The Critical Role of Neutral Cholesterol Ester Hydrolase 1 in Cholesterol Removal From Human Macrophages

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Rationale: Hydrolysis of intracellular cholesterol ester (CE) is the key step in the reverse cholesterol transport in macrophage foam cells. We have recently shown that neutral cholesterol ester hydrolase (Nceh1) and hormone-sensitive lipase (Lipe) are key regulators of this process in mouse macrophages. However, it remains unknown which enzyme is critical in human macrophages and atherosclerosis.

Objective: We aimed to identify the enzyme responsible for the CE hydrolysis in human macrophages and to determine its expression in human atherosclerosis.

Methods and Results: We compared the expression of NCEH1, LIPE, and cholesterol ester hydrolase (CES1) in human monocyte-derived macrophages (HMMs) and examined the effects of inhibition or overexpression of each enzyme in the cholesterol trafficking. The pattern of expression of NCEH1 was similar to that of neutral CE hydrolase activity during the differentiation of HMMs. Overexpression of human NCEH1 increased the hydrolysis of CE, thereby stimulating cholesterol mobilization from THP-1 macrophages. Knockdown of NCEH1 specifically reduced the neutral CE hydrolase activity. Pharmacological inhibition of NCEH1 also increased the cellular CE in HMMs. In contrast, LIPE was barely detectable in HMMs, and its inhibition did not decrease neutral CE hydrolase activity. Neither overexpression nor knockdown of CES1 affected the neutral CE hydrolase activity. NCEH1 was expressed in CD68-positive macrophage foam cells of human atherosclerotic lesions.

Conclusions: NCEH1 is expressed in human atheromatous lesions, where it plays a critical role in the hydrolysis of CE in human macrophage foam cells, thereby contributing to the initial part of reverse cholesterol transport in human atherosclerosis. (Circ Res. 2010;107:1387-1395.)

Key Words: neutral cholesterol ester hydrolase ■ reverse cholesterol transport ■ macrophage ■ atherosclerosis ■ KIAA1363

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. Lipid-rich plaques, which are characterized by a plethora of cholesterol ester (CE)-laden macrophage foam cells, are prone to rupture.1 Esterification of cholesterol in macrophages is mediated by acyl-coenzyme A cholesterol acyltransferase 1 or sterol O-acyltransferase 1 (SOAT1).2 Conflicting results have been reported as to the effects of genetic ablation of SOAT1 on atherosclerosis in mice.3,4 Furthermore, it has not been successful to demonstrate the efficacy of nonselective inhibitors of SOAT to clinically prevent the atherosclerosis in humans.5,6 On the other hand, the hydrolysis of intracellular CE is the initial step of reverse cholesterol transport.7 As the hydrolysis of CE preceding reverse cholesterol transport takes place at neutral pH, the enzymes catalyzing it have been collectively called neutral CE hydrolases. Because this step is rate-limiting, particularly in macrophage foam cells,8,9 it is important to clarify the mechanisms that mediate the hydrolysis of CE in foam cells. To date, 3 enzymes have been proposed to serve as neutral CE hydrolases in macrophages: hormone-sensitive lipase (LIPE)10; cholesteryl ester hydrolase (CEH),11 which is identical to human liver carboxylesterase 1 (CES1, hCE-1)12 or...
macrophage serine esterase 1 (HMSE1), also known as a human ortholog of triacylglycerol hydrolase\(^1\); and neutral cholesterol ester hydrolase 1 (NCEH1), which is also known as KIAA1363 or AADACL1 (arylacetamide deacetylase-like).\(^2\) Lipase is expressed in mouse macrophages and its overexpression inhibits the accumulation of CE in THP-1 macrophages.\(^3\),\(^4\) However, mouse peritoneal macrophages (MPMs) of Lipase-deficient (Lipe\(^{-/-}\)) mice in a mixed genetic background still retain substantial neutral CE hydrolase activity.\(^5\),\(^6\) indicating the presence of additional neutral CE hydrolase(s). Ghoosh reported CES1 as a promising candidate for a neutral CE hydrolase,\(^1\) because its overexpression reduced CE contents in macrophage foam cells.\(^7\),\(^8\) Moreover, its macrophage-specific overexpression driven by the promoter of macrophage scavenger receptor-1 protected against diet-induced atherosclerosis in low-density-lipoprotein receptor-deficient mice.\(^9\) However, the effects of loss-of-function of CES1 on neutral CE hydrolase activity in macrophages have not been reported. Furthermore, a mouse ortholog of CES1, triacylglycerol hydrolase, was barely detectable in MPMs and possessed negligible neutral CE hydrolase activity.\(^10\) 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 its knockdown or knockout significantly reduces neutral CE hydrolase activity of MPMs.\(^11\),\(^12\) We have also shown that Nceh1 is more responsible for the hydrolysis of CE in MPMs than in immortal cell line such as RAW 264.7.\(^13\) Furthermore, ablation of Nceh1 accelerated atherosclerosis in mice.\(^14\) Therefore, Nceh1 is more likely to be involved in the hydrolysis of CE in mouse macrophages including MPMs. However, NCEH1 in human macrophages has yet to be characterized. Furthermore, although Lipase contributes to neutral CE hydrolase activity in MPMs, previous reports showed that expression of Lipase in human macrophages is extremely low.\(^15\),\(^16\) Thus, there seems to be great differences in the hydrolysis of CE among macrophages from different species, and it is unknown which enzyme is the dominant neutral CE hydrolase in human macrophages. To solve this question and translate the findings to clinical application, we aimed to identify the enzyme responsible for CE hydrolysis in human macrophages and to determine its expression in human atherosclerotic lesions.

