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Article

Ablation of Neutral Cholesterol Ester Hydrolase 1 Accelerates Atherosclerosis

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SUMMARY

Cholesterol ester (CE)-laden macrophage foam cells are the hallmark of atherosclerosis, and the hydrolysis of intracellular CE is one of the key steps in foam cell formation. Although hormone-sensitive lipase (LIPE) and cholesterol ester hydrolase (CEH), which is identical to carboxylsterase 1 (CES1, hCE1), were proposed to mediate the neutral CE hydrolase (nCEH) activity in macrophages, recent evidences have suggested the involvement of other enzymes. We have recently reported the identification of a candidate, neutral cholesterol ester hydrolase 1 (Nceh1). Here we demonstrate that genetic ablation of Nceh1 promotes foam cell formation and the development of atherosclerosis in mice. We further demonstrate that Nceh1 and Lipe mediate a comparable degree of nCEH activity in macrophages and together account for most of the activity. Mice lacking both Nceh1 and Lipe aggravated atherosclerosis in an additive manner. Thus, Nceh1 is a promising target for the treatment of atherosclerosis.

INTRODUCTION

Atherosclerotic cardiovascular diseases are the leading causes 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. Lipid-rich plaques, which are characterized by a plethora of cholesterol ester (CE)-laden macrophage foam cells, are prone to rupture (Libby, 2002). The hydrolysis of intracellular CE is the initial step of reverse cholesterol transport (RCT) (Brown et al., 1979). As the hydrolysis of CE preceding RCT takes place at neutral pH, the enzymes catalyzing it have been collectively called neutral CE hydrolases (nCEHs) to distinguish them from lysosomal acid lipase, the CE hydrolase in lysosomes whose optimal pH is acidic. Since this step is rate-limiting particularly in macrophage foam cells (Ishii et al., 1992; Kritharides et al., 1998), it is important to clarify the mechanisms that mediate the hydrolysis of CE in foam cells.

To date, three enzymes have been proposed to serve as nCEHs in macrophages: hormone-sensitive lipase (LIPE) (Yeaman, 2004); cholesteryl ester hydrolase (CEH)(Ghosh, 2000), which is identical to human liver carboxylesterase1 (CES1, hCE-1) (Long et al., 1991) or macrophage serine esterase1 (HMSE1) (Munger et al., 1991), also known as a human ortholog of triacylglycerol hydrolase (TGH) (Lehner and Vance, 1999); and neutral cholesterol ester hydrolase 1(Nceh1, MGI ID 2443191) (Okazaki et al., 2008), which is also known as KIAA1363 or arylacetamide deacetylase-like 1 (Aadacl1) (Nomura et al., 2005). Lipe is expressed in macrophages, and its overexpression inhibits the accumulation of CE in THP-1 macrophages (Escary et al., 1998; Okazaki et al., 2002). However, peritoneal macrophages (MPMs) of Lipe-deficient (*Lipe*$^{-/-}$) mice in a mixed genetic background still retain substantial nCEH activity (Osuga et al., 2000), indicating the presence of additional nCEH(s). Ghosh reported CES1 as a promising candidate for an nCEH, because its macrophage-specific overexpression driven by the promoter of macrophage scavenger receptor-1 protected against diet-induced atherosclerosis in low-density-lipoprotein receptor (Ldlr)-deficient (*Ldlr*$^{-/-}$) mice (Zhao et al., 2007). However, the effects of loss of function of CES1 on nCEH activity in macrophages have not been reported. Furthermore, a mouse ortholog of CES1, TGH, was expressed at a low level in MPMs (Okazaki et al., 2008) and possessed negligible nCEH activity (Okazaki et al., 2006).

In contrast, Nceh1 is robustly expressed in MPMs as well as in atherosclerotic lesions. Its overexpression inhibits the accumulation of CE in THP-1 macrophages and the knockdown of Nceh1 by short interference RNA significantly reduced nCEH activity in MPMs (Okazaki et al., 2008). Therefore, Nceh1 is more likely to be involved in the hydrolysis of CE in MPMs. In the present study, we took advantage of gene targeting to underscore the importance of Nceh1 as well as Lipe in the atherogenesis.

RESULTS

Nceh1 Mediates nCEH Activity in Macrophages

The targeting strategy was designed so that exon 4, which encodes the catalytic domain, of the endogenous *Nceh1* gene

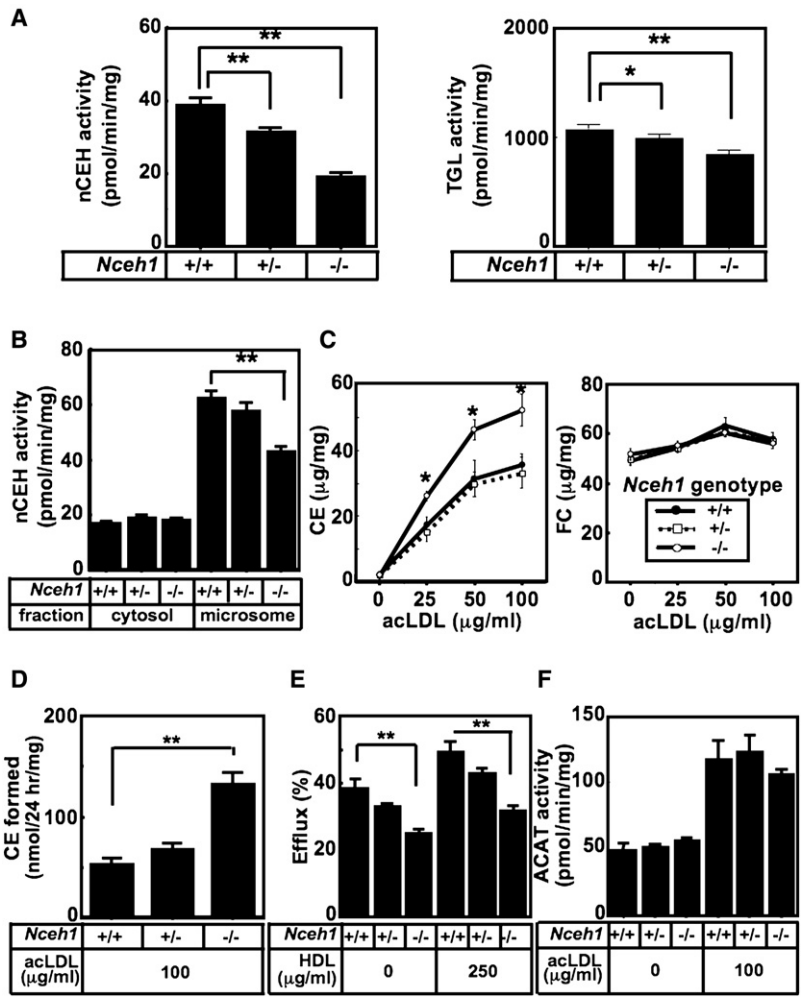

Figure 1. nCEH Activity and Cholesterol Trafficking in *Nceh1*$^{-/-}$ Macrophages

(A) Peritoneal macrophages (n = 7–8) were incubated with DMEM containing 10% FCS. Neutral cholesterol ester hydrolase (nCEH) activity or triglyceride lipase (TGL) activity of whole-cell lysates were measured.

(B) The nCEH activity after subcellular fractionation (n = 8).

(C)Intracellular levels of cholesterol ester (CE) or free cholesterol (FC) in macrophages. Peritoneal macrophages (n = 7–8) were incubated with increasing concentrations of acLDL for 24 hr (symbol definitions given in the inset, n = 7–8).

(D) CE formation. Peritoneal macrophages (n = 7) were incubated with acLDL and [1-^{14}C]oleate-albumin complex for 24 hr, and the incorporation of [1-^{14}C]oleate into CE fraction was measured.

