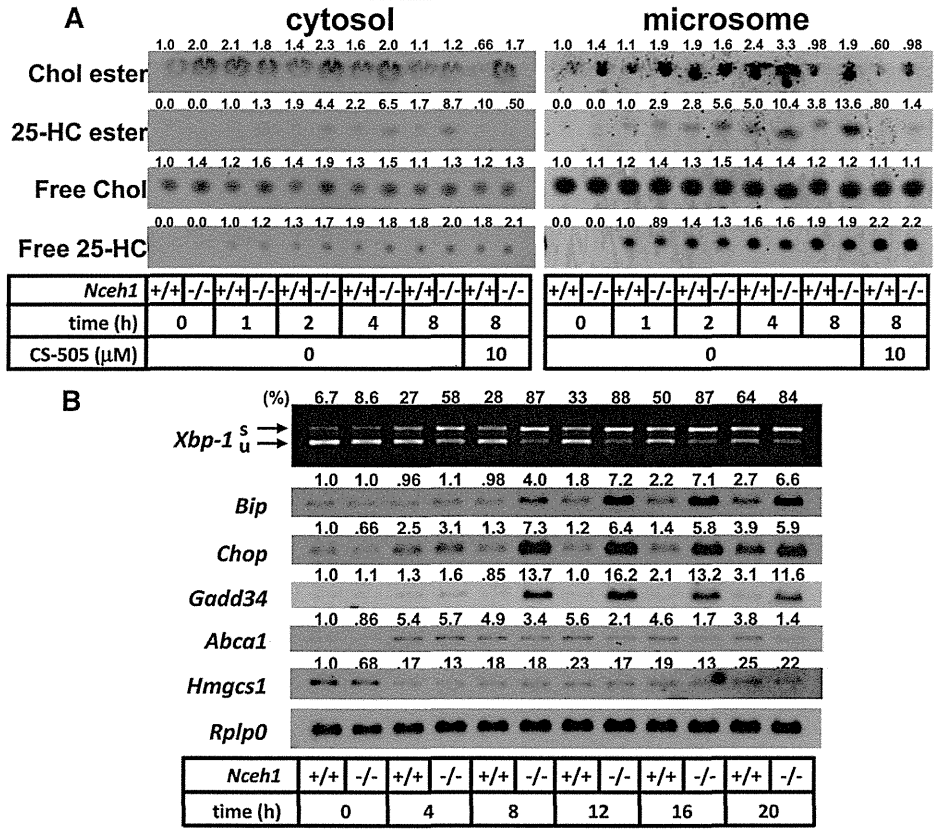

Fig. 3. Involvement of activation of ER stress signaling is preceded by accumulation of 25-HC ester. A: TGEMs were incubated with 25-HC (10 μg/ml) for the indicated length of time in the presence or absence of CS-505 (10 μM). After cell fractionation, the lipids extracted from the cytosolic fraction (100 μg of protein) and microsomal fraction (50 μg of protein) were separated by TLC. Chol, cholesterol. B: Expression profile of ER stress markers. Cells were incubated with 25-HC (10 μg/ml) in the presence or absence of CS-505 (10 μM) for 12 h. s, spliced; u, unspliced. Values above each band/spot indicate the fold difference evaluated by densitometry. Xbp-1 splicing was assessed as the percent spliced Xbp-1 [spliced Xbp-1/(spliced Xbp-1 plus unspliced Xbp-1)].

macrophages. Although the nonelicited *Nceh1*$^{-/-}$ cells showed slightly higher expression of *Chop* than the nonelicited WT cells (supplementary Fig. IA), there was no significant difference in the number of apoptotic cells between the two types of cells after treatment with 25-HC (supplementary Fig. IB). These results indicate that nonelicited macrophages are more susceptible to 25-HC-induced ER stress and apoptosis than elicited TGEMs. The susceptibility may be conferred by both the lower expression of *Nceh1* and higher expression of *Acat1* (supplementary Fig. IC, D).