In the present study, we demonstrate for the first time that NCEH1 is expressed in macrophage foam cells in human atheromatous plaques and accounts for the majority of the neutral CE hydrolase activity of human monocyte-derived macrophages (HMMs). These findings should provide a novel paradigm for understanding the pathogenesis of human atherosclerosis as well as for developing new drugs for its treatment.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Western Blot Analyses**

Western blotting analyses were performed essentially as described previously.\(^1\)

**Enzyme Assays**

\(\mu\)Nitrophenyl butyrate (PNPB)-hydrolyzing activity was determined as described previously.\(^11\),\(^15\) Neutral CE hydrolase activity was determined as described by Hajjar et al.,\(^2\) using a reaction mixture containing 6.14 \(\mu\)mol/L cholesterol \([1-14C]\)oleate (48.8 \(\mu\)Ci/\(\mu\)mol; 1 \(\mu\)Ci = 37 kBq).

**Cholesterol Determination**

Cellular cholesterol contents were determined by enzymatic fluorometric microwasuy.\(^4\)

**Cholesterol Formation**

CE formation from \([14C]\)oleate was measured.\(^29\)

**Samples for Immunohistochemistry**

Tissue samples for immunohistochemistry analysis were obtained from autopsy cases. Arteriosclerotic lesions of the aorta were from 20 autopsy cases (Table). After macroscopic inspection of the intimal surface, several tissue specimens were removed from the thoracic or abdominal aorta of these cases. Biopsy specimens were fixed in buffered formalin, embedded in paraffin wax, and serially sectioned onto 4-\(\mu\)-thick microscopic slides.

**Immunohistochemistry**

The method is described in detail in the Online Data Supplement.

**Results**

To determine which enzyme(s) is closely related to the neutral CE hydrolase activity at various stages in the differentiation of human macrophages, we compared the pattern of expression of NCEH1, CES1, and LIPE with that of neutral CE hydrolase activity of HMMs and 2 immortal lines: THP-1 and U937 (Figure 1). Human NCEH1 protein was recognized as duplets with molecular mass of 40 and 45 kDa. HMMs showed a robust increase in neutral CE hydrolase activity during the differentiation from monocytes up to day 8 (14.9-fold) (Figure 1A). PNPB hydrolysis activity in HMMs showed a similar, but less robust, increase (3.2-fold) (Figure 1B). The manner of its induction during the differentiation from monocytes up to day 8 of differentiation by estimating the density of band of NCEH1

<table>
<thead>
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Non-standard Abbreviations and Acronyms: acLDL, acetylated low-density lipoprotein; CE, cholesterol ester; GM-CSF, granulocyte/macrophage colony-stimulating factor; HDL, high-density lipoprotein; HMM, human monocyte-derived macrophage; LDL, low-density lipoprotein; M-CSF, macrophage colony-stimulating factor; MPM, murine peritoneal macrophage; moi, multiplicity of infection; PNPB, p-nitrophenyl butyrate; SOAT1, sterol O-acyltransferase 1; WAT, white adipose tissue.
or CES1 in HMMs, using GST-fused proteins, which were also used as antigens to produce anti-NCEH1 or CES1 antiserum, as standards (Online Figure II). The molar ratio of NCEH1 to CES1 was calculated to be 10. However, NCEH1 was barely detectable, even at the differentiated stage, in either THP-1 cells or U937 cells, although the differentiation of these cells accompanied increases in neutral CE hydrolase activity. The level of NCEH1 to CES1 protein was greatest. The increased activity of NCEH1, CES1, and LIPE and neutral CE hydrolase activity in HMMs are more relevant to the pathogenesis of human atherosclerosis.

We examined whether differences in cytokines used for differentiation of macrophages affect the expression of NCEH1, CES1, and LIPE and neutral CE hydrolase activity in HMMs. Treatment by macrophage colony-stimulating factor (M-CSF) or granulocyte/macrophage (GM)-CSF greatly increased the amounts of NCEH1 protein (M-CSF, 2.2-fold; GM-CSF, 3.4-fold). In parallel, it increased neutral CE hydrolase activity (M-CSF, 2.4-fold; GM-CSF, 3.4-fold) and PNPB hydrolase activity (B, and protein expression of NCEH1, CES1, and LIPE (C) in peripheral blood monocytes, THP-1, and U937, before and after differentiation, was examined. Mo indicates monocytes; Mφ, macrophages. Human liver (L) and adipose tissue (A) were used for comparison. Ten micrograms of cell lysate were subjected to Western blotting. Data are presented as means±SD of 6 measurements. *P<0.05, **P<0.01 (determined by ANOVA followed by the Bonferroni post hoc analysis).

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AMI indicates acute myocardial infarction; AML, acute myelogenous leukemia; AP, atheromatous plaque; DCM, dilated cardiomyopathy; DIT, diffuse intimal thickening; DM, diabetes mellitus; FS, fatty streak; GVHD, graft-versus-host disease; HCC, hepatocellular carcinoma; HL, hyperlipidemia; HT, hypertension. Ratio of CD68- or NCEH1-positive cells in 200 nucleated cells of representative view was calculated.

Figure 1. Endogenous neutral CE hydrolase in human macrophages. Neutral CE hydrolase activity (A), PNPB hydrolase activity (B), and protein expression of NCEH1, CES1, and LIPE (C) in peripheral blood monocytes, THP-1, and U937, before and after differentiation, was examined. Mo indicates monocytes; Mφ, macrophages. Human liver (L) and adipose tissue (A) were used for comparison. Ten micrograms of cell lysate were subjected to Western blotting. Data are presented as means±SD of 6 measurements. *P<0.05, **P<0.01 (determined by ANOVA followed by the Bonferroni post hoc analysis).
the cellular CE content (Figure 2D), as well as in the rate of formation of CE from oleate (Figure 2E). However, overexpression of CES1 did not significantly reduce the cellular CE accumulation. The decreased CE formation was not accompanied by changes of level of SOAT1 protein (Figure 2F). Similarly, overexpression of NCEH1 or LIPE significantly decreased CE content in THP-1 cells, which had been loaded with oxidized LDL, aggregated LDL, or VLDL (Online Figure IV).

