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.

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

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/3lABkFQ/-3′; *Lipe* forward, 5′-CATATCCGCTCTCCAGTTGACC-3′; *Lipe* reverse, 5′-CCTATCTTCTCCATCGACTACTCC-3′; *Lipe* probe, 5′-/56-FAM/CGAGGCTCC/ZEN/CTTTCCCCGAG/3lABkFQ/-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

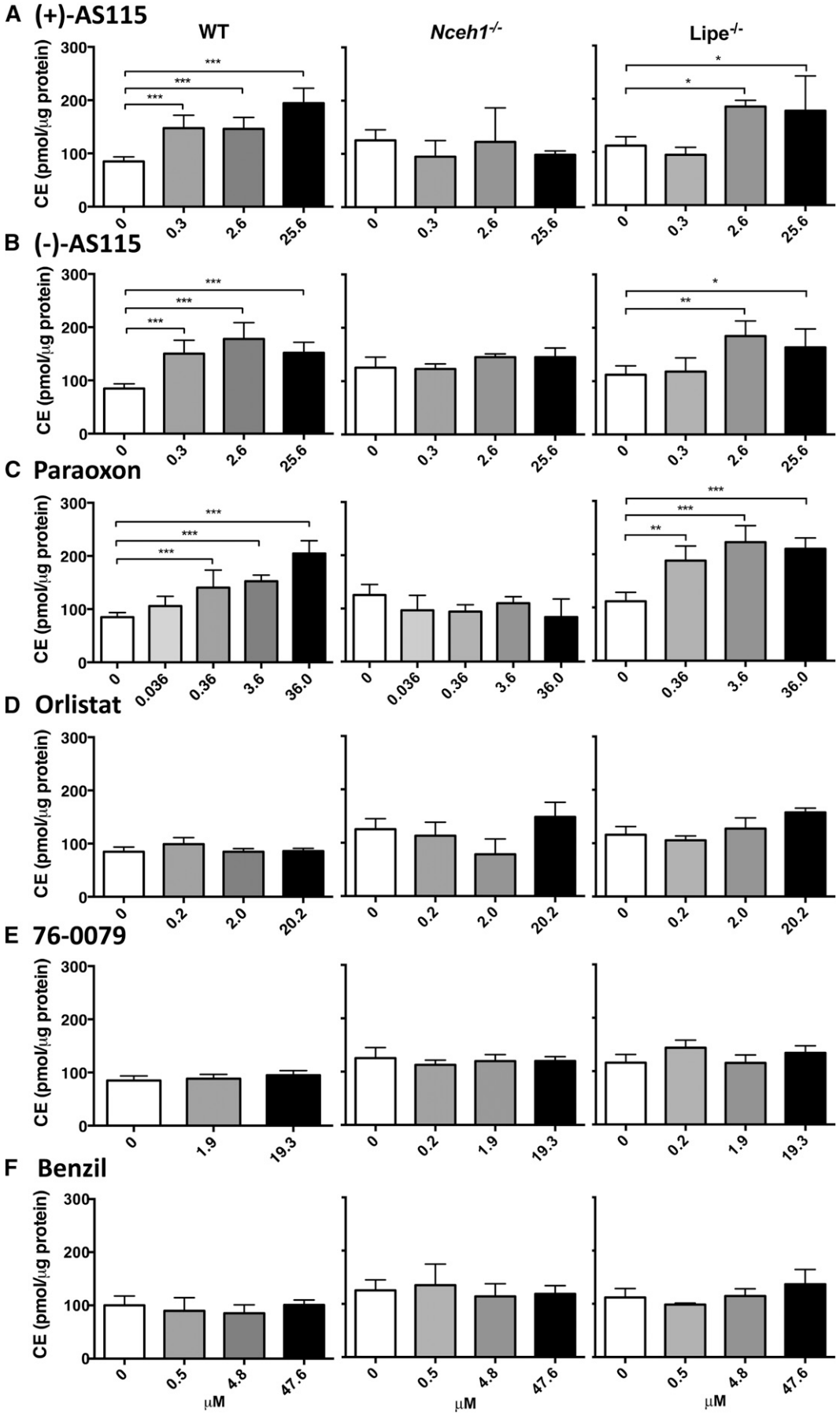
To examine whether inhibition of the hydrolysis of CE by each compound affected cholesterol trafficking, we labeled MPMs with oleic acid and measured the amounts of cholesteryl oleate after exposure to each compound. Treatment with (+)-AS115 or (−)-AS115, which are nonselective inhibitors, and with paraoxon, 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).


A (+)-AS115
WT
Nceh1-/-
Lipe-/-
CE (pmol/μg protein)
300
200
100
0
***
***
***
*
*
0
0.3
2.6
25.6
B (-)-AS115
**
C Paraoxon
0.036
0.36
3.6
36.0
D Orlistat
0.2
2.0
20.2
E 76-0079
1.9
19.3
F Benzil
0.5
4.8
47.6
μM

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).