(E) Cholesterol efflux. After labeling of cells (n = 5) with radiolabeled acLDL, the medium was changed to DMEM/FCS containing acLDL with or without HDL_3. After 24 hr, the radioactivity of an aliquot of the medium and of the cell lysate was measured. The percent efflux was calculated as (media dpm)/(cell + media dpm) × 100.

(F) Microsomal ACAT activity (n = 8). ACAT activity in microsomes was determined by the rate of incorporation of [1-^{14}C]oleoyl-CoA into the CE fraction. Data are expressed as the mean ± SEM. * and ** denote $p < 0.05$ and $p < 0.01$, respectively, as determined by ANOVA followed by the Tukey-Kramer posthoc test.

could be replaced with a gene encoding neo resistance (Figures S1A and S1B). Analysis of progeny showed that mice heterozygous (+/−) and homozygous (−/−) with respect to the disrupted *Nceh1* allele were born in numbers consistent with Mendelian inheritance of the mutant allele, survived normally, and were fertile. Nceh1-deficient (*Nceh1*$^{-/-}$) mice maintained on normal chow exhibit a normal metabolic phenotype (Table S1).

To elucidate the contribution of Nceh1 to the nCEH activity in macrophages, MPMs were examined in vitro. Successful deletion of Nceh1 was confirmed at both the protein and mRNA levels (Figures S1C and S1D). Nceh1 deficiency reduced nCEH activity by 50%, and triacylglycerol lipase (TGL) activity by 20%, respectively (Figure 1A).

Consistent with our previous report that Nceh1 is a microsomal enzyme (Igarashi et al., 2009), Nceh1 deficiency selectively reduced the microsomal nCEH activity but did not affect the cytosolic nCEH activity (Figure 1B).

Nceh1 Is Involved in Foam Cell Formation

Foam cells were produced in vitro by incubation of MPMs with acetylated LDL (acLDL). The incubation with an increasingly higher concentration of acLDL resulted in an increase in intracellular CE content by 50% in *Nceh1*$^{-/-}$ macrophages compared with wild-type macrophages, whereas the intracellular free cholesterol (FC) content was not affected by genotype (Figure 1C). The formation of CE was also monitored by the incorporation of [^{14}C]oleate into cholesterol oleate, and the intracellular [^{14}C]cholesterol oleate was accumulated in *Nceh1*$^{-/-}$ macrophages approximately 2.5-fold compared with wild-type macrophages (Figure 1D). Cholesterol efflux was reduced by 35% in *Nceh1*$^{-/-}$ macrophages compared with wild-type macrophages in the presence or absence of high-density lipoproteins $(HDL)_3$ (250 μg/ml) as an exogenous lipid acceptor (Figure 1E). Upon exposure to acLDL, excess cholesterol in the endoplasmic reticulum (ER) becomes esterified by acyl-CoA:cholesterol acyltransferase (ACAT) and stored in cytoplasmic lipid droplets (LDs) (Chang et al., 2001). The ACAT activity did not differ between wild-type and *Nceh1*$^{-/-}$ macrophages, and the ACAT activity did not explain the excess accumulation of CEs in *Nceh1*$^{-/-}$ macrophages (Figure 1F).

We also investigated the expression profiles of genes involved in the metabolism of cholesterol and formation of foam cells. Nceh1 deficiency did not alter the expression profile, while acLDL loading altered the expression of several genes such as the *Ldlr*, ATP-binding cassette transporter A1(*Abca1*), 3-hydroxy-3-methyl-glutaryl-CoA synthase (*Hmgcs1*), and class B scavenger receptor (*Scarb1*) (Figure S2A). The expression of Lipe was not affected by Nceh1 deficiency at the mRNA or protein level (Figures S2B–S2D).

To clarify the importance of Nceh1 in multiple receptor pathways, we investigated the effects of two other kinds of modified lipoproteins: beta very low-density lipoproteins (VLDL) and

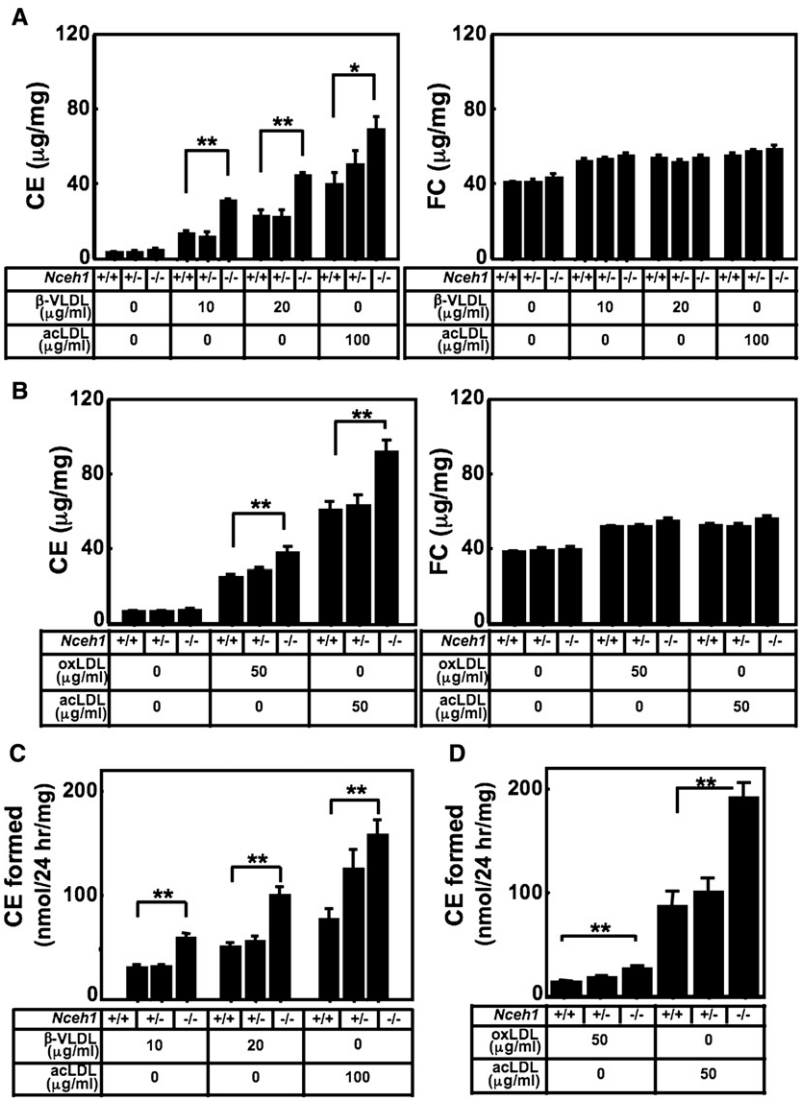

Figure 2. Foam Cell formation Induced by beta-VLDL or Oxidized LDL

(A) Peritoneal macrophages (n = 5–6 for each genotype) were incubated with beta-VLDL (10 or 20 μg/ml) for 24 hr, and intracellular CE content and FC content were determined. As a control, the same cells were also incubated with acLDL (100 μg/ml) simultaneously.

(B) Peritoneal macrophages (n = 7 for each genotype) were incubated with oxLDL (50 μg/ml) or acLDL (50 μg/ml) for 24 hr, and intracellular CE content and FC content were determined.