Cholesterol efflux was examined in THP-1 macrophages that overexpressed NCEH1, CES1, or LIPE (Online Figure V). Overexpression of NCEH1 and LIPE significantly promoted cholesterol efflux in the presence of high-density lipoprotein (HDL) (Online Figure V, B) or apolipoprotein A-1 (Online Figure V, C). Addition of 10 μmol/L, CS-505, a SOAT1 inhibitor, inhibited the CE formation completely (Online Figure V, A) but did not affect cholesterol efflux in the cells overexpressing NCEH1, CES1, or LIPE (Online Figure V, B). Dibutyryl cAMP promoted cholesterol efflux in the cells overexpressing LIPE as described previously but did not affect cholesterol efflux in the cells overexpressing NCEH1 or CES1, in accordance with our previous report (Online Figure V, D). Overexpression of NCEH1, CES1, or LIPE did not affect the expression of ABCG1 protein, whereas overexpression of NCEH1 or LIPE slightly increased the expression of ABCA1 protein (Online Figure V, E). These results indicate that human NCEH1 is primarily involved in CE hydrolysis and that its overexpression promotes cholesterol efflux without affecting SOAT1 activities probably by increasing the expression of ABCA1.

To determine whether LIPE or NCEH1 is involved in the hydrolysis of CE in HMMs, we used 76-0079, a LIPE
inhibitor, or AS115, a KIAA1363 inhibitor. 76-0079 was an inhibitor of both LIPE and CES1, and AS115 was an inhibitor of LIPE, NCEH1, and CES1 (Figure 3A and 3B). Although 76-0079 and AS115 are nonspecific inhibitors, we can estimate the contribution of NCEH1 by the difference between the effects of those inhibitors. First, we examined the effects of 76-0079 and AS115 on the neutral CE hydrolase (Figure 3C and 3E) or PNPB hydrolase (Figure 3D and 3F) activity in the whole cell lysate of HMMs, THP-1 macrophages, and U937 macrophages. 76-0079 did not significantly inhibit the neutral CE hydrolase activity of HMMs. In contrast, AS115 inhibited the neutral CE hydrolase activity by 85%. 76-0079 inhibited the PNPB hydrolase activity of HMMs, THP-1 cells, and U937 macrophages by 23%, 96%, and 80%, respectively. On the other hand, AS115 inhibited the PNPB hydrolase activities of these macrophages by 98%, 100% and 83%, respectively. Furthermore, whereas AS115 significantly decreased cholesterol efflux from HMMs that had been loaded with CE, 76-0079 did not (Figure 3G). These results support the notion that the neutral CE hydrolase activity of HMMs is primarily mediated by NCEH1 but not by CES1 or LIPE.

To determine whether NCEH1 or CES1 is involved in the hydrolysis of CE in HMMs, we used an RNA-silencing technique (Figure 4). Infection with Ad-shNCEH1 reduced the amounts of NCEH1 protein as compared with Ad-shLacZ (by 41% at 250 mos and by 66% at 750 mos). In parallel, it decreased neutral CE hydrolase activity as compared with Ad-shLacZ (by 47% at 250 mos and by 50% at 750 mos). To the contrary, although infection with Ad-shCES1 reduced the amounts of CES1 protein as compared with Ad-shLacZ (by 60% at 250 mos and by 51% at 750 mos), it did not decrease...
the neutral CE hydrolase activity further from that attained with Ad-shLacZ. Infection with increasingly higher doses of Ad-shLacZ nonspecifically reduced the protein expression (Figure 4A) and neutral CE hydrolase activity (Figure 4B). To rule out the possibility that infection of Ad-shLacZ, Ad-shNCEH1, or Ad-shCES1 is cytotoxic, we performed MTT assay (Figure 4C). There was no significant difference in MTT activity among the cells infected with 3 viruses. Viability of cells infected 750 mos of adenovirus slightly decreased compared with no treatment cells, but the difference was not significant. Although there is a nonspecific effect of adenovirus infection, these results support that neutral CE hydrolase activity in HMMs is primarily mediated by NCEH1, not by CES1 or LIPE, in combination with the results of inhibition of human NCEH1 in HMMs by AS115 (Figure 3).

Finally, we investigated the expression of NCEH1 in human aortas (Table). We stained sections of aortas from 20 cases of autopsy whose clinical characteristics are summarized in Table. The lesions were morphologically classified into 3 types: diffuse intimal thickening, fatty streak lesions, and atheromatous plaques. Atheromatous plaques were rich in cells positive for CD68, a pan-macrophage/dendritic cell marker, as well as NCEH1-positive cells compared with fatty streak lesions or diffuse intimal thickening. NCEH1-positive cells were absent in diffuse intimal thickening. Figure 5 shows representative sections from atheromatous plaques. This region contains acellular necrotic core (Figure 5A). The subintimal area between the intima and the necrotic core contained many CD68-positive cells (Figure 5B). A majority of the CD68-positive cells were also positive for NCEH1 (Figure 5C and the Table). The colocalizing characteristics of CD68 and NCEH1 were more obvious at a higher magnification (Figure 5G and 5H). In contrast, CES1 was weakly positive for some CD68-positive cells (Figure 5D). LIPE protein was barely detectable in HMMs in the same condition (Figure 5E). ACTA2 was strongly positive in most of the
cells in the media and weakly positive for some of the cells in the intima (Figure 5F). Furthermore, we show representative sections from fatty streak lesions (Online Figure VII, A through C). Although the expression of CD68 and NCEH1 in fatty streak lesions decrease compared with atherosclerotic plaque, many of the CD68-positive cells were also positive for NCEH1 (Online Figure VII, B and C). CES1 was weakly expressed and LIPE was not expressed (data not shown). Thus, NCEH1 is the major enzyme that is specifically expressed in macrophages of human atherosclerotic lesions.