ACAT inhibitors suppress Nceh1-dependent macrophage apoptosis

Membrane-bound enzyme ACAT, which is responsible for the intracellular esterification of cholesterol, is known to be strongly activated by oxysterols (35). Inasmuch as we have reported that Nceh1 catalyzes the intracellular hydrolysis of CE (2), we speculated that Nceh1 also catalyzes the intracellular hydrolysis of esterified oxysterols and that cycles of esterification-hydrolysis could play a pivotal role in the underlying molecular processes. Indeed, Nceh1 hydrolyzed 25-HC oleate in vitro (supplementary Fig. II). K_m of Nceh1 for 25-HC oleate was comparable to that for cholesteryl oleate: 6.4 ± 0.9 μM versus 6.9 ± 2.3 μM. As expected, nonselective ACAT inhibitor, CS-505, significantly inhibited the Nceh1-dependent augmentation of enhanced 25-HC-induced apoptosis (Fig. 2A). There are two ACAT isozymes: ACAT1 and ACAT2. To determine which isozyme mediated the 25-HC-induced apoptosis, we further compared the effects of ACAT1-specific inhibitor, K-604, and ACAT2-specific inhibitor, PPPA. As expected, the augmentation of the 25-HC-induced apoptosis was specifically inhibited by K-604, but not by PPPA, corroborating the fact that ACAT1 is the major isozyme of TGEMs (36). A similar phenomenon was also observed for 7-KC.

Microsomal accumulation of 25-HC ester precedes activation of ER stress signaling in *Nceh1*-deficient macrophages

Because inhibition of ACAT successfully reduced the augmentation of the 25-HC-induced apoptosis in *Nceh1*$^{-/-}$ TGEMs, we initially focused on the intracellular esterified oxysterol content to explore the underlying mechanism. Intracellular free 25-HC was already detectable after 1 h of incubation, and thereafter the free 25-HC content appeared

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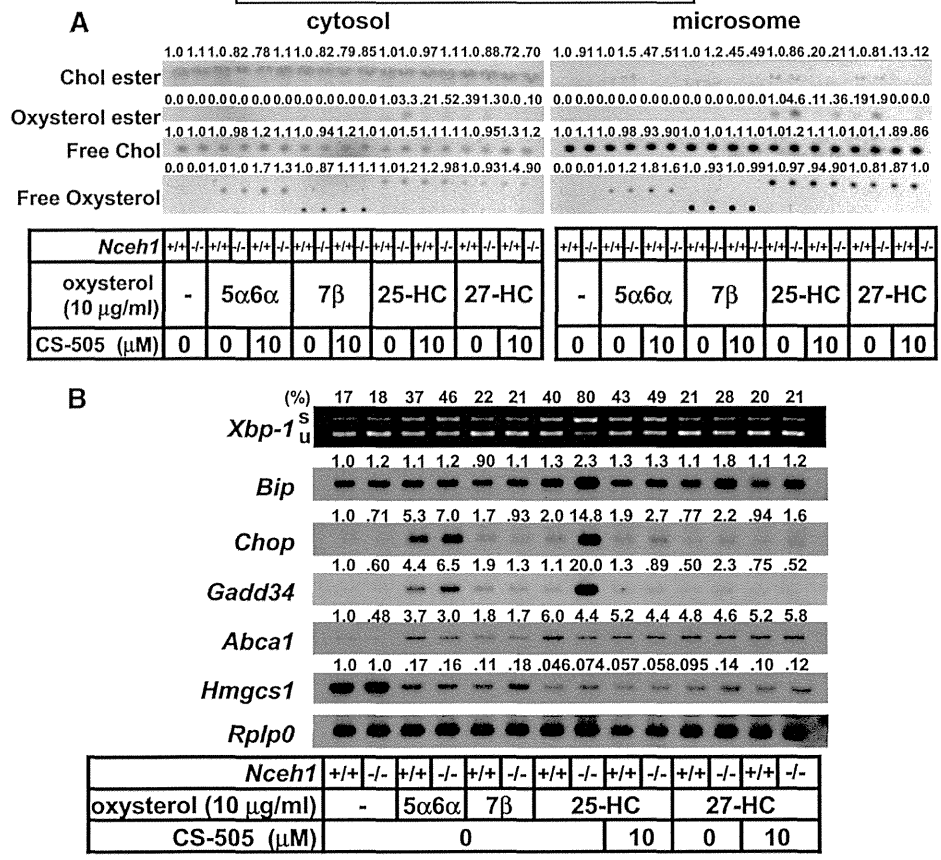

