Biochimica et Biophysica Acta 1840 (2014) 2289–2298

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Ascorbic acid deficiency affects genes for oxidation–reduction and lipid metabolism in livers from SMP30/GNL knockout mice

Keita Takahashi [a,b], Yuki Kishimoto [a,b], Tomokazu Konishi [c], Yasunori Fujita [d], Masafumi Ito [d], Kentaro Shimokado [b], Naoki Maruyama [a], Akihito Ishigami [a,*]

[a] Molecular Regulation of Aging, Tokyo Metropolitan Institute of Gerontology, Tokyo 173-0015, Japan
[b] Department of Geriatrics and Vascular Medicine, Tokyo Medical and Dental University, Tokyo 113-8510, Japan
[c] Faculty of Bioresource Sciences, Akita Prefectural University, Akita 010-0195, Japan
[d] Research Team for Mechanism of Aging, Tokyo Metropolitan Institute of Gerontology, Tokyo 173-0015, Japan

ARTICLE INFO

Article history:
Received 1 January 2014
Received in revised form 21 February 2014
Accepted 28 March 2014
Available online 3 April 2014

Keywords:
Ascorbic acid
Bile acid
Cyp7a1
Lipid metabolism
Microarray
Nrf2

ABSTRACT

Background: We sought to elucidate the effect of an ascorbic acid (AA) deficiency on gene expression, because the water soluble antioxidant AA is an important bioactive substance in vivo.
Methods: We performed microarray analyses of the transcriptome in the liver from senescence marker protein-30 (SMP30)/gluconolactonase (GNL) knockout (KO) mice, which are unable to synthesize AA in vivo.
Results: Our microarray analysis revealed that the AA deficiency increased gene expression related to the oxidation–reduction process, i.e., the *nuclear factor, erythroid derived 2, like 2* (*Nrf2*) gene, which is a reactive oxygen species-sensitive transcriptional factor. Moreover, this AA deficiency increased the expression of genes for lipid metabolism including the *cytochrome P450, family 7, subfamily a, polypeptide 1* (*Cyp7a1*), which is a late-limiting enzyme of the primary bile acid biosynthesis pathway. Although an AA deficiency increased the Cyp7a1 protein level, bile acid levels in the liver and gallbladder decreased. Since Cyp7a1 has a heme iron at the active site, AA must function as a reductant of the iron required for the continuous activation of Cyp7a1.
Conclusions: This experimental evidence strongly supports a role for AA in the physiologic oxidation–reduction process and lipid metabolism including bile acid biosynthesis.
General significance: Although many effects of AA supplementation have been reported, no microarray analysis of AA deficiency in vivo is available. Results from using this unique model of AA deficiency, the SMP30/GNL-KO mouse, now provide new information about formerly unknown AA functions that will implement further study of AA in vivo.

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1. Introduction

Many animals have the ability to synthesize L-ascorbic acid (AA, vitamin C). However, humans, nonhuman primates, and guinea pigs are unable to synthesize AA in vivo because of multiple mutations in the L-gulono-γ-lactone oxidase (*Gulo*) gene, which is responsible for producing an enzyme active in the last step of AA synthesis pathway [1]. Therefore, consumption of exogenous AA is essential for animals that cannot synthesize AA; in its absence a long-term AA deficiency develops resulting in scurvy [2]. AA is a water-soluble hexonic sugar acid and acts as an antioxidant [3]. That capacity for antioxidation enables AA to eliminate reactive oxygen species (ROS) such as superoxide, singlet oxygen, and hydroxyl radicals [4–6]. ROS oxidize lipids, proteins, and DNA thereby leading to tissue and cell damage [7].

Moreover, AA acts as a co-factor in reactions catalyzed by several metal-dependent oxygenases, e.g., Cu^{+}-dependent mono-oxygenases including peptidylglycine α-amidating mono-oxygenase, which is

Abbreviations: AA, ascorbic acid; ANOVA, analysis of variance; AREs, antioxidant response elements; CAT, catalase; Cyp7a1, cytochrome P450, family 7, subfamily a, polypeptide 1; DHA, dehydroascorbic acid; ECD, electrochemical detector; EDTA, ethylenediaminetetraacetic acid; Fasn, fatty acid synthase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GNL, gluconolactonase; GO, Gene Ontology; Gulo, L-gulono-γ-lactone oxidase; HO-1, heme oxygenase-1; HPLC, high performance liquid chromatography; KO, knockout; Keap1, Kelch-like ECH-associated protein 1; MPA, metaphosphoric acid; Nqo1, NAD(P)H dehydrogenase, quinone 1; Nrf2, nuclear factor, erythroid derived 2, like 2; ODS, osteogenic disorder Shionogi; PC, principal component; qPCR, quantitative polymerase chain reaction; ROS, reactive oxygen species; SEM, standard error of the mean; SMP30, senescence marker protein-30; SOD, superoxide dismutase; SREBP, sterol regulatory element binding protein; SVCT, sodium-dependent vitamin C transporter; γ-GCSc, γ-glutamylcysteine synthetase heavy subunit; WT, wild-type

* Corresponding author at: Molecular Regulation of Aging, Tokyo Metropolitan Institute of Gerontology (TMIG), 35-2 Sakae-cho, Itabashi-ku, Tokyo 173-0015, Japan. Tel.: +81 3 3964 3241.
E-mail address: ishigami@tmig.or.jp (A. Ishigami).

http://dx.doi.org/10.1016/j.bbagen.2014.03.019
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involved in peptide hormone synthesis [8,9], dopamine β-hydroxylase, a participant in norepinephrine synthesis [10,11], and Fe^{2+}/α-ketoglutarate-dependent dioxygenases including prolyl and lysyl hydroxylases, both of which contribute to collagen synthesis [12–15], and asparaginyl hydroxylase, which modifies hypoxia-inducible factor 1 [16,17]. These oxygenases require divalent metal ions for enzymatic activity, and this activity depends absolutely on the divalent status of the metal ions.

Senescence marker protein-30 (SMP30) is a 34-kDa protein whose tissue levels in the liver, kidney, and lung decrease with aging [18–20]. Previously, we identified SMP30 as the lactone-hydrolyzing enzyme gluconolactonase (GNL) (EC 3.1.1.17) [21]. The reaction of lactonase with L-gulono-γ-lactone is the penultimate step in the AA biosynthesis pathway. The essential role of SMP30/GNL in this synthetic process was verified by a nutritional study using SMP30/GNL-knockout (KO) mice, indicating that SMP30/GNL-KO mice are unable to synthesize AA in vivo [21]. Previously, we reported that SMP30/GNL-KO mice with a prolonged AA deficiency developed an increased rate of pulmonary emphysema [22], susceptibility to ultraviolet radiation-induced cataracts [23], epidermal atrophy, and extensive ultraviolet B-induced skin pigmentation [24] as well as a decrease in skin collagen content and hair growth [25]. Moreover, as we also noted, an AA deficiency increased superoxide formation in the brain [26,27], enhanced expression of the sodium-dependent vitamin C transporter (SVCT) 1 and SVCT2, and heightened uptake of AA in the liver [28].