Discussion

Previously, we have reported that both Lipe and Nceh1 are involved in the hydrolysis of CE to a comparable degree in mice. Circumstantial evidence, however, suggests that there are great differences in the hydrolysis of CE between human and mouse macrophages. To understand the pathogenesis of atherosclerosis in humans, therefore, it is essential to determine the major enzyme that mediates the hydrolysis of CE hydrolysis in foam cell macrophages of human atherosclerotic lesions. In the present study, we show for the first time that NCEH1 is primarily involved in the hydrolysis of CE in human macrophages, thereby constituting the initial step toward reverse cholesterol transport in atherosclerotic lesions. We also show that contribution of CES1 and LIPE is trivial. Involvement of NCEH1 in atherosclerosis is further supported by its expression in situ in CD68-positive macrophages, which are abundant in human atheromatous plaques with cholesterol crystal in its necrotic core.

Recently, we have identified Nceh1 as a microsomal enzyme that mediates the hydrolysis of CE in mouse macrophages. In that report, we showed that the mRNA of a human ortholog of Nceh1 was markedly increased during the differentiation from human monocytes to mature macrophages. The present results confirm that the expression of NCEH1 protein was also markedly increased (Figure 1C). This level of induction was similar to the pattern of increase in neutral CE hydrolase activity (Figure 1A). Adenovirus-mediated overexpression of human NCEH1 in CE-loaded THP-1 macrophages decreased the cellular CE content by stimulating cholesterol efflux along with the expression of ABCA1 (Figure 2D; Online Figure V). On the other hand, inhibition of human NCEH1 by AS115, a KIAA1363 inhibitor, or RNA interference in HMMs significantly decreased neutral CE hydrolase activity (Figure 3E and 4B). Furthermore AS115 significantly decreased cholesterol efflux from HMMs which had been loaded with CE (Figure 3G). This silencing did not reduce the neutral CE hydrolase activity of HMMs (Figure 4B). Crow et al recently reported similar results: a recombinant CES1 protein did not possess neutral CE hydrolase activity. Interestingly, they found that treatment of cholesterol-loaded THP-1 macrophages with ether paraoxon (a nonspecific CES inhibitor) or benzil (a specific CES inhibitor) caused enhanced retention of intracellular CE. This puzzling finding led them to speculate that the retention of CE in human CE hydrolysis is due in part to inhibition of enzymes other than CES1. Indeed, KIAA1363, an ortholog of NCEH1, is potently inhibited by many of organophosphates (OP) including paraoxon.

There is a great difference in the ability to mobilize cholesterol between different types of macrophages. For example, THP-1 cells are known to be relatively ineffective at mobilizing cholesterol, primarily owing to slow hydrolysis of CE. In addition to the weak expression of Lipe, the absence of NCEH1 may explain the characteristics of THP-1 macrophages (Figure 1C). The expression of Lipe as a neutral CE hydrolase in human macrophages is also supported by the finding that 76-0079, a reportedly specific inhibitor of LIPE, did not significantly inhibit neutral CE hydrolase activity in HMMs (Figure 3C) and cholesterol efflux from HMMs which had been loaded with CE (Figure 3G). This can be extrapolated to other immortal cell lines of human macrophages: THP-1 and U937 macrophages. In mice, on the other hand, LIPE contributes to neutral CE hydrolase activity in MPMs. Thus, there seems to be a species difference in terms of the relative role of LIPE in the hydrolysis of CE in macrophages.

The rediscovery of CES1 in human macrophages provided a second twist in the history of the investigation of neutral CE hydrolase in macrophages. CES1 was originally identified as a human carboxylesterase in both liver and macrophages. The use of different names for CES1 has complicated matters. Ghosh found neutral CE hydrolase activity in CES1. Ghosh and colleagues subsequently reported that overexpression of CES1 inhibits the accumulation of CE in macrophages. We tried to reproduce their findings in our model. So far, we have been unable to detect neutral CE hydrolase activity when overexpressed either in HEK293 cells (Online Figure III) or in THP-1 macrophages (Figure 2B). Overexpression of CES1 did not reduce the amounts of CE in the cells even in the presence of the SOAT inhibitor (Online Figure V, B). Thus, it is unlikely that CES1 stimulates reesterification of cholesterol directly or indirectly, thereby mitigating the effect of increased hydrolysis of CE on the cholesterol efflux. Although CES1 expression was recognized to a level comparable to that in the liver or WAT (Figure 1C), its silencing did not reduce the neutral CE hydrolase activity of HMMs (Figure 4B). Crow et al recently reported similar results: a recombinant CES1 protein did not possess neutral CE hydrolase activity.

There is a great difference in the ability to mobilize cholesterol between different types of macrophages. For example, THP-1 cells are known to be relatively ineffective at mobilizing cholesterol, primarily owing to slow hydrolysis of CE. In addition to the weak expression of LIPE, the absence of NCEH1 may explain the characteristics of THP-1 macrophages (Figure 1C). There are many other examples of differences in neutral CE hydrolysis activity. It is tempting to speculate that these differences are attributable to the difference in the expression level of NCEH1. Furthermore, we examined the difference of the expression of NCEH1, CES1 and LIPE by M-CSF or GM-CSF treatment. NCEH1...
protein and neutral CE hydrolase activity proportionally increased (Online Figure II). In those M-CSF– or GM-CSF–treated HMMs, it is thought that NCEH1 greatly contributes to neutral CE hydrolase. It is further study to investigate difference of the contribution of NCEH1, CES1, and LIPE in macrophages stimulated by other cytokines or in different macrophages subclasses.