Fig. 4. 25-HC is a highly specific trigger for accumulation of esterified oxysterol and activation of ER stress in *Nceh1*-deficient TGEMs. A, B: TGEMs were incubated with various kinds of oxysterols (10 μg/ml) in the presence or absence of CS-505 (10 μM) for 12 h. Lipids were separated by thin layer chromatography (A), and the expression profile was examined (B). s, spliced; u, unspliced; Chol, cholesterol. Oxysterols are abbreviated as follows: 5α6α, 5α6α-epoxycholesterol; 7β, 7β-hydroxycholesterol. Values above each band/spot indicate the fold difference evaluated by densitometry. Xbp-1 splicing was assessed as the percent spliced Xbp-1 [spliced Xbp-1/(spliced XBbp-1 plus unspliced Xbp-1)].

to remain relatively constant (**Fig. 3A**). A small amount of 25-HC ester was detected after 1 h of incubation mainly in the microsomal fraction of *Nceh1*$^{-/-}$ TGEMs, and thereafter the intracellular 25-HC ester content increased gradually in *Nceh1*$^{-/-}$ TGEMs. 25-HC ester was not detectable in cells coincubated with CS-505.

The isolated microsomal fraction contains the ER, where Nceh1 resides, and changes in ER lipid composition are known to initiate ER stress (13, 37). The prolonged activation of ER stress signaling is also known to lead to apoptotic cell death (38). Therefore, we examined whether ER stress mediates the 25-HC-induced apoptosis in *Nceh1*$^{-/-}$ TGEMs. Indeed, the accumulation of 25-HC ester preceded increased expression of ER stress markers such as Chop, Bip, and the spliced form of X-box-binding protein 1 (Xbp-1) (Fig. 3B). Oxysterols inhibit the processing of sterol regulatory element binding protein (Srebp)-2 by binding to an anchor protein called insulin induced genes (Insigs), and block cholesterol biosynthesis (39). Indeed, oxysterols decreased the gene expression of an enzyme involved in cholesterol biosynthesis, *Hmgcs1*, though the expression was not affected by the *Nceh1* genotype. The induction of the *Chop* expression was inhibited by K-604 and CS-505, but not by PPPA (Fig. 2B). These results further indicate that ACAT1 mediates the augmentation of the 25-HC-induced ER stress. A similar phenomenon was observed for 7-KC.

The augmentation of ER stress signaling in Nceh1 deficiency was observed specifically for 25-HC, because there was no such robust induction of ER stress for other oxysterols (**Fig. 4**).

We also investigated whether the cholesterol biosynthetic pathway is involved in the apoptotic process using the HMG-CoA reductase inhibitor, simvastatin. Simvastatin treatment increased macrophage apoptosis as reported previously (40), but this was not influenced by the presence or absence of Nceh1 (Fig. 5). Furthermore, we investigated the potential contribution of a nuclear oxysterol receptor, liver X receptor (LXR), which regulates cholesterol homeostasis (41). Although a representative target of LXR, *Abca1*, was upregulated by oxysterols, the levels did not correlate with the frequency of apoptosis (Fig. 3B, Fig. 4B).

To test whether the pathways proposed by Sinensky et al. for oxysterol-induced apoptosis are involved in the Nceh1-dependent augmentation of 25-HC-induced apoptosis

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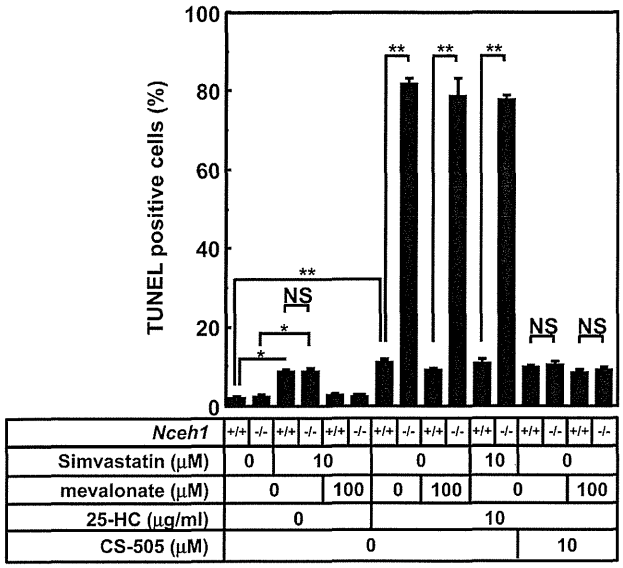

Fig. 5. The evaluation of the contribution of cholesterol biosynthetic pathway. Four wells of TGEMs were incubated in DMEM containing 10% LPDS with compound(s) indicated below the graph for 24 h, and the apoptotic cells were detected by TUNEL method. Data are expressed as the mean ± SEM. $*P < 0.05$ and $**P < 0.01$, as determined by ANOVA followed by the Tukey-Kramer post hoc test. NS, nonsignificant difference.

