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Regulation of nicotinamide N-methyltransferase by oxidative stress in hepatocytes

조선대학교 대학원

글로벌바이오융합학과

신 은 진

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산화적 스트레스에 의한
니코틴아마이드 N-메틸전이효소의 발현 조절 연구

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이 논문을 이학 석사학위 신청 논문으로 제출함.

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ABBREVIATIONS

BHA	Butylated hydroxyanisole
DCFH-DA	2',7'-Dichlorofluorescein diacetate
DMSO	Dimethylsulfoxide
DPI	Diphenyleneiodonium chloride
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	N-acetyl-L-cysteine
NAD	Nicotinamide adenine dinucleotide
NNMT	Nicotinamide N-methyltransferase
NR	Nicotinamide riboside
ROS	Reactive oxygen species
t-BHP	*tert*-Butyl hydroperoxide

ABSTRACT

Regulation of nicotinamide N-methyltransferase by oxidative stress in hepatocytes

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Oxidative stress caused by an imbalance of antioxidant and reactive oxygen species, plays a pathophysiological role in liver disease. Nicotinamide N-methyltransferase (NNMT) is a methyltransferase catalyzing nicotinamide methylation and degradation. However, there is a scarcely information availabling the modulation of NNMT under oxidative stress and its association with liver diseases. In the present study, it was found that the level of NNMT was decreased in *tert*-butylhydroperoxide (*t*-BHP)-exposed HepG2 cell. Downregulation of NNMT by t-BHP was also observed in primary murine hepatocytes, and another hepatocyte cell line L02. However, the lowered level of NNMT could be restored by treatment with antioxidants, including N-acetyl-L-cysteine, butylated hydroxyanisole, or diphenyleneiodonium chloride. Furthermore, *t*-BHP-mediated downregulation of NNMT was not response to either changed mRNA level or protein degradation. Therefore, we investigated a possible involvement of microRNAs (miRNAs, miRs) that putatively binding to the 3'-untranslated region of NNMT. Among miRNA candidates, miR-182-5p was significantly elevated in *t*-BHP-treated HepG2 cells. Furthermore, it was discovered that NNMT did not affect *t*-BHP-induced ROS generation as well as cytotoxicity. Interestingly, *t*-BHP-mediated ATP reduction was reversed by nicotinamide riboside treatment, which was further reduced by incubation with NNMT inhibitors. In addition,

it was revealed that NNMT overexpression increased the levels of SIRT-1, SIRT-3, and SIRT-5. Taken together, these results demonstrate that NNMT may be a candidate molecule for attenuating susceptibility to oxidative stress-induced liver diseases.

1. INTRODUCTION

Reactive oxygen species (ROS), including superoxide anions, hydrogen peroxide, and hydroxyl radicals, are a family of chemically reactive molecules harboring oxygen that can interact with various biomolecules. ROS levels are tightly controlled by antioxidant genes and detoxifying enzymes to keep intracellular homeostasis between oxidants and antioxidants. When the balance between ROS and antioxidants is disrupted, oxidative stress generates. Oxidative stress induces intracellular damage and subsequently activates pathways causing change of cellular metabolism [1]. Particularly, when ROS are produced in hepatocyte, which is a susceptible cell type in liver to oxidative stress, it could affect energy metabolism subsequently provoking liver injury [2]. Indeed, oxidative stress is regarded as a mediator of hepatocyte injury from cytokines, hepatitis virus and ischemia reperfusion hepatitis virus [3-6]. Unfortunately, there is little established signaling pathway that is connected with cell metabolism and ROS. Hence, a better understating of the molecule and related mechanism involved in this process might be beneficial to treat hepatocyte damage as well as hepatic injury against ROS.

Since ROS generation and its scavenging systems are dependent on a NADH/NAD^+ ratio, redox homeostasis is significantly affected by changes in NAD^+ levels [7]. Among the molecules of the NAD^+-related signaling pathway, nicotinamide N-methyltransferase (NNMT), a cytosolic methyltransferase, catalyzes SAM-linked methylation and degradation of nicotinamide, a major precursor for NAD^+ synthesis [8, 9]. A number of studies have illustrated that NNMT plays a critical role in the malignant potential of cancers via cell proliferation, migration, invasion and regulation of metabolism [10-13]. Besides, NNMT and its metabolites have implicating a potential role in the occurrence and progression of liver diseases by recently been connected with various liver diseases such as alcoholic liver disease, non-alcoholic fatty liver disease, liver cirrhosis [14-16]. Nevertheless, whether and how oxidative stress influences NNMT expression, and how altered NNMT contributes to oxidative stress-induced hepatocyte metabolism remain unanswered question.