Finally, the expression of NCEH1 in CD68–positive macrophages in human atherosclerotic lesions in situ substantiates the role of NCEH1 in the development of atherosclerosis proposed above (Figure 5). NCEH1 was expressed in CD68–positive macrophages, but not in other cells such as smooth muscle cells and endothelial cells. This pattern of expression is very similar to that of SOAT1.35 The number of NCEH1–positive cells was generally larger in atheromatous plaques, which contain a necrotic center with a fibrous cap (Table). In contrast, samples with diffuse intimal thickening lacked NCEH1–positive cells. The abundance of these cells was moderate in fatty streak lesions (Online Figure VII, A through C). In spleen as representative of other organs, NCEH1 was abundantly expressed in CD68–positive cells in the marginal zone around white pulp (Online Figure VII, D and E).

Interestingly, the expression level of NCEH1 was not homogeneous in CD68–positive macrophages in those atherosclerotic lesions and spleen. Some CD68–positive macrophages weakly expressed NCEH1. Does this heterogeneity represent the presence of distinct subpopulations of macrophages? Does it just reflect the different stages of differentiation? These questions need to be answered to elucidate the precise pathogenesis of atherosclerosis.

In conclusion, we demonstrate for the first time that NCEH1 is quantitatively the most important neutral CE hydrolase in human macrophages and atherosclerosis. We also clearly show that the contribution of LIPE and CES1 is negligible compared with that of NCEH1, implying that we should be careful when findings in cholesterol metabolism of mice are extrapolated to those of humans. These findings indicate that NCEH1 is the only enzyme that requires attention when dealing with neutral CE hydrolase activity in human macrophages. Given its high levels in CD68–positive macrophages in initial fatty streaks, as well as in more advanced atheromatous plaques of human aortas, NCEH1 is a promising target for the treatment of atherosclerosis.

Acknowledgments
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Disclosures
None.

References
NCEH1 in Human Macrophages and Atherosclerosis

What Is Known?

- Intracellular hydrolysis of cholesterol ester (CE) is the initial and rate-limiting step for removal of cholesterol from macrophage foam cells that predominate in fatty streak atherosclerotic lesions.
- Hormone-sensitive lipase (LIP) and neutral cholesterol ester hydrolase (NCEH1) have comparable roles in CE hydrolysis in mouse macrophages, but the relative contribution of the hydrolytic enzymes in human macrophages is unknown.

What New Information Does This Article Contribute?

- NCEH1 is abundantly expressed in CD68-positive macrophages in cholesterol crystal-rich regions of human atherosclerotic lesions.
- NCEH1 is a promising therapeutic target for promoting reverse cholesterol transport, and provide a novel paradigm for understanding the pathogenesis of human atherosclerosis.
The Critical Role of Neutral Cholesterol Ester Hydrolase 1 in Cholesterol Removal From Human Macrophages

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**Supplemental Materials**

**Materials**—Phorbol 12-myristate 13-acetate (PMA), lecithin, bovine serum albumin fraction V (BSA), leupeptin, p-nitrophenyl butyrate, p-nitrophenol, apolipoprotein A-1 from human plasma were purchased from Sigma-Aldrich (St. Louis, MO). Cholesterol esterase from Pseudomonas sp., horseradish peroxidase, cholesterol oxidase, p-hydroxyphenylacetic acid, sodium taurocholate, recombinant human M-CSF and recombinant human GM-CSF were purchased from Wako Pure Chemicals (Osaka, Japan). Dibutyryl-cAMP sodium salt was purchased from Tocris Bioscience (Missouri, USA). AS115 was purchased from Cayman Chemical (Ann Arbor, MI). ‘76-0079’ (NNC 0076-0000-0079) was a gift from Novo Nordisk (Denmark). CS-505, a SOAT1 inhibitor, was provided by Sankyo and Kyoto Pharmaceuticals (Tokyo, Japan). Cholesterol [1-14C]oleate and [1-14C]oleic acid were purchased from Applied Biosystems (GE Healthcare UK Ltd., England).

**Antibodies**—Amino acid residues of human NCEH1 (83-164, 207-249 or 344-419) or CES1 (437-477) were expressed in bacteria as a glutathione S-transferase (GST)-fused protein, which was purified by glutathione affinity chromatography, and used for the immunization of rabbits according to standard protocols. From serum samples with high antibody titers, IgG
fractions were isolated using a protein G column (GE Healthcare, England). They were used as anti-NCEH1 or CES1 antibody. Serum of non-immunized rabbits was used as a control. We used three kinds of rabbit anti-LIPE polyclonal antibodies. One was used in the previous report \(^2\) and others were from Abcam (Cambridge, UK) and Cell Signaling Technology (Danvers, MA). The rabbit anti-human beta actin (ACTB) polyclonal antibody and the rabbit anti-human ABCG1 polyclonal antibody were from Abcam. The rabbit anti-human ABCA1 polyclonal antibody was from Novus Biologicals (Littleton, CO). The mouse anti-human CD68 Macrophage (clone: KP1) monoclonal antibody and the mouse anti-alpha 2 actin (ACTA2) monoclonal antibody were from DAKO (Tokyo, Japan). The rabbit anti-CES1 antibody used in immunocytochemistry and immunohistochemistry was described previously \(^3\). The rabbit anti-human SOAT1 polyclonal antibody was a generous gift from Dr. T.Y.Chang.

**Human tissue preparation**—Human liver and adipose tissue samples for Western blotting and quantitative real-time PCR were obtained at surgery with informed consent. Liver tissue was from the non-tumor tissue of a subject with chronic hepatitis B and hepatocellular carcinoma. Adipose tissue was from the great omentum near the surgical incision close to the umbilicus of a subject with umbilical hernia. Adipose tissue and liver tissue were respectively
homogenized in buffer A and used for Western blotting. Total RNA was isolated from those tissues using TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA) and used for quantitative real-time PCR. The protocol of the experiments was approved by the institutional ethics committee of Jichi Medical University.