(C and D) CE formation. Peritoneal macrophages (n = 6–7 for each genotype) were incubated with beta-VLDL (10 or 20 μg/ml, C) or oxidized LDL (oxLDL) (50 μg/ml, D) in the presence of [1-^{14}C]oleate-albumin complex for 24 hr. Intracellular lipids were separated by thin layer chromatograpy (TLC), spots corresponding to the CE fraction were scraped off, and radioactivity was measured. As a control, the same cells were incubated with acLDL. Data are expressed as the mean ± SEM. * and ** denote $p < 0.05$ and $p < 0.01$, respectively, as determined by ANOVA followed by the Tukey-Kramer posthoc test.

oxidized low-density lipoproteins (oxLDL). When *Nceh1*$^{-/-}$ macrophages were exposed to beta-VLDL or oxLDL, the intracellular CE content was increased by 194%–231% and 150%, respectively, compared with wild-type macrophages (Figure 2A for beta-VLDL and Figure 2B for oxLDL). As predicted, [^{14}C]oleate's incorporation into intracellular CE was also increased in *Nceh1*$^{-/-}$ macrophages by 195% and 190%, respectively, upon exposure to beta-VLDL and oxLDL (Figure 2C for beta-VLDL and Figure 2D for oxLDL).

Nceh1 Deficiency Aggravates Atherosclerosis

To evaluate the impact of a deficiency of Nceh1 on the development of atherosclerotic lesions, *Nceh1*$^{-/-}$ mice were backcrossed to the C57BL6/J background for at least five generations and cross-bred with Apoe-deficient (*Apoe*$^{-/-}$) mice. The offspring were fed a western-type diet containing 0.15% (w/w) cholesterol and 20% (w/w) fat starting at 5 weeks of age for 12 or 16 weeks, and the degree of atherosclerosis was determined either by quantifying sudanophilic en face lesions in pinned out aortas (Figures 3A and 3B) or by quantifying Oil Red O-stained lesions in cross-sections from the aortic root (Figures 3C and 3D). The areas of the aortic surface covered by atherosclerotic lesions were increased in *Nceh1*$^{-/-}$;*Apoe*$^{-/-}$ mice compared to *Nceh1*$^{+/+}$;*Apoe*$^{-/-}$ mice (2.2-fold for males and 2.3-fold for females at 17 weeks of age, and 1.7-fold for males and 2.4-fold for females at 21 weeks of age). The results of the en face analysis were confirmed by quantification of Oil Red O-stained aortic root sections (1.8-fold for male and 1.2-fold for female at 17 weeks of age, and 1.8-fold for male and 1.6-fold for female at 21 weeks of age). It is known that the correlation between the morphometric assessment of lesions and the content of FC and CE is high (Veniant et al., 2000); therefore aortic lipids were extracted and measured (Figure 3E). Consistent with the increased lesion area in *Nceh1*$^{-/-}$:*Apoe*$^{-/-}$ mice, FC as well as CE content was significantly higher in *Nceh1*$^{-/-}$;*Apoe*$^{-/-}$ aortas than *Nceh1*$^{+/+}$;*Apoe*$^{-/-}$ aortas (1.5-fold and 1.3-fold increase for male and female FC content, respectively, and 2.0-fold and 1.3-fold increase for male and female CE content, respectively). Next, we determined whether the deficiency of Nceh1 in *Apoe*$^{-/-}$ mice influenced whole-body metabolism. None of the metabolic parameters (plasma total cholesterol and triglycerides concentrations, blood glucose level, and body weight) was affected by the deficiency (Table S2).

Furthermore, it was confirmed that the deficiency of Nceh1 in *Apoe*$^{-/-}$ mice had no effect on plasma lipoprotein profiles evaluated by high performance liquid chromatography (HPLC) (Table S3). Taken together, Nceh1 deficiency accelerated the development of atherosclerosis without affecting whole-body metabolism. Immunohistochemistry using the macrophage marker F4/80 was carried out, and the percentage of macrophage-infiltrated area per unit lesion area was not significantly different between *Nceh1*$^{+/+}$;*Apoe*$^{-/-}$ and *Nceh1*$^{-/-}$;*Apoe*$^{-/-}$ mice (Figures 3F and 3G).

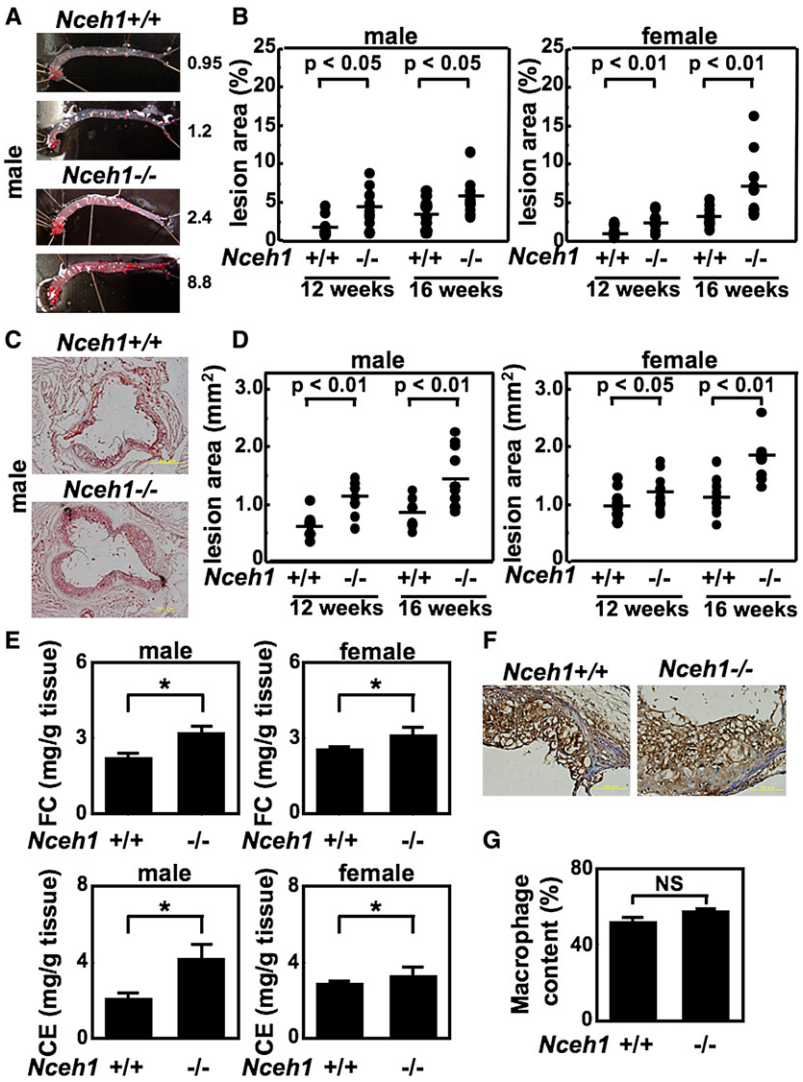

Figure 3. Atherosclerosis in *Nceh1*$^{-/-}$;*Apoe*$^{-/-}$ Mice

(A) Nceh1-deficient mice were crossed with Apoe-deficient mice, and fed a Western-type diet starting at 5 weeks of age for 12 weeks. Representative images of pinned Sudan-IV stained aortas from mice (en face analysis, Nceh1 genotype, and quantification values indicated).

(B) Quantification of the surface area occupied by the lesions (% area occupied by the lesions, n = 10–11). The duration of the dietary load (12 weeks or 16 weeks) and Nceh1 genotype are indicated below the graphs in (B) and (D).

(C) Representative photomicrographs of aortic root cross-sections stained with Oil-Red O. Nceh1 genotype is indicated above the images.

(D) Quantification of the area occupied by lesions using cross-sections of aortic roots (n = 10–15). For analyzing the area occupied by atherosclerotic lesions, both the Mann-Whitney U test and two-tailed Student's t test were used. The P values generated by the two analyses were consistent.

(E) Extraction of aortic wall lipids. After 12 weeks on a western-type diet, mice (n = 11–12 for males and n = 14–16 for females) were euthanized and the aortas were prepared.

(F) Representative photomicrographs of F4/80-stained aortic root cross-sections from male mice fed a western-type diet for 12 weeks.

(G) The quantification of the F4/80-stained area (n = 10 for each genotype). The macrophage lesion area is indicated as a percentage of the total lesion area. Data are expressed as the mean ± SEM. * denotes $p < 0.05$ as determined with the two-tailed Student's t test for (E) and (G) (NS denotes nonsignificant difference).