Upon analyzing gene expression by microarray, Duarte et al. [29] reported that cultured human skin fibroblasts supplemented with AA had an increase of gene expression that was related to the cell cycle and DNA replication. Jiao et al. [30] also documented that AA supplementation increased or decreased, respectively, the expression of genes that regulated stress reactions as well as exclusively/predominantly hepatocyte genes in livers from Gulo-deficient mice. Yu et al. [31] found a change of gene expression during the differentiation of mesencephalic precursor cells into dopaminergic neurons after treatment with AA. On the other hand, Horio et al. [32,33] wrote that an AA deficiency stimulated hepatic genes to express an inflammatory chemokine, cytokine-induced neutrophil chemoattractant-1, and acute phase proteins in scurvy-prone osteogenic disorder Shionogi (ODS) rats; however, no microarray analysis was performed. To advance those results, we used microarray analysis to discern the effect of an AA deficiency on gene expression in livers of SMP30/GNL-KO mice. Accordingly, we found that these animals, when deficient in AA, undergo significant changes in the oxidation–reduction process and lipid metabolism.

2. Materials and methods

2.1. Animals

SMP30/GNL-KO mice were generated by the gene targeting technique as described previously [34]. Since the SMP30/GNL gene is located in the p11.3 to q11.2 segments of the X chromosome, heterozygous male mice do not exist [35]. Female SMP30/GNL-KO (*SMP30/GNL*$^{-/-}$) mice were mated with male SMP30/GNL-KO (*SMP30/GNL*$^{Y/-}$) mice to produce SMP30/GNL-KO males (*SMP30/GNL*$^{Y/-}$) and females (*SMP30/GNL*$^{-/-}$). SMP30/GNL-KO mice were weaned at four-weeks-old, at which time ten male SMP30/GNL-KO mice were divided into the following two groups: AA (−) and AA (+) each of which had five mice. The AA (+) group had free access to water containing AA (1.5 g/L) and 10 μM ethylenediaminetetraacetic acid (EDTA), whereas the AA (−) group had free access to water without AA for four weeks. EDTA was added to stabilize AA in drinking water, which retained that stable state for at least four days. Water bottles were changed every three or four days. Other than the water, all mice were fed an AA-free diet (CL-2, CLEA Japan, Tokyo, Japan) ad libitum. Five male wild-type (WT) (C57BL/6NCrSlc, *SMP30/GNL*$^{Y/+}$) mice at four weeks old were purchased from Japan SLC (Shizuoka, Japan). WT mice were fed an AA-free diet and had free access to water without AA for four weeks. Throughout the experiments, animals were maintained on a 12-h light/dark cycle in a controlled environment. All experimental procedures using laboratory animals were approved by the Animal Care and Use Committee of the Tokyo Metropolitan Institute of Gerontology.

2.2. Preparation of plasma and liver

Mice were sacrificed by cervical dislocation, and their blood was collected from the inferior vena cava. Blood was gently mixed with EDTA and centrifuged at 880 ×*g* for 15 min at 4 °C. The resulting supernatants were used as plasma for further analysis. Afterward, mice were perfused systemically with ice-cold phosphate buffered saline through the left ventricle to wash out the remaining blood cells. Livers were collected and stored at −80 °C until use.

2.3. Determination of AA and dehydroascorbic acid (DHA)

AA and DHA, an oxidized form of AA, were measured by using high performance liquid chromatography (HPLC) and electrochemical detection (ECD) according to the methods described previously [36]. Liver was homogenized in 14 volumes of 5.4% metaphosphate (MPA) containing 1 mM EDTA and centrifuged at 21,000 ×*g* for 10 min at 4 °C. Plasma was mixed with equal volumes of 10% MPA containing 1 mM EDTA and centrifuged at 21,000 ×*g* for 10 min at 4 °C. The supernatants obtained were rapidly frozen and kept at −80 °C until use. For determination of total AA (AA plus DHA), the centrifugal supernatants were reduced with tris (2-carboxyethyl) phosphine hydrochloride for 2 h on ice. After reduction, the reaction mixture was diluted with 5% MPA/EDTA and analyzed for total AA by HPLC coupled with ECD. Separation was achieved on an Atlantis dC18 5 μm column (4.6 × 150 mm) combined with an Atlantis dC18 5 μm guard column (4.6 × 20 mm) from Nihon Waters (Tokyo, Japan). The mobile phase consisted of 50 mM phosphate buffer (pH 2.8), 540 μM EDTA, and 2% methanol at a flow rate of 1.3 mL/min, and electrical signals were recorded by using an ECD with a glassy carbon electrode at +0.6 V. All electrical signal data from the electrochemical detector were collected by using Waters Empower 2 software (Nihon Waters). The value of DHA was determined by subtracting AA from AA plus DHA.

2.4. Extraction of total RNA and cDNA synthesis

Total RNA was extracted by using ISOGEN® (Wako Pure Chemical, Osaka, Japan) [37]. Briefly, each liver was homogenized with a teflon-pestle homogenizer in ISOGEN, and total RNA was extracted according to the manufacturer's protocol. The final RNA pellet was dissolved in diethyl pyrocarbonate-treated H_2O. RNA concentrations were determined and confirmed as free from protein contamination by measuring absorbance at 260 and 280 nm. Then, cDNA was synthesized using SuperScript II Reverse transcriptase (Invitrogen) following the manufacturer's protocol. The cDNA was stored at −80 °C until use.

2.5. Microarrays and data normalization

Hepatic transcriptomes were compared among three groups, AA (+) and AA (−) SMP30/GNL-KO mice, and WT mice with two biological repeats per group. For each sample, total RNA was measured using Affymetrix Mouse Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, USA). The perfect match data were normalized by using the SuperNORM data service (Skylight Biotech Inc., Akita, Japan) according to a three-parameter lognormal distribution model [38]; the normalized data as well as the raw data were deposited in Gene Expression Omnibus (accession code: GSE41365). To check reproducibility, two-way analysis of variance (ANOVA) [39] was performed; p-values of 6914 genes out of 27,541 contents were less than 0.005, and only those genes were used for further analysis.

2.6. Principal component (PC) analysis

To observe characteristics of the groups, a PC analysis was performed on the ANOVA positive genes. To reduce the effect of individual differences among samples, the axes were estimated in a matrix of samples' means of each group and applied to the whole set of data. The mean data for group AA (+) SMP30/GNL-KO mice was used as a reference. To classify the genes, PCs for the genes were categorized by k-means clustering into five groups. Then PCs for genes and the samples were presented in a biplot. In the biplot, PCs for groups were enlarged by two for visual clarity.

2.7. Frequency analysis of biological functions in annotations of the genes selected

To test significance in the categories of biological functions that appear in the selected genes' annotations, binominal tests were performed on frequencies found in the Gene Ontology (GO) Biological Process [38].

