Biochemical and Biophysical Research Communications 404 (2011) 254–260

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Abrogation of neutral cholesterol ester hydrolytic activity causes adrenal enlargement

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ARTICLE INFO

Article history:
Received 22 November 2010
Available online 25 November 2010

Keywords:
Adrenal glands
Nceh1
Lipe
Cholesterol ester

ABSTRACT

We have previously demonstrated that neutral cholesterol ester hydrolase 1 (Nceh1) regulates foam cell formation and atherogenesis through the catalytic activity of cholesterol ester hydrolysis, and that Nceh1 and hormone-sensitive lipase (Lipe) are responsible for the majority of neutral cholesterol ester hydrolase activity in macrophages. There are several cholesterol ester-metabolizing tissues and cells other than macrophages, among which adrenocortical cells are also known to utilize the intracellular cholesterol for steroidogenesis. It has been believed that the mobilization of intracellular cholesterol ester in adrenal glands was facilitated solely by Lipe. We herein demonstrate that Nceh1 is also involved in cholesterol ester hydrolysis in adrenal glands. While Lipe deficiency remarkably reduced the neutral cholesterol ester hydrolase activity in adrenal glands as previously reported, additional inactivation of Nceh1 gene completely abrogated the activity. Adrenal glands were enlarged in proportion to the degree of reduced neutral cholesterol ester hydrolase activity, and the enlargement of adrenal glands and the accumulation of cholesterol esters were most pronounced in the Nceh1/Lipe double-deficient mice. Thus Nceh1 is involved in the adrenal cholesterol metabolism, and the cholesterol ester hydrolytic activity in adrenal glands is associated with the organ enlargement.

1. Introduction

Adrenocortical cells require constant supply of cholesterol as a precursor for the conversion of steroid hormones. Cholesterol delivery in the adrenal glands involves three major processes: (1) uptake of lipoprotein-derived cholesterol via low density lipoprotein receptor (LDLR) mediated endocytic pathways and scavenger receptor class B member 1 (SCARB1)-mediated "selective" uptake pathways (2) endogenous cholesterol biosynthesis in endoplasmic reticulum (ER) (3) cholesterol mobilization from intracellular cholesterol esters (CEs) stored in lipid droplets [1]. Regarding the cholesterol uptake, adrenal glands of rodents are largely dependent on SCARB1-mediated selective uptake pathway while LDLR pathway plays a pivotal role in human adrenal cholesterol metabolism [2–4]. In the selective uptake process, lipoproteins bind to SCARB1 through which CEs are delivered into the cells. The delivered CEs should be hydrolyzed to be utilized for steroidogenesis by non-lysosomal neutral lipase(s). Therefore, CE hydrolysis plays a pivotal role not only in the break down of stored lipids but also in the lipoprotein uptake and utilization. The resultant unesterified cholesterol is transported to mitochondria by the steroidogenic acute regulatory protein (StAR) [5], where it is converted into the different steroid hormones by a battery of oxidative enzymes [6].

Hormone-sensitive lipase (Lipe) was reported to catalyze the liberation of free cholesterol from its esterified form in adrenal glands and to be involved in adrenal steroidogenesis [7]. Lipe is an intracellular neutral lipase which catalyzes the hydrolysis of triacylglycerol (TG), diacylglycerol, monoacylglycerol, CEs, phospholipids, retinyl ester and other lipids in a wide variety of tissues and cells [8–11]. Among the cells expressing Lipe, the hydrolysis of intracellular CEs in macrophages has been a matter of debate,

Abbreviations: LDL, low density lipoprotein; CE, cholesterol ester; Scarb1, scavenger receptor class B member 1; ER, endoplasmic reticulum; nCEH, neutral cholesterol ester hydrolase; StAR, steroidogenic acute regulatory protein; Nceh1, neutral cholesterol ester hydrolase 1; Lipe, hormone-sensitive lipase; ACTH, adrenocorticotropic hormone; Hmgcs1, 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1; Abca1, ATP-binding cassette sub-family A member 1; Soat1, sterol O-acyltransferase 1; Soat2, sterol O-acyltransferase 2.

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doi:10.1016/j.bbrc.2010.11.103

and Lipe has been one of the candidates of macrophage CE hydrolases. However, circumstantial evidences have suggested the existence of unknown lipase(s). We recently identified a promising lipase, neutral cholesterol ester hydrolase 1 (Nceh1) [12], and demonstrated that Nceh1 and Lipe are the major CE hydrolases in macrophages taking advantage of targeted gene deletion [13].