Both Nceh1 and Lipe Mediate nCEH Activity

To characterize the remaining nCEH activity in *Nceh1*$^{-/-}$ macrophages, we examined whether the expression of Lipe was induced in *Nceh1*$^{-/-}$ macrophages. The expression of Lipe was not altered at either the mRNA or protein level irrespective of the incubation with acLDL (Figures S2A–S2C). We also performed a western blot analysis of individual samples. There was no significant difference in the expression of Lipe protein between wild-type and *Nceh1*$^{-/-}$ macrophages (data not shown). Next, we separated microsomal and cytosolic subcellular fractions by ultracentrifugation. Nceh1 deficiency did not affect the expression of Lipe protein in the cytosolic or microsomal fraction (Figure S2D). To test whether Lipe is responsible for the remaining nCEH activity in *Nceh1*$^{-/-}$ macrophages, *Nceh1*$^{-/-}$ mice were cross-bred with Lipe-deficient (*Lipe*$^{-/-}$) mice to produce mice deficient in both Nceh1 and Lipe (*Nceh1*$^{-/-}$;*Lipe*$^{-/-}$). The nCEH activity in *Nceh1*$^{-/-}$;*Lipe*$^{-/-}$ macrophages was dramatically reduced, approximately to 10% of that in wild-type macrophages, and Nceh1 and Lipe were responsible for approximately 50% of the nCEH activity, respectively (Figure 4A). Thus, these two lipases were responsible for the majority of the nCEH activity in MPMs. Upon subcellular fractionation, Nceh1 contributed solely to the microsomal nCEH activity, while Lipe contributed to both cytosolic and microsomal nCEH activity (Figure 4C). Meanwhile, substantial TGL activity remained even in the *Nceh1*$^{-/-}$;*Lipe*$^{-/-}$ macrophages (Figure 4B) (72% without acLDL and 83% with acLDL, of that in wild-type macrophages). To investigate the roles of Nceh1 and Lipe in foam cell formation, the macrophages of each genotype were incubated with acLDL. Upon exposure to acLDL, Nceh1 and Lipe deficiency resulted in an increase of intracellular CE content by 29% and 51% compared with the wild-type, respectively (Figure 4D). Interestingly, Nceh1 and Lipe contributed in an additive manner to the intracellular CE content of *Nceh1*$^{-/-}$;*Lipe*$^{-/-}$ macrophages (an increase of 70% compared with the wild-type, Figure 4D). The extent of foam cell formation for each genotype was also confirmed by the incorporation of [^{14}C]oleate into the cellular CE (Figure 4E).

Although the nCEH activity of *Nceh1*$^{-/-}$ macrophages was almost identical to that of *Lipe*$^{-/-}$ macrophages, *Lipe*$^{-/-}$ macrophages were less prone to form foam cells than *Nceh1*$^{-/-}$ macrophages. Nceh1 and Lipe also contributed in an additive manner to cholesterol efflux (Figure 5A, reduction by 11%–5%, 23%–21%, and 43%–45% in *Nceh1*$^{+/+}$;*Lipe*$^{-/-}$, *Nceh1*$^{-/-}$;*Lipe*$^{+/+}$,

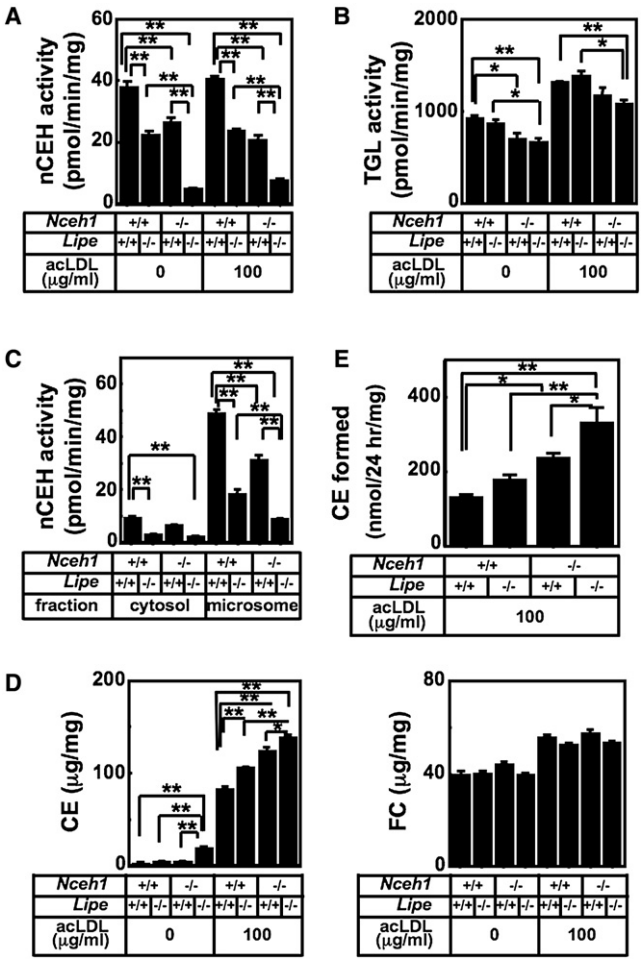

Figure 4. nCEH Activity and Foam Cell Formation in *Lipe*$^{-/-}$, *Nceh1*$^{-/-}$, or *Nceh1*$^{-/-}$;*Lipe*$^{-/-}$ Macrophages

(A and B) Peritoneal macrophages (n = 6–7 for each genotype) were incubated with or without acLDL (100 μg/ml) for 24 hr, and nCEH (A) and TGL (B) activities were determined.

(C) The nCEH activity after subcellular fractionation (n = 6–7).

(D) Peritoneal macrophages (n = 8) were incubated with or without acLDL for 24 hr, and intracellular CE content and FC content were measured.

(E) CE formation. Peritoneal macrophages (n = 7–8) were incubated with acLDL in the presence of [1-^{14}C]oleate-albumin complex for 24 hr, and the incorporation of [1-^{14}C]oleate into CE fraction was measured. Data are expressed as the mean ± SEM. * and ** denote $p < 0.05$ and $p < 0.01$, respectively, as determined by ANOVA followed by the Tukey-Kramer posthoc test.

and *Nceh1*$^{-/-}$;*Lipe*$^{-/-}$ macrophages compared with wild-type macrophages, respectively). The basal efflux to bovine serum albumin (BSA) alone without an extracellular lipoprotein acceptor or serum was also examined (Figure 5B). To eliminate the possibility that the constant cycle of hydrolysis and resynthesis of CE modulates cholesterol efflux, the experiment was conducted in the presence or absence of an ACAT inhibitor, CS-505. Since it was reported that the simultaneous cholesterol-loading and ACAT inhibition led to cytotoxicity in macrophages (Tabas, 2005), macrophages were loaded with acLDL reconstituted with [^{3}H]cholesteryl linoleate for 24 hr, and the medium was changed to that without acLDL in the presence or absence of CS-505 or HDL. Under such conditions, cholesterol efflux was very slow in the absence of HDL and strikingly facilitated by HDL, and the FC was effluxed from macrophages of each genotype in a quite reproducible fashion (Figure 5C). The contributions of Nceh1 and Lipe to the cholesterol efflux were further verified in these additional experiments.

ACAT1 is an allosteric enzyme regulated by FC (Chang et al., 2001), and the hydrolysis of cellular CE would supply substrate for ACAT. Consistent with this assumption, microsomal ACAT activity was reduced in *Nceh1*$^{-/-}$;*Lipe*$^{-/-}$ macrophages when they were loaded with acLDL (45% reduction compared with wild-type macrophages) (Figure 5D).