2.8. Quantitative polymerase chain reaction (qPCR)

Using the qPCR super mix-UDG-with ROX (Invitrogen) following the manufacturer's protocol, qPCR reactions were performed. The primers for cytochrome P450, family 7, subfamily a, polypeptide 1 (Cyp7a1) (Assay ID: Mm00484152_m1), cytochrome P450, family 4, subfamily a, polypeptide 14 (Cyp4a14) (Assay ID: Mm00484135_m1), cytochrome P450, family 2, subfamily c, polypeptide 39 (Cyp2c39) (Assay ID: Mm04207909_g1), cytochrome P450, family 2, subfamily c, polypeptide 38 (Cyp2c38) (Assay ID: Mm00658527_m1), fatty acid synthase (Fasn) (Assay ID: Mm00662319_m1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Assay ID: Hs99999905_m1) came from an inventory of TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). As an endogenous control gene, TaqMan human GAPDH Control Reagents (Applied Biosystems) were used. The reactions were performed by using the real-time PCR equipment (Applied Biosystems 7300 Real Time PCR System) with singlet. The amplification protocol consisted of denaturation at 95 °C for 2 min and 45 cycles of 95 °C for 15 s, annealing temperature for 3 min, and 60 °C for 30 s. For quantitative analysis of each mRNA expression level, a standard curve method was designed; that is, an aliquot from each experimental sample was used to generate standard curves. The correlation coefficient of the standard curve was more than 0.999. The mRNA expression level of each gene was evaluated, and the ratio in AA (+) SMP30/GNL KO mice was considered as 1.0.

2.9. Western blotting analysis

For Western blotting analysis of Cyp7a1, livers from each of five AA (+) and AA (−) SMP30/GNL-KO mice were homogenized with a teflon-pestle homogenizer in homogenate buffer containing 20 mM Tris–HCl (pH 7.4), 0.25 M sucrose, and complete EDTA-Free Protease Inhibitor (Roche Diagnostics, Tokyo, Japan). The cytoplasmic fraction containing the microsome fraction was obtained as supernatant by centrifugation at 8000 ×*g*. For Western blotting analysis of nuclear factor, erythroid derived 2, like 2 (Nrf2) in the nuclear and cytoplasmic fractions, livers from each of five AA (+) and AA (−) SMP30/GNL-KO mice were homogenized with a teflon-pestle homogenizer in homogenate buffer contained 0.1 mM Tris–HCl (pH 7.4), 0.25 M sucrose, and complete EDTA-Free Protease Inhibitor. Nuclear fractions and cytoplasmic fractions were divided by centrifugation at 600 ×*g*. For Western blotting analysis of NAD(P)H dehydrogenase, quinone 1 (Nqo1), γ-glutamylcysteine synthetase heavy subunit (γ-GCSc), catalase (CAT), superoxide dismutase (SOD) 1, and SOD2, Kelch-like ECH-associated protein 1 (Keap1), sterol regulatory element binding protein (SREBP)-1, and SREBP-2, livers from each of five AA (+) and AA (−) SMP30/GNL-KO mice were homogenized with a Polytron homogenizer in homogenate buffer containing 0.1% SDS, 140 mM NaCl, 10 mM Tris–HCl (pH 7.4), and complete EDTA-Free Protease Inhibitor. Protein concentrations were measured by the Lowry method [40] using bovine serum albumin as a standard. Each sample (10 μg protein) was electrophoresed on a 10 or 12% polyacrylamide gel by the method of Laemmli [41], with some modifications. After electrophoresis, proteins were transferred to PVDF membranes. Mouse anti-Cyp7a1 monoclonal antibody (1:1000, Cosmo Bio, Tokyo, Japan) [42], goat anti-Nrf2 polyclonal antibody (1:250, Santa Cruz Biotechnology, Texas, USA) [43], goat anti-Nqo1 polyclonal antibody (1:200, Santa Cruz Biotechnology) [44], rabbit anti-γ-GCSc polyclonal antibody (1:200, Santa Cruz Biotechnology) [44], goat anti-CAT polyclonal antibody (1:200, Santa Cruz Biotechnology) [45], rabbit anti-SOD1 polyclonal antibody (1:200, Santa Cruz Biotechnology) [46], rabbit anti-SOD2 polyclonal antibody (1:200, Santa Cruz Biotechnology) [46], goat anti-Keap1 polyclonal antibody (1:200, Santa Cruz Biotechnology) [47], mouse anti-SREBP-1 monoclonal antibody (1:500; Thermo, Kanagawa, Japan) [48], rabbit anti-SREBP-2 polyclonal antibody (1:500; Novus Biologicals, Littleton, CO, USA) [49], and rabbit anti-β-actin polyclonal antibody (1:1000; Cell Signaling technology, Danvers, MA, USA) [44] were used as primary antibodies. HRP-conjugated goat anti-mouse IgG antibody (1:5000; Thermo), HRP-conjugated goat anti-rabbit IgG antibody (1:5000; Thermo), and HRP-conjugated donkey anti-goat IgG antibody (1:5000; Santa Cruz Biotechnology) were used as secondary antibodies. Densitometric analyses were performed by ImageJ, version 1.45s (National Institutes of Health, Bethesda, Maryland, USA). Nqo1, γ-GCSc, CAT, SOD1, SOD2, and Keap1 protein levels were evaluated relative to the levels of β-actin, and protein levels in the liver from AA (−) SMP30/GNL-KO mice were expressed as ratios when compared to AA (+) SMP30/GNL-KO mice. To confirm the reproducibility, Western blotting analyses were performed twice for each antibody.

2.10. Extraction of bile acid from liver and gallbladder

Bile acid in the liver was extracted by ethanol [50]. Briefly, livers from each of five AA (+) and AA (−) SMP30/GNL-KO mice were homogenized with a teflon-pestle homogenizer in ultrapure water, and incubated with ethanol (pH 11) at 60 °C for 2 h. After incubation, samples were centrifuged at 21,000 ×*g*, and the precipitate was washed in ethanol (pH 11) at 60 °C for 2 h. Bile acid was obtained by evaporating the supernatant to dryness at 60 °C with N_2 gas and dissolution in ethanol. In addition, bile acid from of each five AA (+) and AA (−) SMP30/GNL-KO mice was obtained from the gallbladders.

2.11. Extraction of lipid from liver

Total lipid in the liver was extracted by the Folch method [51]. Livers from each of five AA (+) and AA (−) SMP30/GNL-KO mice were homogenized with a teflon-pestle homogenizer in ultrapure water, and homogenates were incubated with chloroform–methanol (2:1) at 37 °C for 1 h. After incubation, samples were centrifuged at 21,000 ×*g*, and precipitates were washed with chloroform–methanol (2:1) at 37 °C for 2 h. Total lipid was obtained by evaporating the supernatant to dryness at 55 °C in N_2 gas and dissolution in 2-propanol.

2.12. Measurement of bile acid and lipids

Amounts of bile acid, free fatty acid, total cholesterol, triglyceride, and phospholipids were determined with a commercial assay kit. Briefly, bile acid from livers and gallbladders was measured by using enzymatic assay kits (Wako, Tokyo, Japan) [52]. Plasma-free fatty acid [53], total cholesterol [54], triglyceride [55], and phospholipid [56] levels were measured by using enzymatic assay kits (Wako, Tokyo, Japan).