(15–17), we examined the role of Ca^{2+} in the medium in this phenomenon. Elimination of Ca^{2+} from the medium significantly suppressed the 25-HC-mediated apoptosis in WT macrophages, but it did not affect Nceh1-dependent augmentation of 25-HC-induced apoptosis (supplementary Fig. IIIA). Next, we examined the effects of 25-HC on Akt degradation. In WT TGEMs, 25-HC decreased the expression of Akt as expected, although we failed to demonstrate that MG-132, a proteasome inhibitor, protects against the 25-HC-dependent degradation of Akt protein. Deletion of *Nceh1* did not further decrease the expression of Akt (supplementary Fig. IIIB). Therefore, it is unlikely that either increased influx of Ca^{2+} or increased degradation of Akt is responsible for the Nceh1-dependent augmentation of 25-HC-induced ER stress and subsequent apoptosis.

Lipe is not involved in 25-HC-induced augmentation of macrophage apoptosis

We have previously demonstrated that Lipe also plays a crucial role in the hydrolysis of cholesterol ester in macrophages, although the potential impact of Lipe on foam cell formation and atherogenesis is less than that of Nceh1 (9). However, the deficiency of Lipe did not affect either the extent of 25-HC-induced apoptosis or the expression of ER stress response markers (**Fig. 6A, C**). Consistent with the findings, 25-HC ester did not accumulate in $Lipe^{-/-}$ TGEMs (Fig. 6B).

Finally, we used LC-MS/MS to measure 25-HC and its esterified form more quantitatively in the TGEMs treated with 25-HC (**Fig. 7**). 25-HC ester contents were profoundly increased in both microsomal (Fig. 7A) and cytosolic (Fig. 7B) fractions of $Nceh1^{-/-}$ and $Nceh1^{-/-}$;$Lipe^{-/-}$ TGEMs. Free 25-HC contents were also significantly increased only in the cytosolic fraction of $Nceh1^{-/-}$ and $Nceh1^{-/-}$;$Lipe^{-/-}$ TGEMs (Fig. 7D). There were no significant differences in the free 25-HC contents of microsomal fractions in the cells treated with exogenous 25-HC (Fig. 7C).

DISCUSSION

In the present study, we show that deletion of *Nceh1* makes TGEMs susceptible to 25-HC-induced apoptosis. Because the increased oxysterol-induced apoptosis was nearly reversed by inhibition of ACAT1, we would ascribe this phenomenon to the defective hydrolysis of esterified oxysterols. The mRNA expression of the target molecules of ER stress signals correlated with the apoptosis-inducing capacity of oxysterols and preceded the occurrence of apoptosis. Moreover, the augmented ER stress was associated with the accumulation of 25-HC ester in the ER, both of which were suppressed by inhibition of ACAT1. These results indicate that the oxysterol-induced apoptosis in *Nceh1*-deficient TGEMs is mediated by ER stress provoked by the accumulation of esterified oxysterols in the ER.

Oxysterols are present in atherosclerotic plaques and may play diverse roles in plaque development (42). Oxysterols profoundly affect cholesterol homeostasis. For example, 25-HC potently inhibits cholesterol biosynthesis and is cytotoxic (43, 44). This property has been utilized to isolate mutant cells which are defective in the molecular pathway mediating the sterol-mediated feedback inhibition of cholesterol biosynthesis (45). Subsequent works have identified molecular defects of these mutant CHO cells, not only in SREBP-2 (46, 47) and SREBP cleavage-activating protein (48), but also in ACAT (49, 50). In addition to defective feedback repression of cholesterol biosynthesis, absence of ACAT may be advantageous to antagonize certain toxic effects of 25-HC through a pathway distinct from 25-HC-mediated suppression of cholesterol biosynthesis. Indeed, Freeman et al. (18) showed that ACAT mediates oxysterol-induced apoptosis in macrophages, which is largely consistent with our findings that the Nceh1-dependent augmentation of 25-HC-induced ER stress and apoptosis were abrogated by ACAT1 inhibition (Fig. 2).