We performed a northern blot analysis of the genes involved in the metabolism of cholesterol during the formation of foam cells. Cholesterol loading by incubation with acLDL increased the mRNA expression of *Cd36*, *Abca1*, and *Abcg1*, while it decreased the mRNA expression of *Ldlr*. However, there was no obvious difference in the mRNA expression of the genes examined among four genotypes under conditions of cholesterol loading (Figure 5E). We also compared the endocytic uptake and degradation of acLDL by macrophages of each genotype (Figure 5F). Supporting the results of the northern blot analysis, there was no significant difference in the internalization and degradation of acLDL among the 4 genotypes.

Atheroprotective Role of Nceh1 and Lipe

Deficiency of Lipe or Nceh1 in whole body may affect atherosclerosis by the systemic phenotypes irrelevant to the local phenotypes in macrophages. Indeed, Lipe deficiency changed glucose tolerance as well as plasma lipoprotein profiles (Table S4). To specifically assess the biological roles of macrophage nCEHs in atherogenesis in vivo, we took advantage of bone marrow transplantation to selectively disrupt the *Nceh1* gene and/or *Lipe* gene in hematopoietic cells. Female Ldlr-deficient (*Ldlr*$^{-/-}$) mice were exposed to total-body irradiation to eliminate the endogenous bone marrow-derived cells, and subsequently transplanted with bone marrow cells isolated from male donor mice of each genotype. Successful reconstitution of recipients with cells of donor origin after bone marrow transplantation was established by PCR-assisted amplification of the *Sry* gene (a male marker) and *Nceh1* or *Lipe* mutant gene (Figure 6A).

During the course of the experiment, a deficiency of Nceh1 and/or Lipe in hematologic cells did not alter serum lipid levels (Table S5). An analysis using HPLC did not detect any difference in the cholesterol and triglycerides levels of each lipoprotein fraction (data not shown). The atherosclerotic surface area were increased by 1.7-fold in *Lipe*$^{-/-}$(BM)/*Ldlr*$^{-/-}$ mice and 3.1-fold in *Nceh1*$^{-/-}$(BM)/*Ldlr*$^{-/-}$ mice compared to wild-type(BM)/*Ldlr*$^{-/-}$ mice. The effect was most pronounced in *Nceh1*$^{-/-}$;*Lipe*$^{-/-}$(BM)/*Ldlr*$^{-/-}$ mice (a 4.3-fold increase compared to wild-type(BM)/*Ldlr*$^{-/-}$ mice) and was approximately additive with respect to the contribution (Figures 6B and 6C). Although the difference between wild-type(BM)/*Ldlr*$^{-/-}$ mice and *Lipe*$^{-/-}$(BM)/*Ldlr*$^{-/-}$ mice was not statistically significant when analyzed by an one-way ANOVA, it was statistically different when analyzed by a two-tailed Student's t test ($p = 0.016$). Quantification of cross-sectional lesion area in aortic root sections further supported the significance of Nceh1 and Lipe in atherogenesis

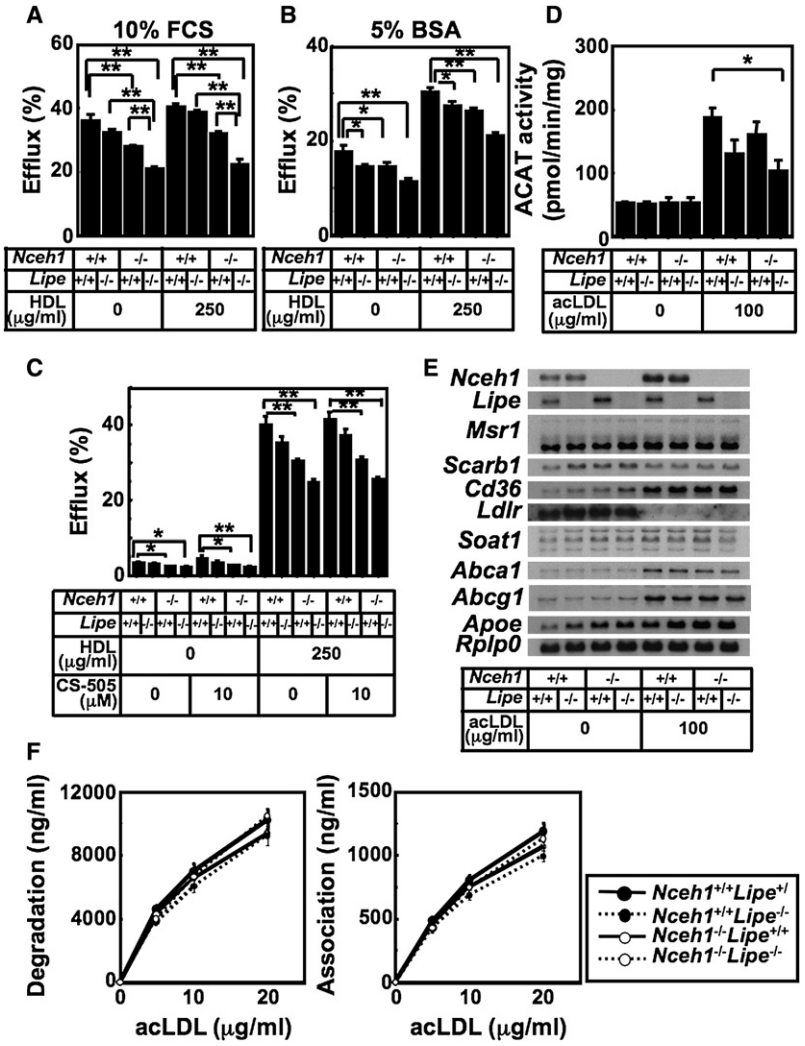

Figure 5. Cholesterol trafficking in *Lipe*$^{-/-}$, *Nceh1*$^{-/-}$, or *Nceh1*$^{-/-}$;*Lipe*$^{-/-}$ Macrophages

(A and B) Cholesterol efflux. Peritoneal macrophages (n = 6, A, n = 6–7, B) were incubated for 24 hr in DMEM containing 10% FCS (A) or 5% BSA (B) with acLDL (100 μg/ml) whose CE was reconstituted with cholesteryl[1,2-^{3}H]linoleate. After labeling of the cells, the medium was changed to DMEM containing 10% FCS (A) or 5% BSA (B) with acLDL (100 μg/ml)in the presence or absence of HDL_3 (250 μg/ml).

(C) Peritoneal macrophages (n = 5–6) were incubated for 24 hr in DMEM containing 5% BSA with 100 μg/ml acLDL whose CE was reconstituted with cholesteryl [1,2-^{3}H]linoleate. After labeling of the cells, the medium was changed to DMEM containing 5% BSA in the presence or absence of an ACAT inhibitor, CS-505 (10 μM) and/or HDL_3 (250 μg/ml). After 24 hr, the radioactivity of an aliquot of the medium and cell lysate was measured. The percent efflux was calculated as (media dpm)/(cell + media dpm) × 100.

(D) Microsomal ACAT activity. Peritoneal macrophages (n = 6–7) were incubated with or without acLDL (100 μg/ml) for 24 hr. ACAT activity in microsomes was determined by the rate of incorporation of [1-^{14}C]oleoyl-CoA into the CE fraction.

(E) Gene expression profile. Peritoneal macrophages (n = 4) were incubated with or without acLDL (100 μg/ml) for 24 hr. *Nceh1*, neutral cholesterol ester hydrolase 1; *Lipe*, hormone-sensitive lipase; *Msr1*, type A scavenger receptor; *Scarb1*, scavenger receptor class B type-I; *Ldlr*, the low-density lipoprotein receptor; *Soat1*, acyl-CoA:cholesterol acetyltransferase 1; *Abca1*, ATP-binding cassette transporter A1; *Abcg1*, ATP-binding cassette transporter G1. Loading was normalized by the expression of *Rplp0* (acidic ribosomal phosphoprotein P0).