2.13. Cyp7a1 activity

Cyp7a1 activity was measured by the method of Horio et al. [57] with some modifications. Briefly, livers from each of four AA (+) and AA (−) SMP30/GNL-KO mice were homogenized with a teflon-pestle homogenizer in homogenate buffer containing 0.1 mM Tris–HCl (pH 7.4), 0.25 M sucrose. The cytoplasmic fraction-containing the microsome fraction was obtained by centrifuging at 8000 ×*g*. The protein concentration was determined by the Lowry method. Samples were incubated with incubation buffer containing 200 μM cholesterol, 2 mM nicotinamide adenine dinucleotide phosphate (NADPH), 20 mM cysteamine, 4 mM $MgCl_2$, 1.5% (w/v) Tween 80, 9990 Bq [7-^{3}H(N)] cholesterol (American Radiolabeled Chemicals Inc., Saint Louis, USA) in 0.1 M Tris–HCl (pH 7.4), and 0.25 M sucrose. After incubation for 15 min, 20% (w/v) trichloroacetic acid was added to stop the reaction, and protein was precipitated by centrifugation. To separate non-metabolized [7-^{3}H(N)] cholesterol, 20 volume chloroform was added; the container was inverted for 1 min followed by centrifugation. ^{3}HOH derived from metabolized [7-^{3}H(N)] cholesterol was counted by Aquasol-2 (PerkinElmer Japan Co., Ltd., Kanagawa, Japan) and a liquid scintillation counter (LSC-6100, Aloka, Tokyo, Japan).

2.14. Statistical analysis

The results are expressed as means ± standard error of the mean (SEM). The probability of statistical differences between experimental groups was determined by one-way ANOVA followed by Dunnett post hoc comparisons and Student's *t*-test using KaleidaGraph software (Synergy Software, Reading, PA, USA). Statistical differences were considered significant at $p < 0.05$.

3. Results

3.1. Body weight comparison

To investigate the effect of an AA deficiency on growth, SMP30/GNL-KO mice were weaned at four weeks of age and divided into two groups designated AA (+) and AA (−). We also used wild-type (WT) mice as a normal control to ascertain the effect of *SMP30/GNL* gene depletion. In this experiment, the AA (+) SMP30/GNL-KO mice were fed an AA-free diet and had free access to water containing AA (1.5 g/L), whereas the AA (−) SMP30/GNL-KO mice received an AA-free diet and had free access to water without AA for four weeks. WT mice were also fed an AA-free diet and had free access to water without AA for four weeks. AA (+) and AA (−) SMP30/GNL-KO and WT mice gained weight to the same degree until four weeks after weaning (Supplementary Fig. S1). The mean body weights of the AA (+) and AA (−) SMP30/GNL-KO and WT mice at four weeks after weaning were 11.3 ± 1.1, 10.1 ± 0.5, and 10.8 ± 0.6 g, respectively, denoting no significant difference among these three groups. Mean rates of water consumption for the AA (+) and AA (−) SMP30/GNL-KO and WT mice were 3.9 ± 0.2, 4.0 ± 0.2, and 4.5 ± 0.1 mL/day, respectively. Moreover, mean levels of dietary consumption for the AA (+) and AA (−) SMP30/GNL-KO and WT mice were 3.4 ± 0.3, 3.3 ± 0.2, and 3.6 ± 0.4 g/day, respectively.

3.2. AA levels in the liver and plasma

To determine the AA levels in the liver and plasma at four weeks after weaning, we measured the total AA (AA plus DHA, an oxidized form of AA) in livers and plasma of AA (+) and AA (−) SMP30/GNL-KO and WT mice. At that time, the total AA levels in the liver and plasma from AA (−) SMP30/GNL-KO mice were less than 2% of the values obtained from the AA (+) SMP30/GNL-KO and WT mice (Supplementary Fig. S2, A, B). Moreover, total AA levels in the livers and plasma of WT mice were 1.2-fold higher than that of AA (+) SMP30/GNL-KO mice. Although, the reason why total AA levels in the livers and plasma of WT mice were higher than that of AA (+) SMP30/GNL-KO mice is still unclear, the cause might be a difference between WT mice, which are able to synthesize AA, and SMP30/GNL-KO mice, which cannot synthesize AA in vivo. On the other hand, the percentage of DHA in the livers and plasma from AA (+) and AA (−) SMP30/GNL-KO and WT mice was not different among these three groups.

3.3. Microarray and PC analysis

To examine the effect of an AA deficiency and *SMP30/GNL* gene deletion on hepatic gene expression, we performed microarray analysis and compared the results among all three groups: AA (+), AA (−) SMP30/GNL-KO, and WT mice with two biological repeats per group. As a result of microarray analysis and two-way ANOVA, 6914 genes that showed p-values less than 0.005 were selected for further analysis out of the total 27,541 content. To observe characteristics of the groups, PC analysis was then performed on the ANOVA-positive genes (Fig. 1). For the PC analysis, we used the AA (+) SMP30/GNL-KO group as a reference. In the resulting scores, genes were separated into five groups by k-means clustering; they formed groups of increased and decreased genes in PC1 and PC2, and stable genes. PC1 and PC2 represented the effect of *SMP30/GNL* gene deletion and AA deficiency, respectively (Fig. 1). That is, PC1-positive symbolizes the increased gene expression levels of WT mice when compared to AA (+) SMP30/GNL-KO mice (Fig. 1, Supplementary Table S1). In addition, PC1-negative symbolizes decreased gene expression levels of WT mice when compared to AA (+) SMP30/GNL-KO mice (Fig. 1, Supplementary Table S2). Similarly, PC2-positive and -negative symbolize increased and decreased gene expression levels of AA (−) SMP30/GNL-KO mice when compared to AA (+) SMP30/GNL-KO mice, respectively (Fig. 1, Supplementary Table S3, S4). Groups I and II in Fig. 1 depict that genes decreased or increased, respectively, by *SMP30/GNL* gene deletion. Groups III and IV in Fig. 1 depict that genes increased or decreased, respectively, by AA deficiency. Zone V consists of stable genes that did not affect either the *SMP30/GNL* gene deletion or the AA deficiency.

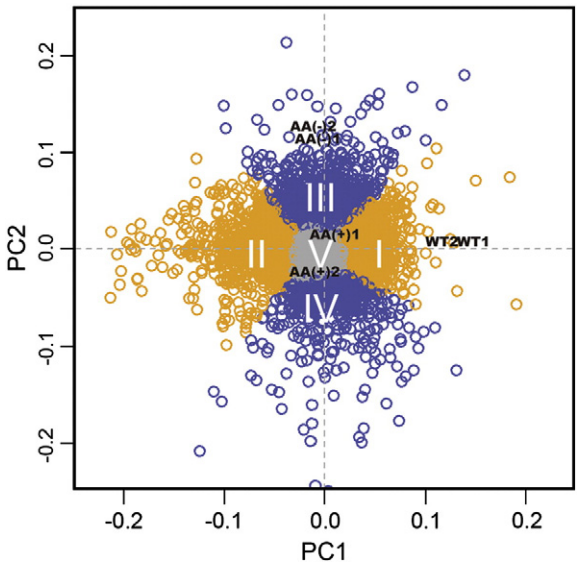

Fig. 1. PC analysis. PC analysis was performed on the ANOVA-positive genes as described in the Materials and methods. Data for hepatic gene expression of AA (+) SMP30/GNL-KO mice was used as a reference. PC1 and PC2 represented the effect of *SMP30/GNL* gene deletion and AA deficiency, respectively. Zones I and II showed the genes decreased or increased, respectively, by *SMP30/GNL* gene deletion. Zones III and IV showed the genes increased or decreased, respectively, by AA deficiency. Zone V showed stable genes. WT, wild-type mice. AA (+), AA (+) SMP30/GNL-KO mice. AA (−), AA (−) SMP30/GNL-KO mice.