Nceh1 is highly expressed in macrophages and the expression is also observed in brain, kidney, heart and other tissues. Nceh1 liberates fatty acids from CEs, and also from TG to a lesser extent [12]. In terms of structural aspects, Nceh1 is inserted into the ER membrane by an N-terminal single-spanning transmembrane domain [14]. Lipe possesses an N-terminal domain which interacts with fatty acid binding protein 4 [15] whereas Nceh1 lacks such a domain. Lipe is also reported to interact with StAR [16] and perilipin [17]. The C-terminal domain of Nceh1 is composed of α/β hydrolase folds that accommodate the catalytic motif which is almost identical to that of Lipe in amino acid sequence. Adenovirus-mediated overexpression of Nceh1 blocked foam cell formation in THP-1 macrophages [12].

We herein demonstrate that Nceh1 is also involved in the adrenal CE metabolism. To the best of our knowledge, this is the first published report that lack of Lipe resulted in a significant increase of adrenal weight, which was more pronounced in adrenal glands in *Nceh1/Lipe* double-deficient mice. Lipe was the major neutral CE hydrolase (nCEH) in adrenal glands while Nceh1 was also responsible for lesser but significant amounts of nCEH activity. The extent of reduction of nCEH activity was correlated with the adrenal enlargement as well as adrenal lipid contents.

2. Materials and methods

2.1. Mice

Nceh1-deficient, Lipe-deficinet, or Nceh1/Lipe double-deficient mice were generated as described previously [13,18]. All mice used in this study were crossed onto the C57BL/6J background for more than 10 generations. Mice were maintained on a 12 h dark/light cycle and were fed a normal chow diet (Lab Diet 5053, PMI Nutrition International). Mice were euthanized at 9 weeks at the beginning of the dark cycle on ad libitum, unless otherwise stated. All experimental procedures handling animals were conducted according to our institutional guidelines.

2.2. Preparation of adrenal glands

Adrenal glands were removed, dissected free of fat under stereoscopic microscope, rinsed in PBS, blotted dry and weighed individually on an electronic platform scale (AG135, Mettler Toledo, Tokyo, Japan).

2.3. Determination of adrenal CE content

Adrenal lipids were extracted by methanol/chloroform, and CE content was determined by an enzymatic fluorometric microassay [13].

2.4. Determination of adrenal DNA content

Adrenal glands were homogenized and sonicated. The DNA content was measured by fluorescent method with Hoechst 33258 [19].

2.5. nCEH activity assay

Enzyme activity was assayed basically as described previously [18]. The homogenates were incubated at 37 °C for 60 min in a final volume of 200 μl of a reaction mixture containing 6.14 μmol/l cholesterol [1-^{14}C] oleate (48.8 μCi/μmol), 23.7 μmol/l lecithin, 12.5 μmol/l sodium taurocholate, and 85 mmol/l potassium phosphate (pH 7.0).

2.6. Histology

Sections were stained with hematoxylin–eosin or immunostained as follows. After incubation with primary antibody (anti-mouse ki-67 clone TEC-3 1:50; DAKO or anti-Nceh1 antibody 1:8000, respectively) for overnight at 4 °C, the sections were incubated with secondary antibody for 30 min at room temperature and then with EnVision plus system (DAKO) or Vectastain elite ABC standard kit (Vector), respectively for 30 min at room temperature. Finally, the sections were developed with DAB (Wako Pure Chemicals Co.) and counterstained with hematoxylin.

2.7. Statistics

Statistical differences between groups were analyzed by one-way ANOVA and a Tukey–Kramer posthoc test, unless stated otherwise.

3. Results

3.1. Genetical inactivation of Nceh1 and/or Lipe results in an increase in adrenal weights

While attempting to reveal as-yet-unidentified physiological roles of Nceh1, we noted the considerable enlargement of adrenal glands in *Nceh1/Lipe* double-deficient mice (Fig. 1A). Consistent with the visual observations, deficiency of Nceh1 and/or Lipe increased adrenal weights although the effect of Nceh1 deficiency was relatively small compared to Lipe deficiency (increased by 10% and 26% in *Nceh1*-deficient mice and *Lipe*-deficient mice, respectively (Fig. 1C). The enlargement of adrenal glands was most pronounced in *Nceh1/Lipe* double-deficient mice (62% increase). Adrenal glands of male mice are normally smaller than those of female mice [20], that was also reproduced in our experimental setting, and the enlargement of adrenal glands caused by the deficiency of lipase(s) was observed in both genders (data not shown). Although the adrenal mass is under systemic hormonal control, it has been reported that several intra-adrenal molecules directly affect adrenal mass [21–23]. Indeed, we were able to detect NCEH1 protein expression in adrenal glands as well as LIPE (Fig. 1B), therefore we attempted to reveal the physiological roles of Nceh1 and Lipe in adrenal glands.