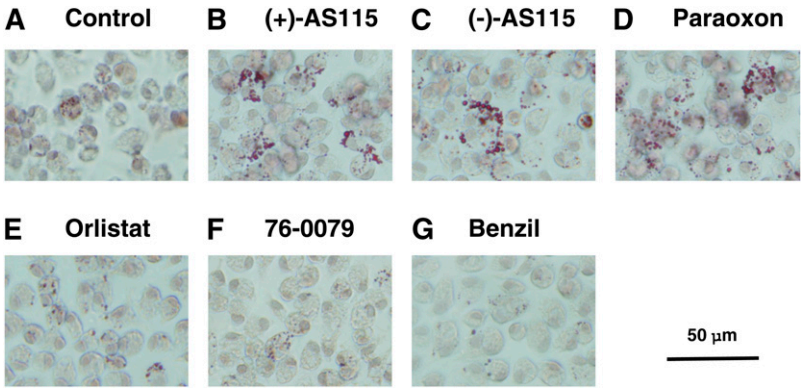


Fig. 2. Effect of inhibitors against Nceh1 and/or Lipe on neutral lipid droplet accumulation in MPMs from WT mice. Intracellular neutral lipids were stained with Oil Red O. A: Control (methanol). B: (+)-AS115, 25.6 μM. C: (−)-AS115, 25.6 μM. D: Paraoxon, 36.0 μM. E: Orlistat, 20.2 μM. F: 76-0079, 19.3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

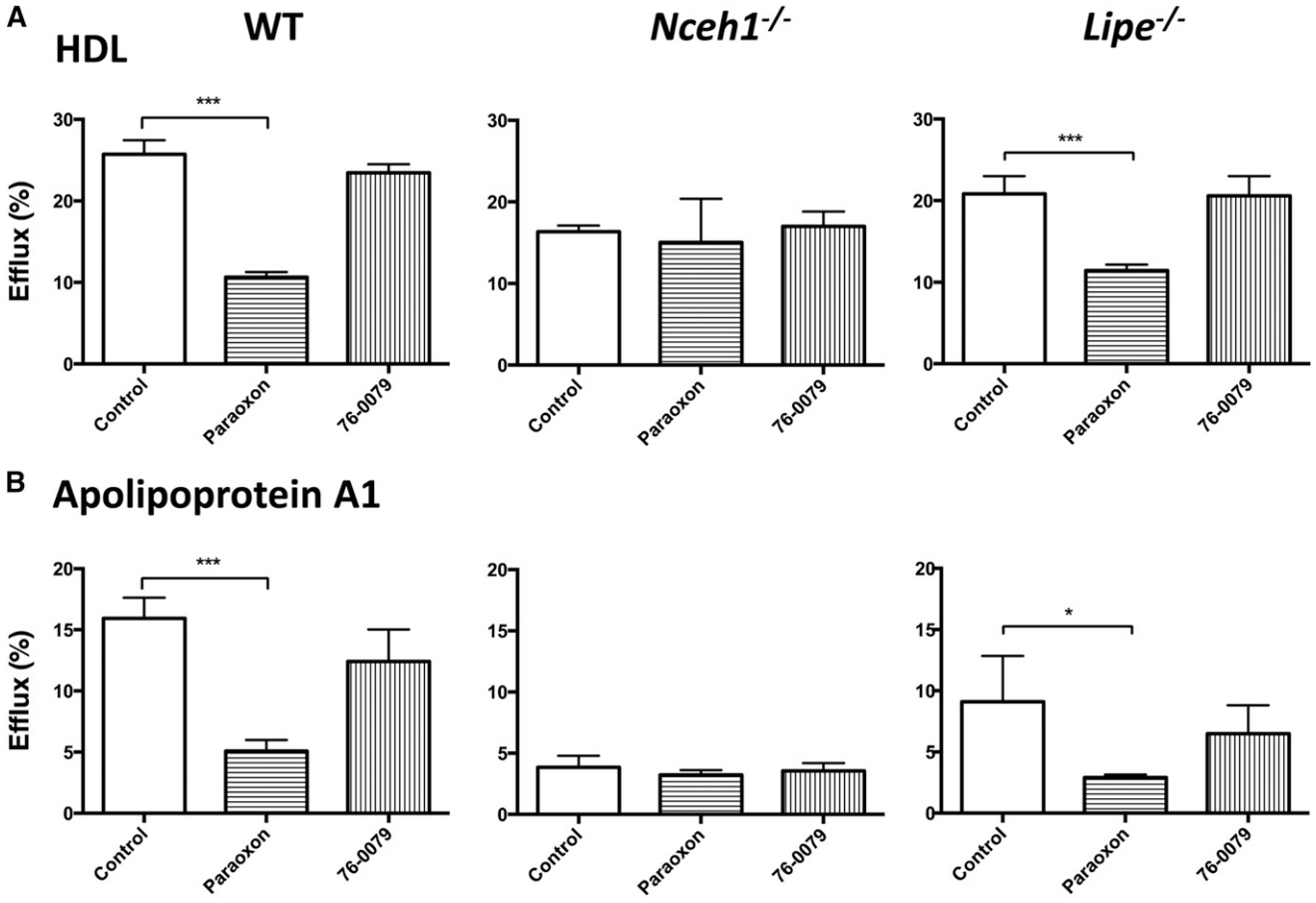


Fig. 3. Effect of inhibitors against Nceh1 or Lipe on cholesterol efflux in MPMs from WT, *Nceh1*$^{-/-}$, or *Lipe*$^{-/-}$ mice. MPMs were loaded with [1,2,6,7-^{3}H(N)]cholesteryl oleate by incubating the cells with acLDL. After 24 h, cholesterol efflux was initiated by the addition of HDL or apoA-1 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).

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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