To chart the characteristics of these groups, each annotation key word was recorded by the frequency of its appearance then subjected to a binominal test for comparison with those found in the GO Biological Process [38] key words. As a result, 400 genes of the 466 in group III (Fig. 1), which represented gene expression increased by AA deficiency, had annotations of the GO biological process. We then counted annotations whose expression ratio was higher than √2 in the livers of AA (−) SMP30/GNL-KO mice when compared to AA (+) SMP30/GNL-KO mice (Supplementary Table S5). Genes related to the oxidation–reduction process had the highest counts (47 counts) produced by AA deficiency. Moreover, an AA deficiency increased the expression of genes related to lipid metabolism, i.e., lipid metabolic (20 counts), steroid biosynthetic (17 counts), lipid biosynthetic (17 counts), sterol biosynthetic (13 counts), cholesterol biosynthetic (11 counts), steroid metabolic (9 counts), cholesterol metabolic (8 counts), isoprenoid biosynthetic (6 counts), acyl-CoA metabolic (6 counts), fatty acid metabolic (6 counts), phospholipid biosynthetic (5 counts), and fatty acid biosynthetic process (5 counts) (Supplementary Table S5).

Next we found that 275 genes of the 328 genes in group IV (Fig. 1), which denoted gene expression decreased by an AA deficiency, had annotations of the GO biological process. Genes related to the acute-phase response had the highest counts (8) yielded by the AA deficiency (Supplementary Table S6); however, those counts were fewer than the highest counts observed in Supplementary Table S5. Of the 343 genes in group I, 296 had genes whose expression was decreased by *SMP30/GNL* gene deletion and also had annotations of the GO biological process. Genes related to the regulation of translation had by far the highest counts (25) generated by *SMP30/GNL* gene deletion (Supplementary Table S7). Also, 364 genes of the 628 genes in group II, the expression levels of which were increased by *SMP30/GNL* gene deletion, had annotations of the GO biological process. The high counts for genes related to the regulation of transcription (65 counts) and to transcription (64 counts) were apparent in the milieu of *SMP30/GNL* gene deletion (Supplementary Table S8). Moreover, *SMP30/GNL* gene deletion increased the expression of genes related to heart morphogenesis (17 counts), embryonic hindlimb morphogenesis (14 counts), ear morphogenesis (13 counts), and inner ear morphogenesis (13 counts) (Supplementary Table S8).

3.4. Validation of microarray analysis by qPCR

The relative values of microarray analysis in comparisons between AA (+) and AA (−) SMP30/GNL-KO mice were validated by qPCR. Gene expression was tabulated for the cytochrome P450 family such as *Cyp7a1*, *Cyp4a14*, *Cyp2c39*, and *Cyp2c38* and lipid metabolism such as *Fasn*. In those comparisons, the gene expression level in AA (−) SMP30/GNL-KO mice was more than 2-fold higher than that of AA (+) SMP30/GNL-KO mice in the microarray analysis (Supplementary Table S3), and those data were validated by qPCR. That is, this independent technique confirmed that all selected genes were increased by the AA deficiency in the same fashion as in the microarray analysis (Supplementary Fig. S3).

3.5. Effect of AA deficiency on the antioxidant system

Since genes related to the oxidation–reduction process were the most increased by an AA deficiency (Supplementary Table S5), we focused on the *Nrf2* gene, which increased 1.57-fold in the presence of an AA deficiency (Supplementary Table S9). Analysis of this gene was chosen, because Nrf2 is a ubiquitous master transcription factor that regulates antioxidant response element (ARE)-mediated expression of antioxidant enzyme and cytoprotective proteins [58]. As shown in Supplementary Table S9, Nrf2 target genes that have AREs [59] include *Nqo1*, *aldehyde oxidase 1* (*Aox1*), *glutathione S-transferase, alpha 2* (*Yc2*) (*Gsta2*), *and glutamate-cysteine ligase*, *catalytic subunit* (*Gclc*), and the AA deficiency prompted increases of those genes by 1.93-fold, 1.77-fold, 1.55-fold, and 1.46-fold, respectively.

3.6. Western blot analysis of nuclear Nrf2 translocation and its target gene products

Nrf2 is known to become activated in response to oxidative stress, detaches from its cytosolic inhibitor, Keap1, translocates to the nucleus, and binds to the AREs in the promoter of target genes [58]. To confirm that Nrf2 is activated by an AA deficiency, we evaluated Nrf2 protein levels in the nuclei and cytoplasm by Western blot analysis (Fig. 2). Indeed, the amount of Nrf2 protein in the nuclei from AA (−) SMP30/GNL-KO mice was 1.6-fold higher than those from AA (+) SMP30/GNL-KO mice (Fig. 2A). However, almost the same Nrf2 protein content was found in the cytoplasm of liver cells from both groups (Fig. 2B). We next evaluated the protein levels of Nrf2 target genes such as *Nqo1* and *γ-GCSc*, and anti-oxidant proteins such as *CAT*, *SOD 1*, *and SOD2*, and *Keap1* (Fig. 3). Only Nqo1 protein in livers from AA (−) SMP30/GNL-KO mice showed 2-fold more of this protein than that from AA (+) SMP30/GNL-KO mice (Fig. 3A). All others of these proteins had almost the same levels in AA (+) and AA (−) SMP30/GNL-KO mice (Fig. 3B–F).

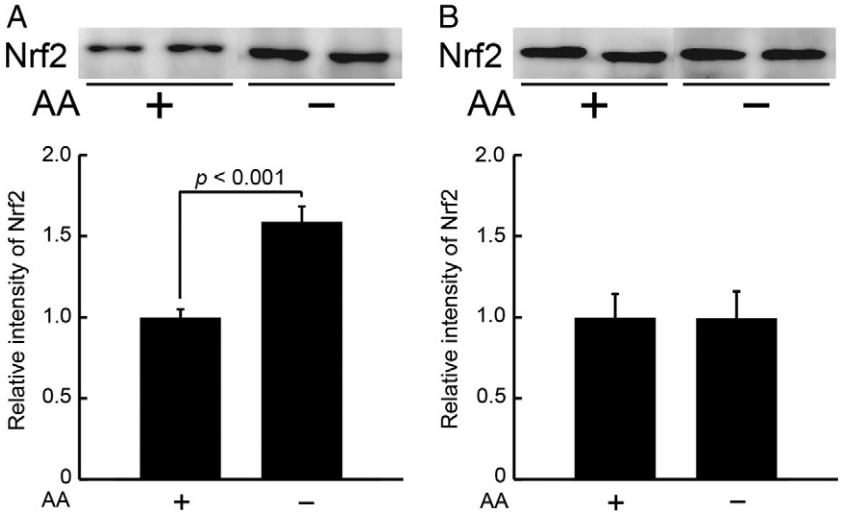

Fig. 2. Western blot analysis of nuclear Nrf2 translocation. Nrf2 protein levels in the nuclear (A) and cytoplasm (B) of the livers from AA (+) and AA (−) SMP30/GNL-KO mice were quantitated with ImageJ, version 1.45s. Nrf2 protein levels from AA (−) SMP30/GNL-KO mice were expressed as a ratio compared with AA (+) SMP30/GNL-KO mice. Values are expressed as means ± SEM of five animals. Statistical differences were considered significant at $p < 0.05$.