Given the pro-apoptotic role of ACAT discussed above, it is quite logical that the deficiency in NCEH1, an enzyme mediating a counteraction against ACAT, plays an anti-apoptotic role as we demonstrate herein. It is noteworthy that 25-HC was extremely potent in inducing apoptosis in $Nceh1^{-/-}$ TGEMs (Fig. 1D). The increases in the amounts of esterified forms of these oxysterols in the ER appear to be proportional to the apoptotic death (Fig. 3). As 25-HC stimulated ACAT activity in fibroblasts (43) as well as in a cell-free system (51), it is plausible that 25-HC ester is most easily accumulated in cells that lack hydrolyzing activity toward 25-HC ester. Indeed, Nceh1 hydrolyzes 25-HC ester in vitro (supplementary Fig. II). In this context, it is reasonable that the catalytic domain of Nceh1 resides in the

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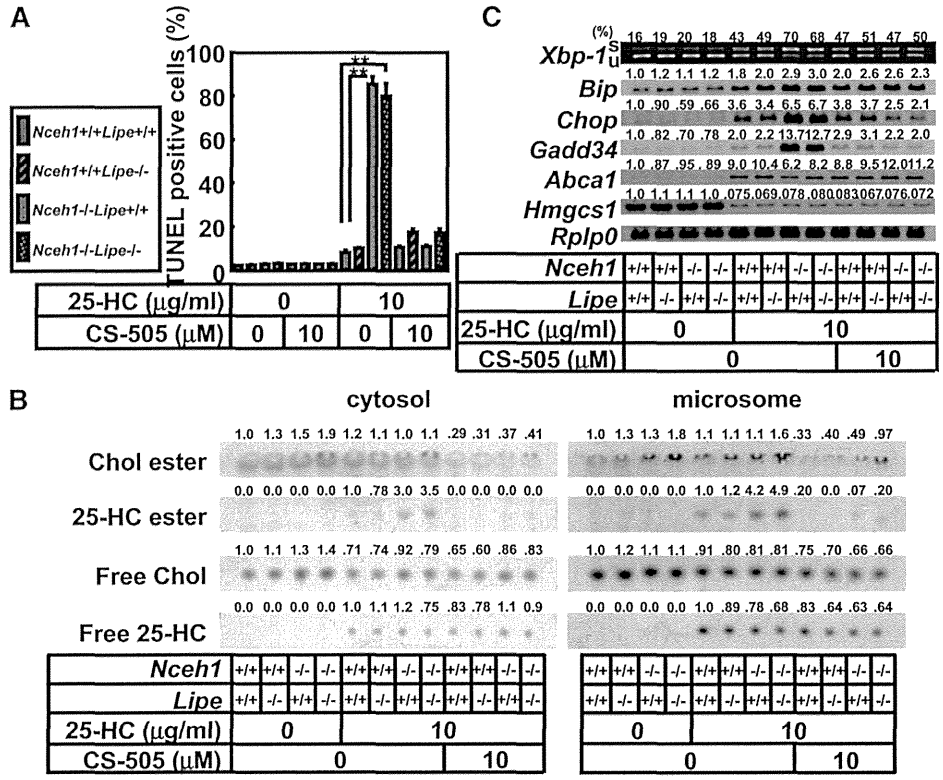

Fig. 6. Lipe is not involved in *Nceh1*-dependent augmentation of 25-HC-induced apoptosis. TGEMs of each genotype (the symbol corresponding to each genotype is given in the inset, four dishes) were incubated with 25-HC (10 μg/ml) in the presence or absence of CS-505 (10 μM) for 24 h, and the apoptotic cells were detected by the TUNEL method. Data are expressed as the mean ± SEM. $^{**}P < 0.01$. B: Lipid separation by TLC. C: Expression profile of ER stress markers. Cells were incubated with 25-HC (10 μg/ml) in the presence or absence of CS-505 (10 μM) for 12 h. s, spliced; u, unspliced; Chol, cholesterol. Values above each band/spot indicate the fold difference evaluated by densitometry. Xbp-1 splicing was assessed as the percent spliced Xbp-1[spliced Xbp-1/(spliced Xbp-1 plus unspliced Xbp-1)].