(F) Cellular uptake and degradation of acLDL. Peritoneal macrophages (n = 5) were incubated with a medium containing varying concentrations of ^{125}I-acLDL with or without a 40-fold excess of unlabeled acLDL for 5 hr at 37°C. The amounts of ^{125}I-acLDL either degraded by or associated with the cells were measured. Data are expressed as the mean ± SEM. * and ** denote $p < 0.05$ and $p < 0.01$, respectively, as determined by ANOVA (A–D) or repeated-measures ANOVA (F) followed by the Tukey-Kramer posthoc test.

(a 1.2-fold increase in *Lipe*$^{-/-}$ (BM)/*Ldlr*$^{-/-}$ mice, a 1.6-fold increase in *Nceh1*$^{-/-}$ (BM)/*Ldlr*$^{-/-}$ mice, and a 2.0-fold increase in *Nceh1*$^{-/-}$;*Lipe*$^{-/-}$ (BM)/*Ldlr*$^{-/-}$ mice compared to wild-type(BM)/*Ldlr*$^{-/-}$ mice, respectively (Figures 6D and 6E).

DISCUSSION

The hydrolysis of cellular CEs is the first committed step in the efflux of cholesterol. Subsequently, cholesterol is transferred to plasma membrane where ABCA1 and/or ABCG1 mediate final transfer to apoA-I and/or HDL, respectively. Under normal circumstances, the efflux of cholesterol mediated by ABC transporters is rate-limiting in the overall process, since the combined deficiency severely impaired cholesterol efflux (Out et al., 2008; Wang et al., 2007; Yvan-Charvet et al., 2007), and since cholesterol efflux was stimulated when the expression of these ABC transporters were induced by the activation of LXR (Venkateswaran et al., 2000; Tazoe et al., 2008). Under conditions where an excess of cholesterol is stored as CE in LDs of macrophage foam cells, the hydolysis of cellular CE may become rate-limiting as was observed in the present study (Figures 1E and 5A–5C). It is of note that the deficiency of Nceh1 caused a more profound decrease in cholesterol efflux than the deficiency of Lipe, even though the reduction in nCEH activity was comparable (Figure 4A). These results suggest that the roles of Lipe and Nceh1 in the mobilization of cholesterol from LDs to the medium are somewhat different: Nceh1 is more efficient than Lipe in this process.

Based on our current results, the contribution of the ortholog of CEH, TGH1, and Ces1, to the nCEH activity of MPM would be marginal. In contrast to MPMs, human monocyte-derived macrophages express CEH, a macrophage serine-esterase. Even in human macrophages, CES1 is not a major enzyme mediating the hydrolysis of CE, and NCEH1 (NM_020792) also plays a central role in the CE hydrolysis in human macrophages (M.I., unpublished data).

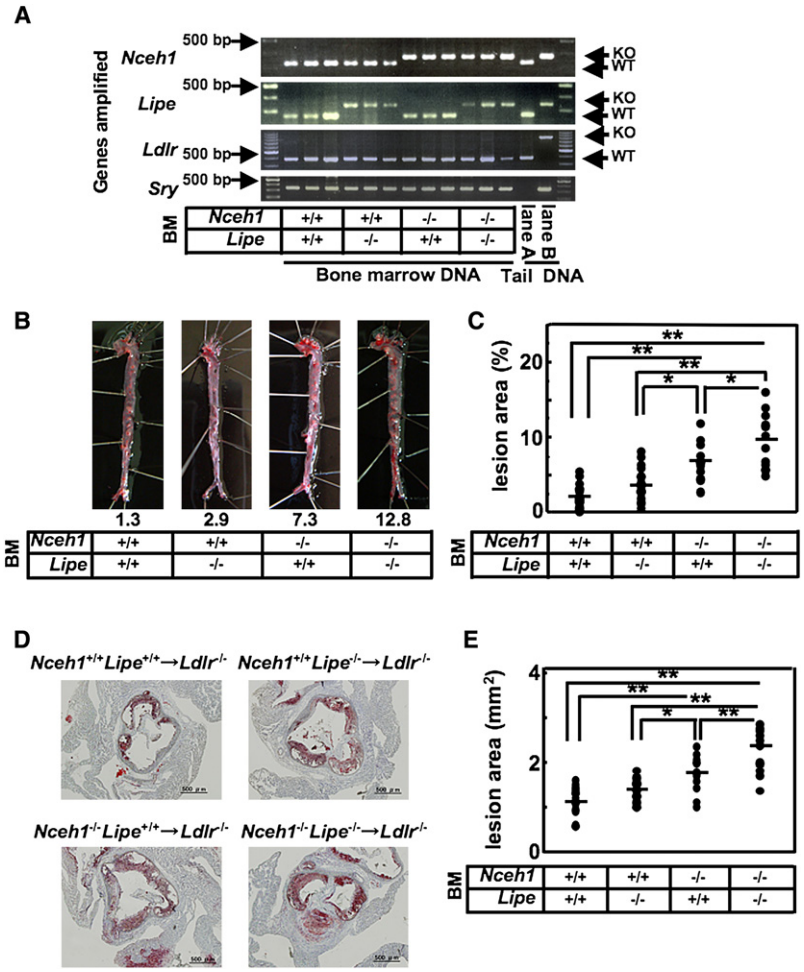

Figure 6. Aherosclerosis in *Ldlr*$^{-/-}$ Mice with *Nceh1*$^{-/-}$, *Lipe*$^{-/-}$, or *Nceh1*$^{-/-}$;*Lipe*$^{-/-}$ Bone Marrow

(A)Irradiated female *Ldlr*$^{-/-}$ mice (age 8 weeks, n = 12–17) were transplanted with bone marrow cells isolated from male wild-type, *Lipe*$^{-/-}$, *Nceh1*$^{-/-}$, *Nceh1*$^{-/-}$;*Lipe*$^{-/-}$ mice (age 8 weeks). Mice were maintained on a chow diet for the first 4 weeks, then switched to a high-cholesterol diet. After 12 weeks on the high-cholesterol diet, the mice were euthanized to assess their lesions. (A) shows verification of successful reconstitution with donor hematopoietic cells by PCR amplification of specific fragments of several genes. Genomic DNA from bone marrow of chimeric mice (the genotype of donor mice is indicated below) or tail was used as a template. The following templates were used as positive and negative controls (lane A and B): Tail DNA from a *Nceh1*$^{+/+}$ and *Nceh1*$^{-/-}$ mouse for *Nceh1* gene amplification, tail DNA from a *Lipe*$^{+/+}$ and *Lipe*$^{-/-}$ mouse for *Lipe* gene amplification, tail DNA from a *Ldlr*$^{+/+}$ and *Ldlr*$^{-/-}$ mouse for *Ldlr* gene amplification, and tail DNA from a female and male C57BL6/J mouse for *Sry* gene amplification in lane A and B, respectively.

(B–E) Quantification by en face analysis (B) (quantification values indicated below the images) (C), and cross-sectional analysis (D and E). The genotypes of donor cells are indicated below the graphs. * and ** denote $p < 0.05$ and $p < 0.01$, respectively, as determined by ANOVA followed by the Tukey-Kramer posthoc test.

Based on the current findings, it is expected that induction of the expression of Nceh1 in macrophages can be used for the treatment or prevention of atherosclerosis. In vitro, we demonstrated that adenovirus-mediated overexpression of Nceh1 decreased foam cell formation (Okazaki et al., 2008). To substantiate these findings, we need to prove the hypothesis in vivo either by transgenic overexpression of Nceh1 or by its pharmacological activation. In this context, it is noteworthy that this hypothesis has been tested by overexpressing LIPE or CEH to hydrolyze CE in a macrophage-specific manner. The results for LIPE are somewhat complicated. Overexpression of LIPE paradoxically increases the development of atherosclerosis (Escary et al., 1998) and the concomitant co-overexpression of a cholesterol acceptor, apoA-IV, is anti-atherogenic(Choy et al., 2003). Moreover, overexpression of CES1 was reported to reduce the atherosclerosis of *Ldlr*$^{-/-}$ mice(Zhao et al., 2007).