To address whether the adrenal enlargement is due to cellular hypertrophy and/or hyperplasia, adrenal DNA content was measured (Fig. 1D). DNA content was mildly increased solely in *Nceh1/Lipe* double-deficient adrenal glands, and weight/DNA ratio, which reflects the average weight per cell, was significantly increased solely in *Nceh1/Lipe* double-deficient adrenal glands (Fig. 1E). These results indicated that the adrenal enlargement in *Nceh1/Lipe* double-deficient mice was caused by both cellular hypertrophy and hyperplasia. Since plasma adrenocorticotropic hormone (ACTH) levels were similar among groups, the adrenal enlargement was not due to the activation of hypothalamic pituitary adrenal axis (Fig. 1F).

3.2. Nceh1 and/or Lipe deficiency reduced adrenal nCEH activity and caused cellular CE accumulation

To explore the underlying mechanism, the adrenal glands of each genotype were examined histologically (hematoxylin–eosin

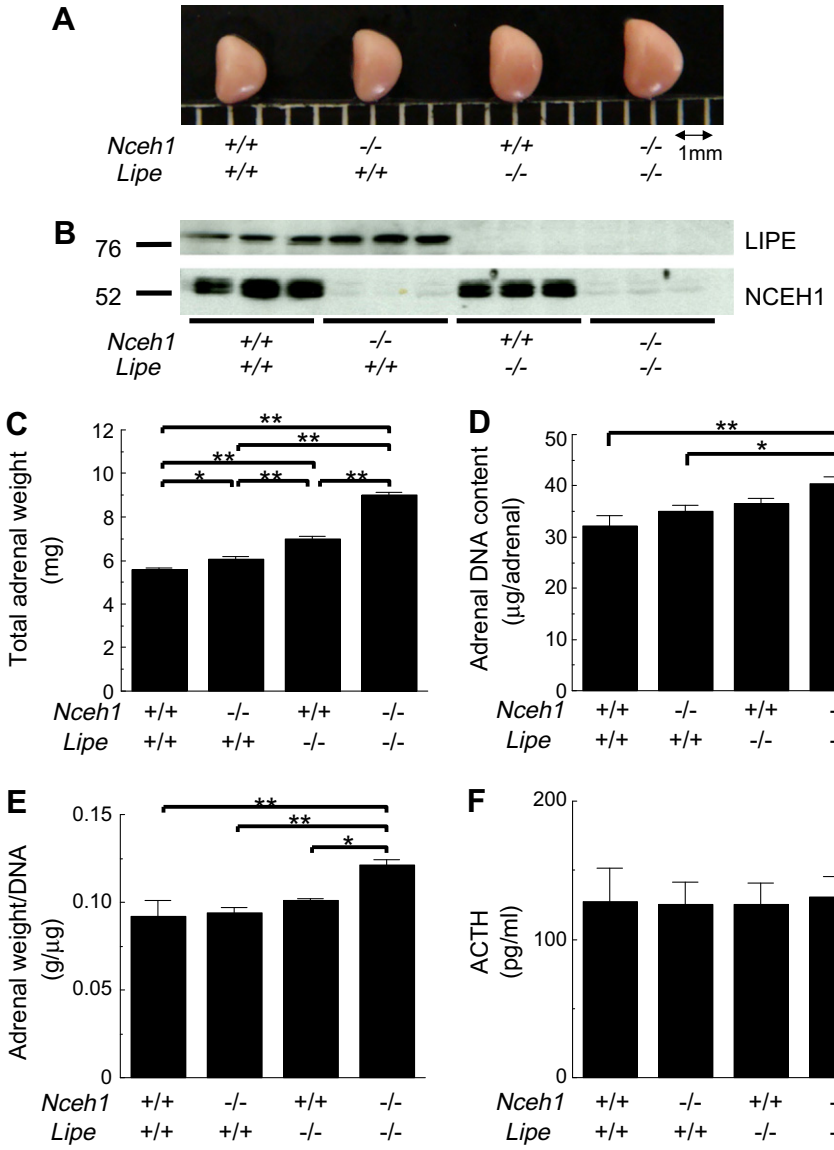

Fig. 1. Adrenal enlargement induced by the deficiency of Nceh1 and/or Lipe. Female mice were euthanized at 9 weeks of age. (**A**): Gross appearance of left adrenal glands from female mice of each genotype. (**B**): Adrenal expression of LIPE and NCEH1. (**C**): The pared adrenal glands removed from each mouse were weighed on an electronic platform scale (n = 16–29). (**D**): The adrenal DNA content was measured by a fluorescent method (n = 9–10). (**E**): The adrenal weight was normalized with respect to DNA content (n = 9–10). (F): Levels of ACTH were determined in serum samples (n = 6–8). Data are expressed as means ± SEM. *and **denote $P < 0.05$ and $P < 0.01$, respectively as determined by ANOVA followed by the Tukey–Kramer posthoc test.

stain). We found a marked accumulation of lipid droplets in zona fasciculata and zona glomerulosa in *Lipe*-deficient adrenal glands as reported previously [24] whereas Nceh1 deficiency had only marginal effects on the accumulation of lipids. However, compared with inactivation of Lipe alone, the additional inactivation of Nceh1 caused most pronounced lipid accumulation in the adrenal glands (Fig. 2A). Since adrenal glands are morphologically heterogeneous and are composed of the outer cortex and inner medulla, we attempted to determine the localization of these lipases. LIPE is known to be expressed in adrenal cortex [25], therefore, the localization of NCEH1 protein in adrenal glands was determined by immunohistochemistry. Immunohistochemistry clearly detected NCEH1 expression virtually exclusively in zona fasciculata while adrenal glands from *Nceh1*-deficient mice gave no positive signal (Fig. 2B). In addition, immunohistochemical staining for Ki-67 antigen was employed to assess the proliferative activity of adrenocortical cells since Ki-67 antigen is present only in the nuclei of cycling cells. Consistent with the assessment of DNA content, Ki-67 positive cells were scarcely detected even in the adrenal glands of *Nceh1/Lipe* double-deficient mice (data not shown). The histological observations were further reinforced by the quantification of the adrenal lipid content (Fig. 3A and B). Nceh1 and/or Lipe deficiency actually increased the cellular CE content although Nceh1 deficiency increased cellular CE to a lesser extent than Lipe deficiency. Consistent with these results, inactivation of Nceh1 and/or Lipe decreased nCEH activity in adrenal glands (Fig. 3C–E).

3.3. The organomegaly was observed in an adrenal-specific manner

Since Nceh1 and Lipe are known to be expressed in a wide variety of tissues and cells [7,8,12], we further examined the nCEH activity in other tissues than adrenal glands. These two lipases were responsible for the majority of the nCEH activity in liver, white adipose tissues, brain, kidney and heart (Fig. 4A–E). In particular, the extent of contribution of Nceh1 and Lipe to the nCEH activity in white adipose tissues, brain and heart was similar to that observed in adrenal glands (Nceh1 and Lipe were responsible for more than 90% of nCEH activity in these tissues). However, no