**Cells**—HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) FBS. THP-1 and U937 cells were cultured in RPMI-1640 medium containing 10% (v/v) FBS and differentiated into macrophages by treatment with 100 nmol/L PMA for 48 hours. Mononuclear cells were isolated from peripheral blood of healthy donors using Lymphoprep (NYCOMED, Roskilde, Denmark), suspended in RPMI-1640 medium and seeded onto 6-cm dishes \((4 \times 10^6 \text{ cells per dish})\). After 1 hour of incubation for adherence, the medium was replaced with RPMI-1640 medium supplemented with 10% FBS. Adhered monocytes were incubated at 37°C in 5% CO\(_2\) for 6-8 days to induce differentiation into macrophages.

**Preparation of lipoproteins**—After an overnight fast, blood was collected from normolipidemic volunteers to isolate plasma. LDL \((d\ 1.019-1.063 \text{ g/mL})\) and HDL \((d\ 1.063-1.21 \text{ g/mL})\) were isolated from the plasma by sequential density ultracentrifugation \(^4\).
LDL was acetylated by repeatedly adding acetic anhydride \(^5\). To prepare oxidized LDL, LDL was incubated for 18 hours at 37°C with 5 mmol/L CuSO\(_4\), followed by the addition of 2.5 mmol/L EDTA \(^6\). Aggregated LDL was generated as described by Khoo et al. \(^7\) just before addition to cultured cells. Sterile LDL (0.2 mg of protein per mL) was subjected to high speed vortexing for 30 seconds. The preparation was immediately diluted to 20 µg of protein per mL with RPMI-1640 medium. β-very-low-density lipoprotein (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.

**cDNA cloning for the expression of recombinant proteins**—Total RNA isolated from THP-1 macrophages and human adipocytes was transcribed into cDNA using Thermoscript RNase H - reverse transcriptase (Invitrogen) following the manufacturer’s protocol. The coding sequences of *NCEH1* (GenBank accession No. NM_020792) and *CES1* (NM_001025195) were amplified by PCR from macrophage cDNA using Amplitaq DNA polymerase (Roche Applied Science, Basel, Switzerland). Likewise the coding sequences of *LIPE* were amplified from human adipose tissue cDNA. The primers used for PCR were as follows:
Recombinant adenovirus for NCEH1, CES1 and LIPE expression (Ad-NCEH1, Ad-CES1 and Ad-LIPE)—NCEH1, CES1 or LIPE cDNA fragment was amplified by PCR from human cDNA containing plasmids (described above) using forward and reverse primers. LacZ cDNA was amplified by PCR from the SV40 β-galactosidase vector (Promega, Madison, WI). The PCR products were ligated into pENTER4 (Invitrogen). Subsequently, NCEH1, CES1 or LIPE cDNA fragment was subcloned into the pAd/CMV/V5-DEST vector by Gateway Technology (Invitrogen). The adenoviral vectors were linearized using restriction enzymes and transfected into HEK293 using Superfect Transfection Reagent. Large-scale production of high-titer recombinant Ad-NCEH1, Ad-CES1, Ad-LIPE, or Ad-LacZ was performed as described previously. The purified viruses were stored in 10% (v/v) glycerol/
phosphate-buffered saline (PBS) at -80 °C.

Recombinant adenovirus expressing short-hairpin (sh) RNA directed against NCEH1 and CES1. —The recombinant adenovirus expressing shRNA for NCEH1 (Ad-shNCEH1) or CES1 (Ad-shCES1) was produced by Gateway Technology (Invitrogen).

The following oligonucleotides were used:

shNCEH1 sense template: 5´- GT GAG TGA CCT GAT CTA CTA CGT GTG CTG TCC GTA GTG GAT CAG GTT ACT CAC -3´.

shNCEH1 antisense template: 5´- GT GAG TAA CCT GAT CCA CTA CGG ACA GCA CAC GTA GTA GAT CAG GTC ACT CAC-3´.

shCES1 sense template: 5´- GC TTC GAG CTG AGA GGA ATT TGT GTG CTG TCC AAA TTC CTT TCA GCT TGA AGC -3´.

shCES1 antisense template: 5´- GC TTC AAG CTG AAA GGA ATT TGG ACA GCA CAC AAA TTC CTC TCA GCT CGA AGC -3´.

An adenovirus containing shRNA for β-galactosidase (Ad-shLacZ) was used as a control.

Human mononuclear cells were seeded onto 6 - cm dishes (4 × 10⁶ cells per dish) and differentiated into HMMs. After transduction with Ad-shNCEH1, Ad-shCES1 or Ad-shLacZ for
48 hours at 37 °C, cells were harvested and whole cell lysate was assayed for enzymatic activities.

**Quantitative Real-Time PCR**—Two micrograms of total RNA were reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed using Light Cycler 480 SYBER Green I Master kit (Rosh Applied Science) in Light Cycler 480 System II (Roche Applied Science). The relative abundance of each transcript was calculated from a standard curve of cycle thresholds for serial dilutions of a cDNA sample. Primer sequences are as follows.

**LIPE**

forward: 5’-CTCCTCATGGCTCAACTCCTTTCC-3’

reverse: 5’-AGGGTCTTGACTATGGGTG-3’

**ACTB**

forward: 5’-AGAGATGGCCACGGCTT-3’

reverse: 5’-ATTTGCAGTGGACGATGAG-3’

**Determination of neutral CE hydrolase activity**

Neutral CE hydrolase activity was determined essentially as described by Hajjar et al. Alternatively, neutral CE hydrolase activity was determined by the method described by Ghosh et al. In brief, the assay mixture contained 100 mmol/L Tris HCl buffer pH 7.5 containing 5
mmol/L 2-mercaptoethanol and 50 µg enzyme protein in a final volume of 500 µl. The reaction was started by the addition of [1-%C]cholesteryl oleate in acetone and incubated for 60 minutes at 37 °C.