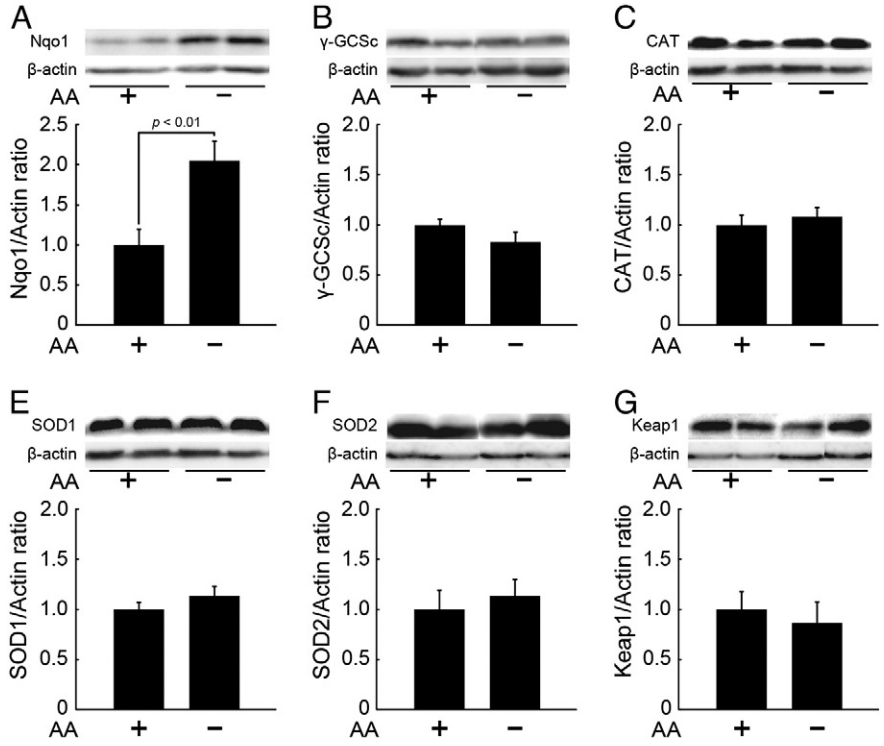

Fig. 3. Western blot analysis of Nrf2 target genes and antioxidant enzymes. Nqo1 (A), γ-GCSc (B), CAT (C), SOD1 (D), SOD2 (E), and Keap1 (F) protein levels in the livers from AA (+) and AA (−) SMP30/GNL-KO mice were quantitated with ImageJ, version 1.45s. Protein levels were evaluated relative to levels of β-actin, and protein levels from AA (−) SMP30/GNL-KO mice are expressed as a ratio compared to AA (+) SMP30/GNL-KO mice. Values are expressed as means ± SEM of five animals. Statistical differences were considered significant at $p < 0.05$.

3.7. Effect of AA deficiency on the primary bile acid biosynthesis pathway

Primary bile acids including cholic acid, taurocholic acid, and glycocholic acid are biosynthesized from cholesterol in the liver (Fig. 4) and saved as bile in the gallbladder. Since expression of the *Cyp7a1* gene, which is related primary bile to acid biosynthesis of main cholesterol metabolism pathway, was increased 3.33-fold by the AA deficiency (Supplementary Table S3), we measured bile acid levels in the liver and gallbladder (Fig. 5). Notably, amounts of bile acid at those sites were 72% and 34%, respectively, lower in AA (−) SMP30/GNL-KO mice than in AA (+) SMP30/GNL-KO mice (Fig. 5A, B).

Since livers and gallbladders from AA (−) SMP30/GNL-KO mice had lower bile acid levels than those from AA (+) SMP30/GNL-KO mice (Fig. 5), we examined Cyp7a1 protein content in livers from AA (+) and AA (−) SMP30/GNL-KO mice by Western blot analysis (Fig. 6A). As shown, livers from AA (−) SMP30/GNL-KO mice had 1.6-fold more Cyp7a1 protein than in AA (+) SMP30/GNL-KO mice. However, the Cyp7a1 enzyme activity in livers from AA (−) SMP30/GNL-KO mice was 35% lower than those from AA (+) SMP30/GNL-KO mice (Fig. 6B).

3.8. Effect of AA deficiency on lipid composition in the liver and plasma

To examine the effect of an AA deficiency on the lipid composition in the liver and plasma, we measured free fatty acid, total cholesterol, triglyceride, and phospholipid levels in the livers and plasma from AA (+) and AA (−) SMP30/GNL-KO mice (Supplementary Fig. S4). All these concentrations were similar in the livers and plasma of AA (+) and AA (−) SMP30/GNL-KO mice (Supplementary Fig. S4A–H).

3.9. Effect of AA deficiency on SREBP-1 and SREBP-2 protein levels

SREBP-1 and SREBP-2 are transcriptional factors present in precursor and activated forms and have the ability to increase gene expression related to lipid biosynthesis [56]. Therefore, we examined SREBP-1 and SREBP-2 protein levels in livers from AA (+) and AA (−) SMP30/GNL-KO mice by using Western blot analysis (Fig. 7). The AA deficiency activated transcriptional protein, SREBP-1 (Fig. 7A); however, SREBP-2 was not activated by an AA deficiency (Fig. 7B).

4. Discussion

As this study documents, a deficiency of AA affected the expression of many genes concerned with the oxidation–reduction process and lipid metabolism in the livers of SMP30/GNL-KO mice, which are unable to synthesize AA in vivo.

Extracting functional annotations from the results of microarray analysis revealed that numerous genes related to the oxidation–reduction process manifested increased expression in an AA-deficient setting. Furthermore, since Nrf2 is a ubiquitous master transcription factor that regulates ARE-mediated expression of antioxidant enzyme and cytoprotective proteins [58], we focused here on scrutinizing Nrf2. Indeed, microarray analysis revealed a pronounced increase in *Nrf2* gene expression and highlighted a number of Nrf2 target genes with AREs whose expression also increased when the liver was AA deficient (Supplementary Table S9). Nrf2 is known to activate in response to oxidative stress, detaches from its cytosolic inhibitor, Keap1, translocates to the nucleus, and binds to the AREs in the promoter of target genes [58]. In this study, Nrf2 protein reached higher levels in the nuclei from AA-deficient animals (Fig. 2A). Therefore, the AA deficiency must activate Nrf2, which becomes translocated to the nuclei and able to induce the expression of its target genes.