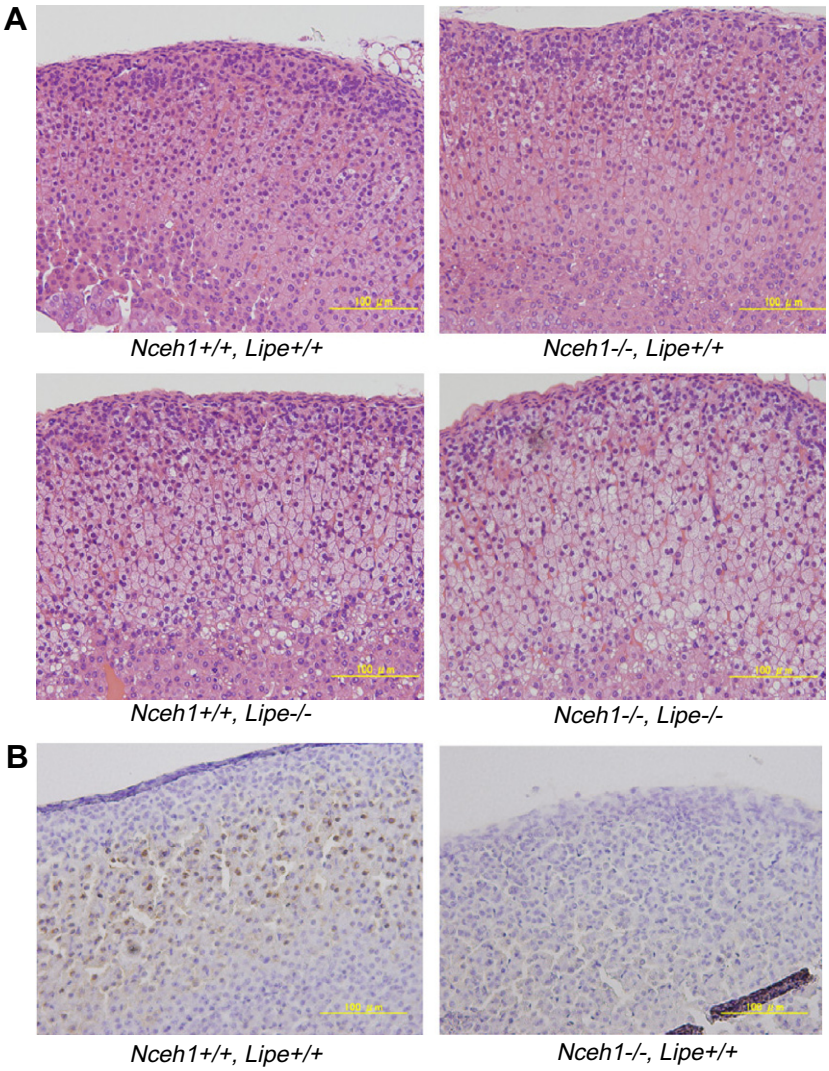

Fig. 2. Histological analysis of adrenal glands. Histological analysis of adrenal glands of female mice (9 weeks of age). (**A**): Representative photomicrographs of hematoxylin/eosin sections of adrenal cortex. (**B**): Immunohistochemical localization of NCEH1 in adrenal cortex. Frozen sections of adrenal glands from wild-type and *Nceh1*-deficient mice were incubated with anti-NCEH1 polyclonal antibody and counterstained with hematoxylin. Objective magnifications are 200×.

organomegaly was observed in the tissues other than adrenal glands (Supplementary Table 1).

3.4. Gene expression analysis

Next, we investigated the levels of expression of key regulators of cholesterol metabolism (Supplementary Fig. 1). Lipe deficiency affected the gene expression in adrenal glands while Nceh1 deficiency had only marginal effects. Lipe deficiency upregulated the genes involved in cholesterol biosynthesis (represented here by 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (*Hmgcs1*)). Lipe deficiency also repressed the expression of ATP-binding cassette sub-family A member 1 (*Abca1*) and *Scarb1*. The expression of the other key molecules involved in steroidogenesis was not affected by genotype (e.g., *Star* and sterol O-acyltransferase 1 (*Soat1*, also known as ACAT1)).

3.5. The adrenal response to ACTH was not affected by the deficiency of *Nceh1* and/or *Lipe*

Since uptake of extracellular lipoprotein is an important source of cholesterol in steroidogenic tissues, we initially evaluated serum lipid profile prior to the evaluation of the effects of lipase deficiency on ACTH-stimulated responses. As we reported elsewhere [13], Lipe deficiency elevated the serum HDL-cholesterol concentrations while Nceh1 deficiency did not significantly alter the serum cholesterol concentrations (data not shown). Although Lipe-mediated CE hydrolysis was reported to play a crucial role in ACTH-stimulated corticosterone secretion, we could not detect any significant effects of Lipe deficiency on adrenal corticosterone production in both ACTH-stimulated [18] and leptin-deficient [26] conditions. In this study, we evaluated the adrenocortical response to ACTH not only in *Lipe*-deficient mice but also in *Nceh1*-deficient and *Nceh1/Lipe* double-deficient mice. However, using an acute single-dose ACTH regimen, there was no significant difference among genotype in both the basal and ACTH-stimulated state, which was totally consistent with our previous reports [18,26] (Supplementary Fig. 2).