**MTT assay**—MTT cell proliferation assay kit was purchased from Cayman Chemical (Ann Arbor, MI). Assay was performed following manufacture’s protocol.

**Immunocytochemistry**—Cells were fixed with 4% paraformaldehyde after being washed with PBS. The samples were incubated in 3% H₂O₂ in PBS to quench endogenous peroxidase activity, and washed with PBS, and non-specific binding sites were blocked by incubation with 3% BSA. The samples were then incubated with affinity-purified anti-NCEH1 polyclonal antibody (1:250) or anti-CES1 polyclonal antibody (1:1,000). After being washed with PBS, the samples were incubated with a biotinylated goat anti-rabbit antibody (1:200, Vector Laboratories, Burlingame, CA). The antibody-specific staining was visualized with a Vecstain ABC reagent (Vector) and diamine benzidine, resulting in a brown precipitate. Cell nuclei were counterstained with methylgreen.

**Immunohistochemistry** — The primary antibodies used were a mouse anti-CD68 monoclonal antibody (1:100 dilution), a rabbit anti-NCEH1 polyclonal antibody (1:2,000 dilution)
dilution), a rabbit anti-CES1 polyclonal antibody (1:100,000 dilution), a rabbit anti-LIPE polyclonal antibody (Abcam, 1:1,200 dilution) and a mouse anti-ACTA2 monoclonal antibody (1:50). For Antigen retrieval, the deparaffinized sections were placed in 10 mmol/L citrate buffer (pH 6.0), heated to 121 °C in an autoclave for 10 minutes. The sections were incubated at 4 °C overnight with either antibody and washed 3 times with TBS. Endogenous peroxidase was blocked with 3% hydrogen peroxide in TBS for 10 minutes and washed with TBS. The sections were incubated with a biotinylated secondary antibody for 30 minutes at room temperature and were then reacted with a streptavidin–biotin peroxidase reagent (Vectastatin Elite ABC Kit, Vector Laboratories, Burlingame, CA). The antigen was visualized with a chromogen, diaminobenzidine, in 3% hydrogen peroxide, and the sections were counterstained with hematoxylin, dehydrated and mounted. Control immunohistochemical staining with nonimmune rabbit IgG showed only background staining.

Statistical analyses—Results are presented as the mean ± S.D.. Statistical differences between groups were analyzed by one-way analysis of variance and the Bonferroni post hoc analysis, or Student's t test. All calculations were performed with STAT view version 5.0 for Macintosh (SAS Institute Inc.).
Supplemental Results

**Determination of neutral CE hydrolase activities**—We determined neutral CE hydrolase activity essentially as described by Hajjar et al.8. This assay gave us a significant activity for LIPE and a slightly significant activity for NCEH1. However, we could not detect a significant increase in the neutral CE hydrolase for CES1 (Online Figure III, B), despite high PNPB hydrolase activity of CES1 (Online Figure III, C). Furthermore, we tried to faithfully employ the method used by Ghosh et al. to measure neutral CE hydrolase activities of the overexpressed CES19-11. The results were similar to those given by the method of Hajjar, which was mainly used in this study (Online Figure III, D). Since Ghosh et al. used a modified method of Chen et al.11, we also used this original method. However, even with this method, neutral CE hydrolase activity of CES1 was not detectable (data not shown).

In summary, method by both Hajjar and Ghosh seem to be optimized to measure neutral cholesterol hydrolase activity of LIPE. As for NCEH1, both methods were sensitive enough to detect its neutral cholesterol hydrolase activity. However, none of these assays was capable to detect neutral cholesterol hydrolase activity of the CES1.
**Specificity of inhibitors**—We tested the specificity of 76-0079 or AS115 (Fig. 3A, B).

We examined the effects of 76-0079 on neutral CE hydrolase (Fig. 3A) or PNPB hydrolase activity (Fig. 3B) in whole cell lysate of HEK293 cells transfected with adenovirus expressing NCEH1, CES1 or LIPE. 76-0079 inhibited the neutral CE hydrolase activity of LIPE almost completely, while it did not inhibit the neutral CE hydrolase activity of NCEH1 (Fig. 3A).

76-0079 inhibited not only the PNPB hydrolase activity of LIPE but also that of CES1 almost completely, while it did not inhibit the PNPB hydrolase activity of NCEH1 (Fig. 3B). Thus, 76-0079 is an inhibitor of both LIPE and CES1. AS115, KIAA1363 inhibitor 12, inhibited not only the neutral CE hydrolase activity and the PNPB hydrolase activity of NCEH1 but also those of LIPE and CES1 almost completely. Thus, AS115 is an inhibitor of LIPE, NCEH1 and CES1. Although 76-0079 and AS115 are non-specific inhibitors, we can estimate the contribution of NCEH1 by the difference between the effects of those inhibitors.

**Specificity of the antibodies**—We tested whether the antibodies specifically recognize the proteins (Online Figure VI). Some of the Hela cells infected with Ad-\textit{NCEH1} were positive
for staining with the anti-NCEH1 antibody, but not with the anti-CES1 antibody (Online Figure VI, A). Vice versa, some of the Hela cells infected with Ad-CES1 were positive for staining with the anti-CES1 antibody, but not with the anti-NCEH1 antibody (Online Figure VI, B). These results indicate that both antibodies against NCEH1 and CES1 are specific. To confirm whether these antibodies are useful to detect the expression of endogenous proteins in human samples, HMMs were immunostained with the anti-NCEH1 or CES1 antibody. Either NCEH1 or CES1 was detected by the respective antibody in HMMs (Online Figure VI, C).