ER lumen (7), where the majority of 25-HC ester accumulates (52). The increased amounts of oxysterol esters in the ER may trigger apoptotic signals. In addition to efficient esterification of 25-HC as mentioned above, other chemical properties of 25-HC which are not shared with other oxysterols might account for the susceptibility to this specific oxysterol. The esterified form of 25-HC might be the most potent inducer of ER stress. Probably, hydroxyl moiety at C25 is important for localization in the ER whereby triggering ER stress.

25-HC ester also significantly accumulated in the cytosolic fraction of TGEMs lacking *Nceh1* (Fig. 7B). The newly synthesized 25-HC ester in the microsome might be readily transported to and stored in lipid droplets (LDs). Interestingly, *Lipe* deficiency did not further increase 25-HC ester in the cytosol. Given that 25-HC ester can be hydrolyzed by Lipe in vitro (supplementary Fig. II), LDs containing an excess of 25-HC ester might have an inhibitory effect on the efficient hydrolysis of 25-HC ester by Lipe. In the cytosol, free 25-HC was also increased in TGEMs lacking *Nceh1* (Fig. 7D). We assume that enlarged LDs have large surface area that can accommodate a large amount of free 25-HC.

It is plausible that ER stress mediates apoptotic signaling in the 25-HC-treated *Nceh1*$^{-/-}$ TGEMs. The mRNA levels of Xbp-1(s), Bip, Chop, and Gadd34 were all significantly elevated in Nceh1-deficient TGEMs exposed to 25-HC (Fig. 3). Xbp-1(s) is a downstream target of inositol-requiring protein-1 (IRE1), and CHOP and GADD34 are downstream targets of protein kinase RNA-like ER kinase (PERK) signaling (37). BIP is an ER chaperone that prevents activating transcription factor-6 (ATF6) from moving to the Golgi-apparatus. Therefore, we presume that activation of both the IRE1 and PERK arms is necessary for the induction of apoptosis. In this context, Shibata et al. (53) have recently reported that 25-HC triggers integrated stress response via activation of GCN2/eIF2/ATF4 in macrophages. It would be intriguing to know whether the same pathway is activated in the Nceh1-dependent augmentation of 25-HC-induced ER stress, although it is beyond the scope of this study.

In our experiments, there was no discernible difference in the expression levels of genes regulating cholesterol homeostasis such as *Abca1* and *Hmgcs1* between the cells with and without *Nceh1*, even though apoptosis is known to be induced either by inhibition of cholesterol biosynthesis (54) or by suppression of cholesterol efflux (55). Furthermore, the Nceh1-dependent augmentation of 25-HC-induced apoptosis does not appear to involve increased Ca^{2+} influx or increased degradation of Akt (supplementary Fig. III).