4. Discussion

It has been reported that Lipe plays multifaceted roles in adrenal steroidogenesis. Lipe was reported to be responsible for the majority of the adrenal nCEH activity and utilize the stored lipid for steroidogenesis [25]. Lipe binds to StAR, which escorts cholesterol across the mitochondrial membrane [16]. It was also

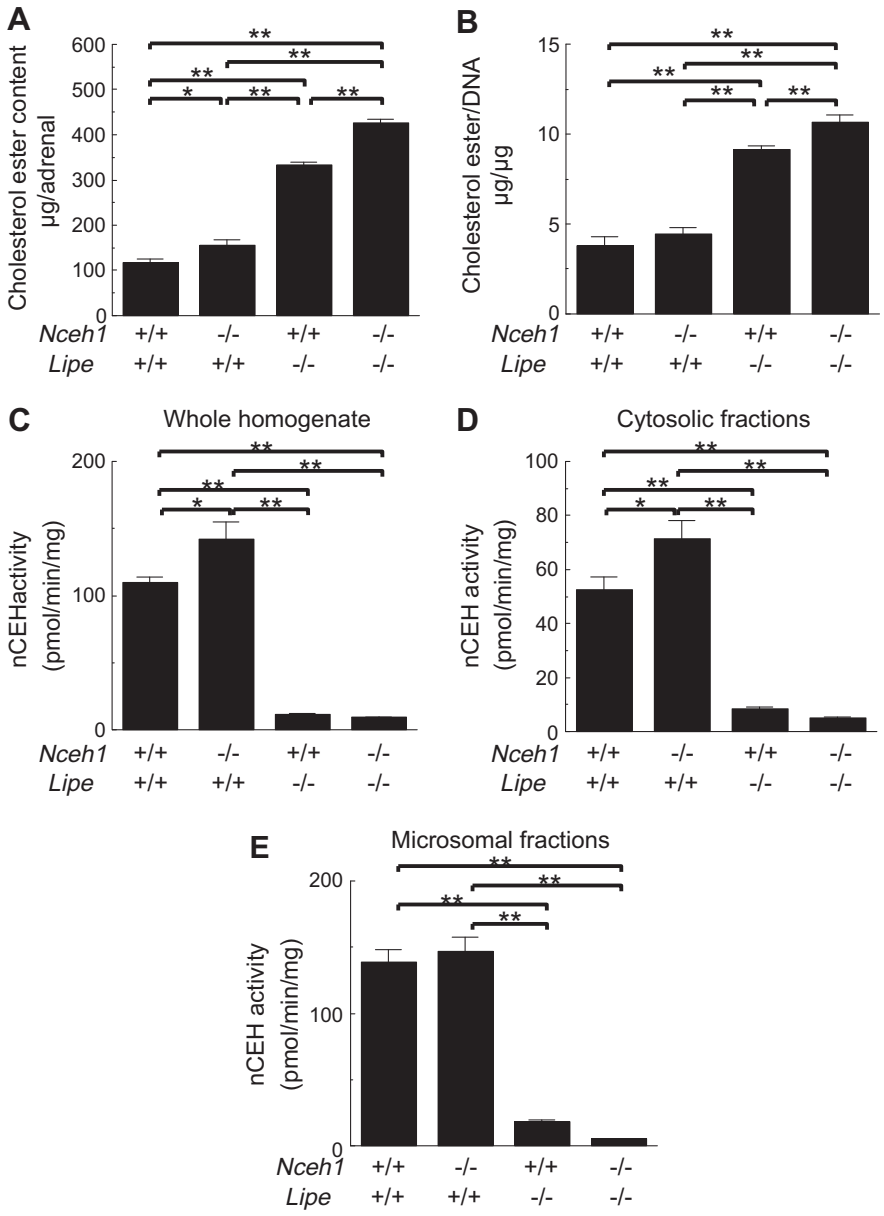

Fig. 3. Adrenal cholesterol ester (CE) content and nCEH activity. (**A**): CE content in adrenal glands ($n = 10$). (**B**): CE content was normalized with respect to DNA content in adrenal glands ($n = 10$). (**C**): nCEH activity in adrenal glands ($n = 6$). (**D**) and (**E**): Adrenal nCEH activity after subcellular fractionation ($n = 6$). Data are expressed as means ± SEM. *and **denote $P < 0.05$ and $P < 0.01$, respectively as determined by ANOVA followed by the Tukey–Kramer posthoc test.

suggested that CE taken up via selective uptake pathway would be hydrolyzed by Lipe to generate free cholesterol [7]. We recently identified and characterized another nCEH, Nceh1, and established its crucial role in foam cell formation and atherogenesis [12,13]. In this report, we further expand our understanding of adrenal CE hydrolysis using *Nceh1/Lipe* double-deficient mice. NCEH1 was expressed in adrenal cortex and involved in CE homeostasis in adrenal glands. In addition, we demonstrated that adrenal enlargement was caused in proportion to the degree of decreased nCEH activity.