Nqo1 is highly susceptible to oxidative stress and induced by the activation of Nrf2 [60]. Among Nrf2 target genes, *Nqo1* gene expression was the most increased by an AA deficiency (Supplementary Table S9), and the Nqo1 protein level was increased a notable two-fold by the AA deficient state (Fig. 3A). Since Nqo1 has multiple antioxidant activities, in particular the scavenging of superoxide [61],

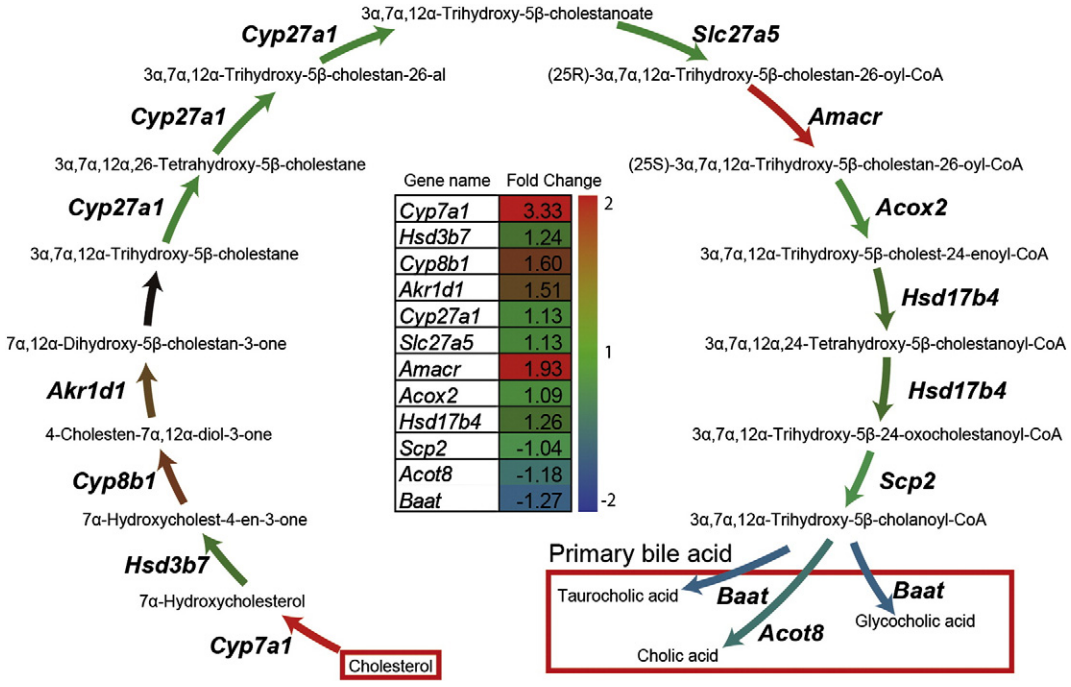

Fig. 4. Effect of AA deficiency on gene expression in the primary bile acid biosynthesis pathway. Primary bile acids including cholic acid, taurocholic acid, and glycocholic acid are synthesized from cholesterol in the liver. Levels of genes for enzymes involved in primary bile acid biosynthesis in livers from AA (−) SMP30/GNL-KO mice were expressed as a ratio compared to those of AA (+) SMP30/GNL-KO mice and are indicated in a color scale from red (increased) to blue (decreased). *Cyp7a1*, cytochrome P450, family 7, subfamily a, polypeptide 1; *Hsd3b7*, hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7; *Cyp8b1*, cytochrome P450, family 8, subfamily b, polypeptide 1; *Akr1d1*, aldo-keto reductase family 1, member D1; *Cyp27a1*, cytochrome P450, family 27, subfamily a, polypeptide 1; *Slc27a5*, solute carrier family 27 (fatty acid transporter), member 5; *Amacr*, alpha-methylacyl-CoA racemase; *Acox2*, acyl-Coenzyme A oxidase 2, branched chain; *Hsd17b4*, hydroxysteroid (17-beta) dehydrogenase; *Scp2*, sterol carrier protein 2; *Acot8*, acyl-CoA thioesterase 8; and *Baat*, bile acid-Coenzyme A: amino acid N-acyltransferase.

Nqo1 might be a major contributor to maintaining the oxidation–reduction process in AA-deficient livers. Another Nrf2 target gene product analyzed here, γ-GCSc, as well as the anti-oxidant proteins CAT, SOD1, and SOD2 remained at almost the same levels in the AA-deficient or -sufficient backgrounds (Fig. 3B–E). Moreover, expression of the Nrf2 target gene, *heme oxygenase-1* (*HO-1*), was decreased by an AA deficiency (Supplementary Table S9). These results realistically indicate the importance of AA in the liver's complicated oxidation–reduction balance.

The expression of genes involved in the primary bile acid biosynthesis pathway, which is a main route of cholesterol metabolism, was clearly altered by the AA deficiency (Fig. 4). Especially, expression of the *Cyp7a1* gene, which is a cytochrome P450 heme enzyme and a rate-limiting enzyme of bile acid biosynthesis, was increased by an AA deficiency. In the AA deficient surroundings, the Cyp7a1 protein level also increased (Fig. 6A); however, Cyp7a1 enzyme activity decreased (Fig. 6B). As a result, AA deficiency caused a reduction of bile acid levels in the liver and gallbladder (Fig. 5). Cyp7a1 is also known as cholesterol 7α-hydroxylase or cholesterol 7-α-monooxygenase, which oxidizes cholesterol using molecular oxygen [62]. For the enzymatic activation of Cyp7a1, ferrous iron (Fe^{2+}) is necessary and must become oxidized to ferric iron (Fe^{3+}), because Cyp7a1 has a heme iron at the active site. Therefore, AA must act as a reductant for the iron, thereby reducing Fe^{3+} to Fe^{2+} for the continuous activation of Cyp7a1 [63]. Actually, several reports noted that bile acid levels and Cyp7a1 activity were reduced in AA-deficient guinea pigs and ODS rats [57,62,64,65].

Bile acid is biosynthesized from cholesterol in the liver, and an AA deficiency increased the expression of genes related to lipid metabolism. Therefore, we checked the free fatty acid, total cholesterol, triglyceride, and phospholipids levels in livers and plasma from AA (+) and AA (−) SMP30/GNL-KO mice; however, no differences resulted from the AA deficiency, even of total cholesterol levels (Supplementary

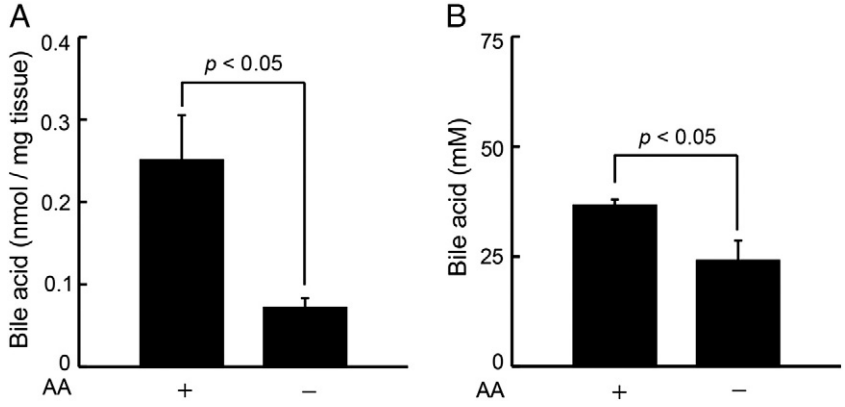

Fig. 5. Effect of the AA deficiency on primary bile acid biosynthesis. Bile acid levels in livers (A) and gallbladders (B) from AA (+) and AA (−) SMP30/GNL-KO mice were measured as described in the Materials and methods. Values are expressed as a means ± SEM of five animals. Statistical differences were considered significant at $p < 0.05$.