**The expression of NCEH1 in spleen**— We investigated the expression of NCEH1 in human spleen (Online Figure VII, D and E). CD68 positive cells and NCEH1 positive cells were particularly abundant in the marginal zone around white pulp. Some of the CD68-positive cells were also positive for NCEH1. CES1 was weakly positive and LIPE protein was barely detectable (data not shown).
Supplemental References


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Online Figure I. NCEH1, CES1 and LIPE expressed in HMMs

(A) Quantification of NCEH1 or CES1 in HMMs. The amino acid residues of NCEH1 (amino acids 83-164) and CES1 (amino acids 437-477) were expressed in bacteria as a glutathione S-transferase (GST) fusion protein. NCEH1-GST or CES1-GST was purified by glutathione affinity chromatography. The theoretical molecular weights of NCEH1-GST and CES1-GST were 48.5 kDa and 44.1 kDa, respectively. The indicated amounts of GST, GST-NCEH1 or GST-CES1 and 5 µg of protein prepared from HMMs were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with anti-NCEH1 antibody or anti-CES1 antibody, which was purified from antiserum raised against the GST-fused proteins. The density of the band which corresponded to HMMs, NCEH1-GST, CES1-GST or GST was quantified using NIH image. A linear correlation was recognized between the amounts of GST-fusion proteins and density of NCEH1-GST minus density of GST, or density of CES1-GST minus density of GST. Moles of NCEH1 or CES1 in HMMs were calculated by using these regression lines as standard curves. (B) LIPE mRNA was measured in HMMs, liver (L) or adipose tissue (A) by quantitative real-time PCR. Data are presented as the mean ± S.D. of 5 measurements. * P < 0.05, ** P < 0.01 (determined by the 2-tailed Student’s t test).
Online Figure II. The effect of M-CSF or GM-CSF in HMMs

Peripheral blood monocytes were differentiated into HMMs with or without 15 ng/ml of M-CSF or GM-CSF. Neutral CE hydrolase activity (A) and protein expression of NCEH1, CES1 and LIPE (B) were examined. Ten micrograms of cell lysate were subjected to Western blotting. Data are presented as the mean ± S.D. of 4 measurements. * P < 0.05, ** P < 0.01 (determined by ANOVA followed by the Bonferroni post hoc analysis).
Online Figure III. Determination of neutral CE hydrolase activity

HEK293 cells were transfected with Ad-LacZ, Ad-NCEH1, Ad-CES1 or Ad-LIPE. Ten micrograms of the sonicated cells were used for Western blot analyses (A) and measurements of neutral CE hydrolase activity (B and D) and PNPB hydrolase activity (C). Neutral CE hydrolase activity was determined by the method by Hajjar et.al. (B) or Ghosh et.al. (D). Data are presented as the mean ± S.D. of 3 measurements. * P < 0.05, ** P < 0.01 (determined by the 2-tailed Student’s t test).
Online Figure IV. Overexpression of NCEH1, CES1 or LIPE in THP-1 macrophages which had been loaded with oxidized LDL, aggregated LDL or β-VLDL.

THP-1 macrophages were incubated with 50 µg/mL of oxidized LDL (A), 20 µg/mL of aggregated LDL (B) or 10 µg/mL of β-VLDL (C) for 24 hours. After infection with Ad-LacZ, Ad-NCEH1, Ad-CES1 or Ad-LIPE at 300 moi, the cells were incubated with a medium containing 50 µg/mL of oxidized LDL, 20 µg/mL of aggregated LDL or 10 µg/mL of β-VLDL and 250 µg/mL of HDL. Three days after the infection, the cells were used for the measurement of CE mass. Data are presented as the mean ± S.D. of 5 measurements. * P < 0.05, ** P < 0.01. Ad-NCEH1 vs. Ad-LacZ, Ad-CES1 vs. Ad-LacZ, or Ad-LIPE vs. Ad-LacZ (determined by ANOVA followed by the Bonferroni post hoc analysis).
**Online Figure V. Cholesterol efflux from cholesterol-loaded THP-1 macrophages overexpressing NCEH1, CES1 or LIPE**

(A) THP-1 macrophages were loaded with cholesterol by incubating the cells for 24 hours with 100 µg/mL aC-LDL in the presence of indicated density of CS-505. Lipids were extracted and cellular CE mass was measured. THP-1 macrophages were loaded with cholesterol by incubating the cells for 24 hours with 100 µg/mL aC-LDL. After 1 day equilibration and adenoviral infection at 300 moi, cholesterol efflux was initiated by the addition of 100 µg/mL HDL (B and D) or 15 µg/mL apolipoprotein A-1 (C), in the presence of 10 µmol/L CS-505 and continued for 12 hours. Efflux was compared in the presence or absence of CS-505 (B) or 300 µmol/L dibutylryl cAMP (D). Lipids were extracted after termination of efflux and cellular CE mass was measured. In the same condition, the prepared cells were used for Western blot analyses for ABCA1 or ABCG1 (E). Data are presented as the mean ± S.D. of 3 (A) or 6 (B, C and D) measurements. * P < 0.05, ** P < 0.01 (determined by ANOVA followed by the Bonferroni post hoc analysis).
Online Figure VI. Specificity of antibodies against overexpressed NCEH1 or CES1 in Hela cells

Hela cells infected with 1 moi of the recombinant adenovirus carrying NCEH1 (A) or CES1 (B) were immunostained by the anti-NCEH1 antibody, anti-CES1 antibody or rabbit IgG as described in Materials and Methods. HMMs were immunostained with anti-NCEH1 antibody, anti-CES1 antibody or rabbit IgG similarly (C). Original magnifications: (A and B), ×40; (C), ×400.
Online Figure VII. Expression of NCEH1 in human tissues
Sections of aorta from Case 3 which contained fatty streak (A, B and C) or spleen (D and E) were used for histological examination. HA staining (A). Immunohistochemistry for CD68 (B and D), NCEH1 (C and E). Original magnifications: ×40 (A through C); ×100 (D and E).