Although Nceh1 was actually involved in adrenal CE hydrolysis, the relative contribution of Nceh1 to adrenal nCEH activity was less than that of Lipe. On the other hand, Nceh1 was the major nCEH in macrophage-derived foam cells [13]. Since both Nceh1 and Soat1 reside in the ER membrane, Nceh1 would preferably hydrolyze the CEs immediately after their synthesis by Soat1, and Nceh1 and Soat1 would be responsible for the continual cycle of CE hydrolysis and re-esterification in the ER. Assuming the notion, Nceh1 could play a crucial role in CE hydrolysis in foam cells where most of the intracellular CEs are continuously generated by Soat1. On the other hand, significant amount of the intracellular CEs in adrenal glands would be derived via the selective uptake pathway without the esterification process by Soat1. Consistent with this assumption, it was reported that de novo synthesis is responsible for 4% of cholesterol in adrenal glands and that the majority of adrenal cholesterol is derived from blood [27]. Two independent groups, including ours, reported the importance of Soat1 in the adrenal cholesterol homeostasis by demonstrating that CEs were markedly diminished in adrenal glands in *Soat*1-deficient mice [23,28]. However, the indirect effects such as alteration in plasma CE concentrations by global inactivation of *Soat1* gene should be considered to evaluate these results. Regarding the relative contribution of Nceh1 and Lipe in adrenal glands, relative expression levels and substrate specificity of these two lipases also deserve further scrutiny.