The human ortholog of Nceh1, KIAA1363, is identical to human AADACL1 (Probst et al., 1994). Recently, Tiwari et al. reported that steryl deacetylase 1 in *S. cerevisiae*, which has homology to mammalian AADAC and AADACLs, controls the export of sterols by regulating the deacetylation/acetylation of cholesterol (Tiwari et al., 2007). In this report, it was shown that human AADAC, but not AADACL1, catalyzed the deacetylation of acetylated cholesterol. Thus, it is not surprising that the substrate of AADACL1 (NCEH1) is CE.

In foam cells, CE is the major lipid component in LDs with triglyceride typically comprising 10% or less of the total lipid. While Nceh1 and Lipe were responsible for the majority of the nCEH activity in macrophages, substantial amount of TGL activity in macrophages was remained even in the *Nceh1*$^{-/-}$;*Lipe*$^{-/-}$ macrophages. In adipose tissue, adipose triglyceride lipase (PNPLA2) was identified as a non-Lipe triglyceride lipase, and Pnpla2 is also expressed in macrophages (Zimmermann et al., 2008). Pnpla2 might be a promising candidate responsible for the remaining TGL activity in *Nceh1*$^{-}$/$^{-}$;*Lipe*$^{-}$/$^{-}$ macrophages.

It was also reported that KIAA1363 hydrolyzes 2-acetyl monoalkylglycerol and the chemical inhibition of KIAA1363 modulates the metabolism phospholipids such as lysophosphatidinic acid and platelet-activating factor (Chiang et al., 2006). Lysophosphatidinic acid and platelet-activating factor are implicated in monocyte adhesion and transmigration, and promote atherosclerosis through their inflammatory activities (Ninio, 2005). Thus, it remains possible that the Nceh1 deficiency aggravates atherosclerosis at least in part by altering the composition of phospholipids, which have pro-atherogenic or anti-atherogenic potentials.

In conclusion, Nceh1 plays a crucial role in the development of both foam cells and atherosclerotic lesions. Nceh1 and Lipe are two major enzymes with nCEH activity in murine macrophages. These findings should provide the basis for understanding the pathophysiology of atherosclerosis and can be exploited to develop an attractive therapeutic approach.

EXPERIMENTAL PROCEDURES

Generation of the *Nceh1*$^{-/-}$ Mice

A replacement-type targeting vector was constructed; the 1 kb short arm spanning intron 3, and the 10 kb long-arm fragment encompassing intron 4–5 were generated by PCR using genomic DNA from the 129/Sv mouse as a template. Primers used were as follows; 5′-ctcgagatcagctcaaggctggagt gtg-3′ and 5′-ctcgagctctttgtaaagtacatgtggccc-3′ for the short arm and 5′-acct ttctcacggggttttctgaagtgggt-3′ and 5′-gcatcctgcctgaatctgcaagtgctgag-3′ for the long arm. After subcloning into pGEM-T easy vector (Promega, Madison, WI), the fragments were cut out and inserted into the *Xho I* and *Not I* sites, respectively, of pPol II -short-neo-bpA-HSVTK as described previously (Ishibashi et al., 1993). Thus, a 1.2 kb region spanning exon 4 of the *Nceh1* gene was replaced by a neomycin-resistant cassette, which was expected to abolish translation of the catalytic motif. After linearization by digestion with SalI, the vector was electroporated into JH1 embryonic stem cells (a gift from Dr. J Herz). Targeted clones, which had been selected in the presence of G418 and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil, were identified by PCR using the following primers; sense (5′-gacttgacctcacagtgtgt gaacc-3′) and antisense (5′- gattgggaagacaatagcaggcatgc-3′).

Homologous recombination was verified by Southern blot analysis after digestion with BglII using a 5′-flanking probe. Targeted embryonic stem cell clones were injected into the C57BL/6J blastocysts yielding two lines of chimeric mice, which transmitted the disrupted allele through the germ line. Genotyping was performed with PCR using specific primers: sense (5′-tccagta gaagagggcacagaggtcc-3′), antisense (5′-ccatcccgtttaccaataaagcttaaggc-3′) and neo (5′-aggattgggaagacaatagcaggcat-3′).

The genetic background used in this study was as follows: (1) experiments in vitro using *Nceh1*$^{-/-}$ mice were performed on matched littermates from 129/Sv-C57BL/6J hybrid descendants (F1 and subsequent generations) unless otherwise stated. (2) *Nceh1*$^{-/-}$ mice were crossed onto the C57BL/6J background for more than five generations and then crossed with *Apoe*$^{-/-}$ mice on a C57BL/6 background (the Jackson Laboratory). (3) *Nceh1*$^{-/-}$ mice were crossed onto the C57BL/6J background for more than five generations and then crossed with *Lipe*$^{-/-}$ mice (crossed onto the C57BL6/J background for more than ten generations) (Osuga et al., 2000) for experiments in vitro using *Nceh1*$^{-/-}$;*Lipe*$^{-/-}$ macrophages. (4) For donor mice in the bone marrow transplantation experiments, mice were crossed onto the C57BL6/J background for more than ten generations. Mice were maintained on a 12 h dark/light cycle and were fed a normal chow diet (Lab Diet 5053, PMI Nutrition International) unless otherwise stated. All experimental procedures involving animals were conducted according to our institutional guidelines.

Bone Marrow Transplantation

To eliminate the endogenous bone marrow-derived cells, female *Ldlr*$^{-/-}$ mice (age 8 weeks) were exposed to a single dose of 9 Gy total body irradiation. Irradiated recipients were transplanted with 5 × 10^6 bone marrow cells, isolated from male wild-type, *Lipe*$^{-/-}$, *Nceh1*$^{-/-}$, and *Nceh1*$^{-/-}$;*Lipe*$^{-/-}$ mice (age 8 weeks). Mice were maintained on a chow diet for the first 4 weeks, and then switched to a high-cholesterol diet containing 1.25% (w/w) cholesterol and 15% (w/w) cocoa butter (Oriental Yeast Company; Tokyo, Japan). After 12 weeks on the high-cholesterol diet, the chimeras were euthanized for the assessment of the lesions. The hematologic chimerism of the *Ldlr*$^{-/-}$ mice was determined in genomic DNA from bone marrow by PCR at 12 weeks posttransplant. Sry-s (5′-gtggtgagaggcacaagt-3′) and Sry-as (5′-gagtacaggtgtgcagct-3′) were used to detect the *Sry* gene specific to the mouse Y chromosome(Mignon et al., 1998). The primers used to detect both the wild-type and the null mutant *Nceh1* or *Lipe* gene were described above or previously (Osuga et al., 2000).

Peritoneal Macrophages

Thioglycorate-elicited macrophages were obtained from 8 week-old mice as described previously (Osuga et al., 2000). The cells were resuspended in Dulbecco's Modified Eagle Medium (DMEM) to give a concentration of 10^6 cells per ml, and 10 ml per dish was plated in 10 cm dishes. Cells were incubated in DMEM containing 10% fetal calf serum (FCS) in an atmosphere of 5% CO_2 and 95% air unless otherwise stated.

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 (Havel et al., 1955). LDL was acetylated by repetitive additions of acetic anhydride (Goldstein et al., 1979). acLDL was radioiodinated with $Na^{125}I$ by the iodine monochloride method (Goldstein et al., 1979). CE in acLDL was reconstituted with [1,2-^{3}H]cholesteryl linoleate as described previously (Roberts et al., 1985). To prepare oxLDL, LDL was incubated for 17 hr at 37°C with 5 mM $CuSO_4$, followed by the addition of 2.5 mM EDTA (Sakai et al., 1996). Beta-VLDL (d = 1.006–1.019 g/ml) was isolated by sequential ultracentrifugation of plasma obtained from overnight fasted male Japanese White rabbits maintained on a cholesterol-enriched diet containing 1% (w/w) cholesterol.