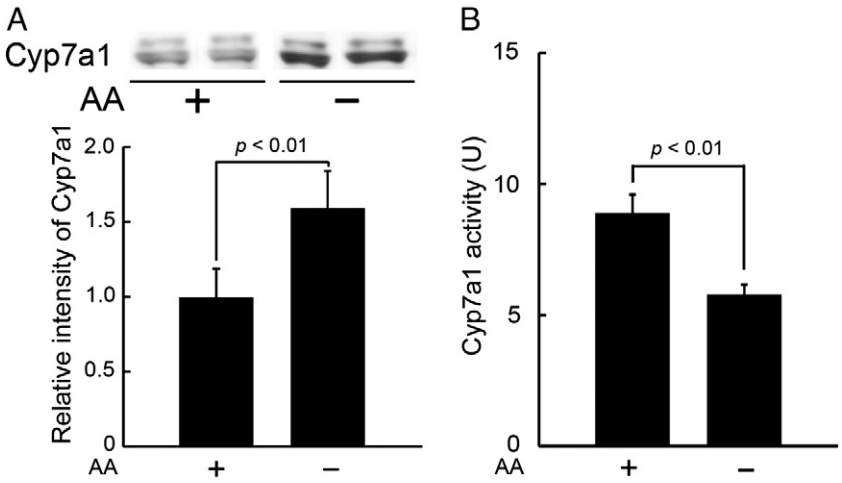

Fig. 6. Western blot analysis of Cyp7a1 and activity of Cyp7a1. (A) Cyp7a1 protein levels in the liver from AA (+) and AA (−) SMP30/GNL-KO mice were quantitated with ImageJ, version 1.45s. Cyp7a1 protein levels from AA (−) SMP30/GNL-KO mice were expressed as a ratio compared to AA (+) SMP30/GNL-KO mice. Values are presented as means ± SEM of five animals. Statistical differences were considered significant at $p < 0.05$. (B) Cyp7a1 activity was measured as described in the Materials and methods using [7-^{3}H(N)] cholesterol. Values are means ± SEM from four animals. Statistical differences were considered significant at $p < 0.05$. U, pmol/min/protein.

Fig. S4). Nevertheless, an AA deficiency activated transcriptional factor, SREBP-1, which enhances the expression of genes for fatty acid, triglyceride, and phospholipid biosynthesis (Fig. 7A, Supplementary Table S3) [66]. However, SREBP-2, which enhances gene expression related to cholesterol biosynthesis, was not activated by an AA deficiency (Fig. 7B). Previously, we reported that triglyceride, total cholesterol, and phospholipids accumulated in livers from 12-month-old SMP30/GNL-KO mice fed an AA-insufficient diet [67]. The overall likelihood, therefore, is that a long-term AA deficiency must lead to an accumulation of lipids in the liver.

The liver houses metabolism of many physiologically critical substances, especially, xenobiotic metabolism of the cytochrome P450 family. Therefore, an AA-deficiency related increase of more than two-fold for the expression of not only *Cyp7a1* but also other genes of the cytochrome P450 family, such as *Cyp4a14*, *Cyp2c39*, *Cyp2c28* is striking (Supplementary Fig. S3). Like Cyp7a1, Cyp4a14, and Cyp2c39, Cyp2c28 proteins also have heme iron at the active site. Therefore, the probable contribution of AA to the activation of cytochrome P450 family members as well as Cyp7a1 might influence xenobiotic metabolism.

Our microarray analyses revealed that AA participates in the expression of numerous genes. For the present, we provide no conclusions about genes for which expression decreased in the AA deficient state, because the results were not consistent (Supplementary Table S4), although genes related to the acute-phase response had the highest counts of annotations (Supplementary Table S6).

Frequency analysis of annotations revealed, though, that genes related to the regulation of translation and transcription had the highest counts in the *SMP30/GNL* gene deletion category (Supplementary Tables S7, S8). The expression of numerous genes was altered by *SMP30/GNL* gene deletion (Supplementary Table S1, S2); however, we found no regularity or consistent mechanism of genes related to translation and transcription. Previously, we reported that SMP30/GNL protein localized in both the nuclei and cytoplasm of mouse hepatocytes

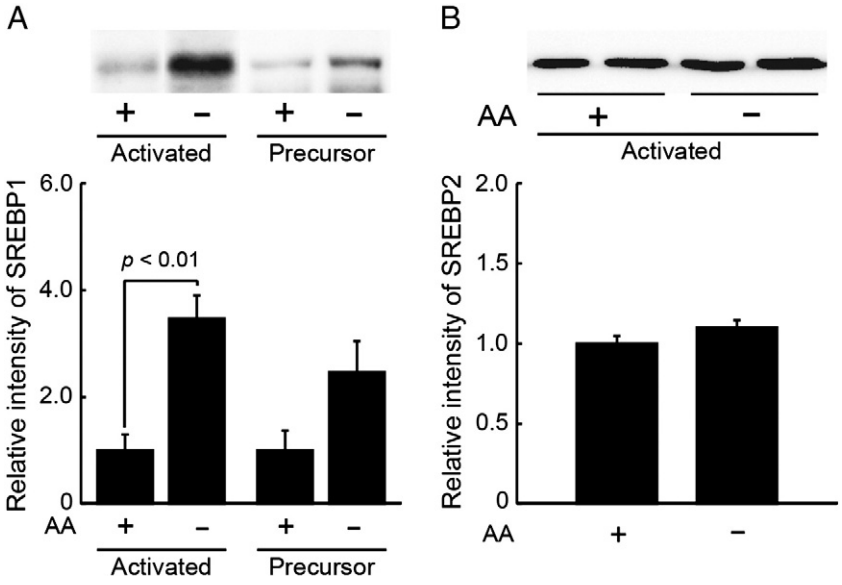

Fig. 7. Western blot analysis of SREBP-1 and SREBP-2. SREBP-1 and SREBP-2, which are transcriptional factors present in precursor and activated forms and have the ability to increase gene expression related to lipid biosynthesis. SREBP-1 and SREBP-2 protein levels in livers from AA (+) and AA (−) SMP30/GNL-KO mice were quantitated with ImageJ, version 1.45s. SREBP-1 and SREBP-2 protein levels from AA (−) SMP30/GNL-KO mice were expressed as a ratio compared to AA (+) SMP30/GNL-KO mice. Values are expressed as means ± SEM of five animals. Statistical differences were considered significant at $p < 0.05$.

[68]. Therefore, the possibility remains that SMP30/GNL is involved in the translation and transcription mechanism.

Finally, this study revealed that AA is an important factor in lipid metabolism including bile acid biosynthesis in liver as well as the oxidation–reduction process. The results from this extensive investigation of SMP30/GNL-KO mice provide us with new and detailed information about formerly unknown AA functions and form the foundation for further study of AA in vivo.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.03.019.

Acknowledgements

This work was supported by JSPS KAKENHI Grant Number 24380073 to A.I. and 23590441 to N.M. We wish to thank Prof. Fumihiko Horio, Department of Agricultural Chemistry, Nagoya University, Japan, for advice and technical help regarding Cyp7a1 activity. We thank Ms. P. Minick for the excellent English editorial assistance. Ascorbic acid powder was kindly provided by DSM Nutrition Japan.

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