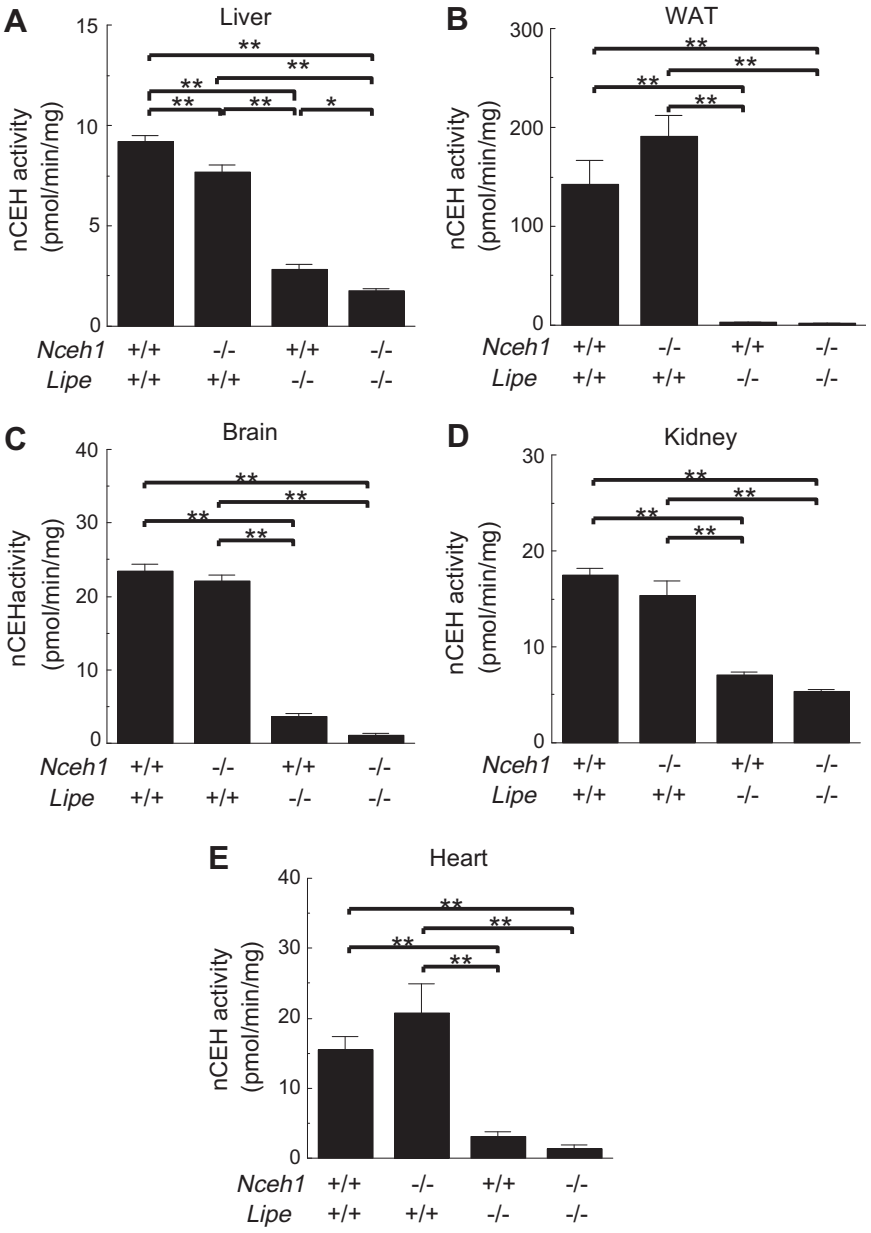

Fig. 4. nCEH activity in several tissues. Tissues were removed from female mice (9 weeks) on ad libitum, and the nCEH activity was determined. (**A**): liver, (**B**): white adipose tissue (WAT), (**C**): brain, (**D**): kidney, (**E**): heart ($n = 5$). Data are expressed as means ± SEM. *and **denote $P < 0.05$ and $P < 0.01$, respectively as determined by ANOVA followed by the Tukey–Kramer posthoc test.

The relationship between CE metabolism and corticosterone production has been a matter of debate. Two independent groups reported that the plasma corticosterone response to exogenous ACTH was suppressed in *Lipe*-deficient mice [24,25] although Kraemer et al. reported there was a statistical significant difference only in female mice. Kraemer et al. also reported the suppressed ACTH-stimulated corticosterone secretion in *Lipe*-deficient adrenocortical cells isolated from both genders. On the other hand, we reported Lipe deficiency had no significant effect on adrenal corticosterone production in both ACTH-stimulated [18] and *leptin*-deficient [26] conditions. In this report, we could not detect any significant differences among genotypes in both genders even in the *Nceh1/Lipe* double-deficient mice.

Several reports have suggested the relationship between adrenal mass and intra-adrenal cholesterol metabolism. Scarb1 deficiency impaired the selective uptake of cholesterol, reduced adrenal CE content, and reduced serum corticosterone levels. The adrenal insufficiency in *Scarb1*-deficient mice increased serum ACTH concentrations, which resulted in adrenal enlargement [22]. Adrenal lipid accumulation was reported in the *Star*-deficient mice although they died soon after birth [29], and they are the model of congenital adrenal hyperplasia. Soat1 deficiency diminished adrenal CE content and reduced adrenal mass [23]. To the best of our knowledge, this is the first report showing the correlation between CE hydrolysis and adrenal mass. We revealed the molecular mechanism controlling neutral CE hydrolysis by demonstrating that Nceh1 was also involved in adrenal