Determination of Cholesterol Content

Cellular lipids were extracted by hexane/isopropyl alcohol, and cholesterol content was determined by an enzymatic fluorometric microassay according to the method of Heider and Boyett, with minor modifications (Heider and Boyett, 1978; Yagyu et al., 2000).

CE Formation

The amounts of CE formed from [1-^{14}C]oleic acid was determined as described previously with minor modifications (Perrey et al., 2001).

TGL and nCEH Activity Assay

Enzymatic activity was assayed as described previously (Osuga et al., 2000; Sekiya et al., 2008). The samples were incubated at 37°C for 30–60 min in a final volume of 200 μl of a reaction mixture containing 10^5 mmol/l tri[^{3}H]oleoylglycerol (99.4 mCi/mmol), 23.7 mmol/l lecithin, 12.5 mmol/l sodium taurocholate, 1 mol/l NaCl, and 85 mmol/l potassium phosphate (pH 7.0). The high concentration of NaCl was included to inactivate lipoprotein lipase. The nCEH activity was measured using a reaction mixture containing 6.14 mmol/l cholesterol [1-^{14}C]oleate (48.8 mCi/mmol).

Cholesterol Efflux

Cholesterol efflux assays were performed as described previously (Roberts et al., 1985). Cells were incubated for 24 hr at 37°C in DMEM containing bovine serum albumin (BSA, 5 mg/ml) or 10% FCS and acLDL (100 μg/ml) whose CE was reconstituted with [1,2-^{3}H]cholesteryl linoleate. After labeling of the cells, the medium was changed to DMEM containing BSA (5 mg/ml) or 10% FCS with acLDL (100 μg/ml) and with/without HDL_3 (250 μg/ml). After 24 hr, 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 0.1 N NaOH, and the radioactivity in an aliquot of the cell lysate was measured. The percent efflux was calculated as (media dpm)/(cell + media dpm) × 100. To precisely evaluate the contribution of nCEH activity to cholesterol efflux, additional assays were conducted in the presence of the ACAT inhibitor CS-505 (provided by Daiichi Sankyo and Kyoto pharmaceutical industries) (Takahashi et al., 2008). After cells were loaded with cholesterol for 24 hr by incubation with acLDL, whose CE was reconstituted with [1,2-^{3}H]cholesteryl linoleate, the medium was changed to DMEM containing BSA (5 mg/ml) in the presence or absence of CS-505 (10 μM) or HDL_3 (250 μg/ml). After incubation for 24 hr, the radioactivity in media and cells was measured.

Microsomal ACAT Activity

Cells were sonicated in buffer A (20 mM Tris-HCl pH 7.0, 250 mM sucrose, 5 μg/ml leupeptin, and 2.8 μg/ml aprotinin), ultracentrifuged at 100,000 × *g* for 45 min at 4°C, and re-ultracentrifuged at 100,000 × *g* for 45 min at 4°C. The precipitates were resuspended and used for the assay. ACAT activity in microsomes was determined by the rate of incorporation of [1-^{14}C]oleoyl-CoA into the CE fraction according to Yagyu et al. (Yagyu et al., 2000).

Cellular Uptake and Degradation of ^{125}I-AcLDL

Peritoneal macrophages were plated in 12-well plates at a density of 1 × 10^6/well and incubated with a medium containing varying concentrations of

^{125}I-AcLDL with or without a 40-fold excess of unlabeled acLDL at 37°C. After 5 hr, the amounts of ^{125}I-AcLDL either degraded by or associated with the cells were measured according to a modified method of Goldstein (Goldstein et al., 1983; Perrey et al., 2001).

Northern Blot Analysis

Total RNA was isolated using Trizol Reagent (Life Technologies; Helgerman, CT). Ten microgram RNA samples equally pooled from each genotype were electrophoresed in a 1% agarose gel containing formaldehyde and transferred to a nylon membrane. The membranes were hybridized with probes, which were labeled with [α-^{32}P]dCTP using a Megaprime DNA Labeling System Kit (Amersham Biosciences; Piscataway, NJ) in Rapid-hyb Buffer (Amersham Biosciences) or Ultra-hyb buffer (for HSL mRNA detection) (Ambion; Austin, TX) and analyzed using a BAS2000 Bioimaging Analyzer (Fuji Photo Film; Tokyo, Japan). Loading was normalized by the expression of *Rplp0* (acidic ribosomal phosphoprotein P0) mRNA.

Quantitative Real-Time PCR

Two micrograms of total RNA was 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*. Primer sequences are available upon request.

Measurement of Atherosclerotic Lesions

Nceh1$^{-/-}$;*Apoe*$^{-/-}$ mice and littermate *Nceh1*$^{+/+}$;*Apoe*$^{-/-}$ mice were fed a western-type diet containing 0.15% (w/w) cholesterol and 20% (w/w) fat (Oriental Yeast Company; Tokyo, Japan). (2) Bone marrow chimeric Ldlr-deficient mice were fed the high-cholesterol diet. After the indicate time, they were euthanized and their hearts and aortas were isolated. The degree of atherosclerosis was assessed by determining lesion sizes on both pinned-open aortas and serial cross-sections through the aortic root (Paigen et al., 1987; Yagyu et al., 2000). The aorta was opened longitudinally along the ventral midline from the iliac arteries to the aortic root. After branching vessels were removed, the aorta was pinned out flat on a black wax surface. The lesions were stained with Sudan IV for 15 min, destained with 70% ethanol, and then fixed in 4% phosphate-buffered formalin. Aortic images were analyzed with Adobe Photoshop 7 software (Adobe Systems, Incorporated; Mountain View, CA). Data are reported as the percentage of the aortic surface covered by lesions (total surface area of the atherosclerotic lesions divided by the total surface area of the aorta). The hearts were perfused with saline containing 4% (w/v) formalin and fixed for more than 48 hr in the same solution. The basal half of the hearts was embedded in Tissue-Tek OCT compound (Miles, Incorporated), and serial sections were captured using a Cryostat microtome (10 μm thick). Four sections were used to evaluate the lesions: two at the end of the aortic sinus and two at the junction of the sinus and ascending aorta. The sections were stained with Oil Red O and counterstained with hematoxylin.

Immunohistochemistry

Sections were incubated with primary antibody to mouse F4/80 (1:20; Serotec, Raleigh, NC) for 2 hr. After washes, the sections were incubated with biotinylated anti-rat antibody for 30 min at room temperature and then with avidin-biotin peroxidase complex (Vector Labs) for 30 min. Finally, the sections were developed with DAB (Wako Pure Chemicals Co.; Osaka, Japan) and counterstained with methylgreen.

Extraction of Aortic Wall Lipids

After 12 weeks on a western-type diet, mice were euthanized and the aortas were prepared. After the removal of connective tissue and fat, lipids were extracted by the chloroform-methanol method, and FC content and CE content were measured.

Statistics

Statistical differences between groups were analyzed by one-way analysis of variance and the post hoc Tukey-Kramer test, unless otherwise stated.

SUPPLEMENTAL DATA

The Supplemental Data include five tables and two figures and can be found with this article online at http://www.cell.com/cellmetabolism/supplemental/S1550-4131(09)00233-2.

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M.S. performed the majority of the experiments, analyzed data, and wrote the manuscript; J.O. supervised the overall project; S.N., M.T., T.O, F.T., T.W., and Y.F. assisted with bone marrow transplantation experiments; M.I. provided helpful data supporting our conclusion; H.O. identified Nceh1 and contributed to the design of targeting construct; S.I. supervised the overall project and wrote the manuscript.

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