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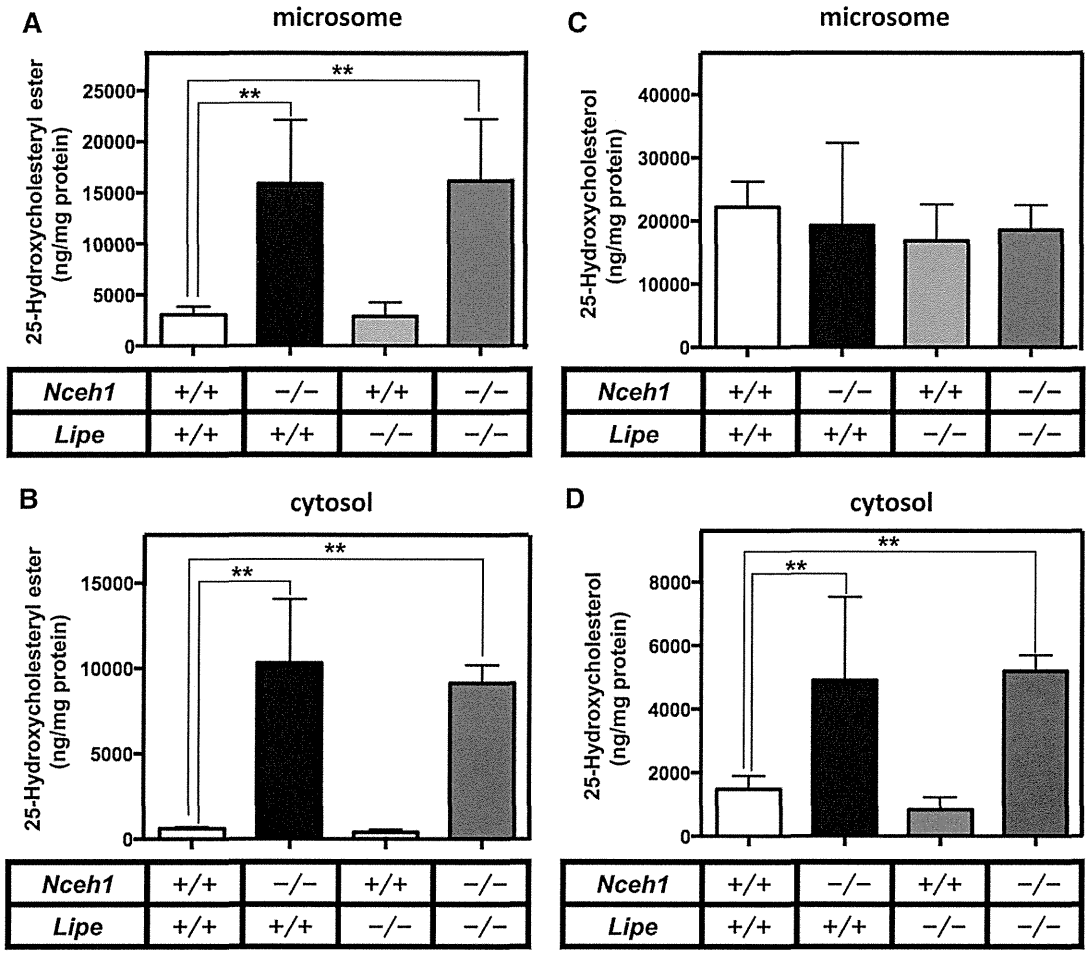

Fig. 7. Quantification of free and esterified 25-HC in subcellular fractions. TGEMs plated at 5×10^6 cells per 6 cm dish in 5–6 dishes were incubated with 10 μg/ml of 25-HC for 12 h. After cell fractionation, the lipids extracted from the cytosolic (B, D) and microsomal fractions (A, C) were subjected to LC-MS/MS to measure free (C, D) and esterified forms (A, B) of 25-HC. Data are expressed as the mean ± SEM. $^{**}P < 0.01$; NS, nonsignificant difference.

As mentioned above, 25-HC is a potent stimulator of ACAT activity (35) and markedly suppresses the SREBP-mediated transactivation of its target genes by binding to Insigs (39). 25-HC also binds other proteins including LXR, cholesterol transporters, and Niemann-Pick C1 (56). In addition to these robust biological effects of 25-HC on cholesterol metabolism, accumulating evidence has suggested that stimulation of Toll-like receptor signaling elicits its endogenous production of 25-HC in macrophages, thereby regulating immune and antiviral functions (57–59). Therefore, it is plausible that hydrolysis of 25-HC ester may be involved in the regulation of the immune system by affecting these pathways.

Surprisingly, nonelicited peritoneal macrophages were highly sensitive to 25-HC even in the presence of *Nceh1* (supplementary Fig. I). Conceivably, both the lower expression of *Nceh1* and the higher expression of *Acat1* render nonelicited macrophages susceptible to 25-HC-induced ER stress and apoptosis. In other words, the inflammatory state provoked by thioglycollate changes the property of macrophages making them more resistant to 25-HC as long as Nceh1 is present. Nceh1 appears to protect inflammatory macrophages from oxysterol-induced apoptosis, thereby perpetuating inflammation into chronic phase, as is observed in atherosclerosis.

In conclusion, Nceh1 plays a protective role in the oxysterol-induced apoptosis of macrophages. The accumulation of oxysterol esters in ER activates ER stress signaling. These findings should provide the basis for understanding the pathophysiology of the development of atherosclerosis and other diseases involving macrophage apoptosis.

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