CE hydrolysis as well as confirming the importance of Lipe. Both Nceh1 and Lipe affected adrenal mass in proportion to their hydrolytic activity. We investigated some of the possible underlying mechanisms. We also showed that genes involved in cholesterol biosynthetic pathway were upregulated in *Lipe*-deficient and *Nceh1/Lipe* double-deficient adrenal glands. Since farnesyl pyrophosphate, and intermediate on the pathway for cholesterol synthesis, serves also as precursor for

synthesis of various non-steroidal isoprenoids [30], it is tempting to speculate that increased prenylated small GTPases could activate proliferative signals in *Lipe*-deficient and *Nceh1/Lipe* double-deficient adrenal glands.

We previously reported that Nceh1 and Lipe are responsible for almost all nCEH activity in macrophages [13]. In this report, we further demonstrated that Lipe and Nceh1 play a crucial role as nCEHs in other tissues. Among the tissues affected by the deficiency of Nceh1 and Lipe, liver would deserve further scrutiny. We have previously reported that Lipe is involved in hepatic cholesterol metabolism [31]. In this study, we further demonstrated that Nceh1 deficiency reduced hepatic nCEH activity by 17%. It was reported that esterification of cholesterol catalyzed by sterol O-acyltransferase 2 (Soat2) is required for the hepatic secretion of apoB-containing lipoproteins [32]. The hydrolysis of stored CEs might supply the substrates for Soat2, and the resultant CEs could be assembled into lipoprotein particles. The involvement of Nceh1 in hepatic VLDL secretion remains to be elucidated.

In conclusion, Nceh1 is also involved in adrenal CE hydrolysis. We also demonstrated that CE hydrolysis is one of the important biological processes linked to adrenal mass.

Acknowledgments

This work was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education and Science (to M. Sekiya). This work was also partially supported by Astrazeneca Research Grant and Suzuken Memorial Foundation (to M. Sekiya).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.11.103.

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