Sirtuins (SIRTs), mammalian homologs of the yeast silent information regulator 2 protein, are family of NAD^+-dependent deacetylases and have been reported to have a correlation with mitochondrial function, DNA damage, and apoptosis by regulating oxidative stress-connected processes. The Sirtuin family is composed of seven subfamilies form SIRT-1 to SIRT-7 and mediates lysine deacetylation of various proteins and enzymes involved in oxidative stress [17]. Until now, only little has been revealed about the relationship between NNMT and SIRTs in hepatic damage accompanying oxidative stress. In particular, nothing is investigated about the role of NNMT in conjugation with SIRTs as an interface between energy metabolism and oxidative stress.

Given the lack of study on the role of NNMT in oxidative stress and the potential connection between NNMT and SIRTs, this study explored whether NNMT is decreased in due to oxidative stress in hepatocytes, if so, what the impact is on liver and how it is regulated. The results obtained by this study show that exposing hepatocytes to ROS such as *tert*-butylhydroperoxide (*t*-BHP) deceased NNMT level, which might be regulated by a specific microRNA (miRNA, miR). In addition, NNMT might affect energy metabolism of hepatocyte in conjunction with SIRTs. All these results suggest that NNMT might exert as a potential target of liver diseases via regulation of oxidative stress-mediated liver metabolism.

2. MATERIALS AND METHODS

2-1. Materials

The nicontinamide N-methyltransferase (NNMT) antibody was purchased from Proteintech (Rosemount, IL, USA). Chloroquine (CQ), Z-Leu-Leu-Leu-al (MG132), Rotenone, *t*-butylhydroperoxide (*t*-BHP), N-acetyl-L-cysteine (NAC), butylated hydroxyanisole (BHA), diphenyleneiodonium chloride (DPI), dimethylsulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), 1-methylnicotinamide (MNA), 2',7'-dichlorofluorescein diacetate (DCFH-DA), and β-actin antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA). Nicotinamide riboside was purchased from GlpBio (Montclair, CA, USA). Anti-mouse and anti-rabbit antibodies were supplied from Invitrogen (Carlsbad, CA, USA). JBSNF-000088 (JB) was procured from MedChem Express (Monmouth Junction, NJ, USA). SIRT-1 antibody was obtained from Novus Biologicals (Centennial CO, CO, USA). Antibodies against SIRT-3 and SIRT-5 was purchased from Cell Signaling (Danversm, MA, USA) and Abcam (Cambridge, MA, USA), respectively.

2-2. Cell culture

HepG2 and L02 cell lines were obtained from ATCC (American Type Culture Collection, Manassas, VA). Cells were plated in 60 mm or 6-well plates (Thermo Scientific, Waltham, MA, USA), and used with 70-80% confluency. Cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) with 50 U/mL penicillin/streptomycin, 10% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO, USA) at 37℃ and a humidified 5% CO_2 atmosphere.

2-3. Primary Hepatocytes isolation

Hepatocytes were separated from the 8-week-old ICR mice (Oriental Bio, Sungnam, South Korea). In brief, after catheter intubation in the hepatic portal vein Hank's balanced saline solution was perfused for 20 min at 37℃. Then, the hepatocytes were further perfused with a solution containing 0.05% collagenase and Ca^{2+} for 20 min to separate. Carefully remove the perfused liver, filtered through 70 μM cell strainer (BD Bioscience, Two Oak Park, Bedford, MA) and centrifuged at 50 x g for 2 min.

2-4. Immunoblot analysis

Preparation of cell lysates, SDS-polyacrylamide gel electrophoresis, and immunoblot analysis were performed previously published methods [18]. Cell lysates were centrifuged at 12,000 x *g* for 10 min to extract proteins and then separated on 7.5% and 12% SDS-polyacrylamide gels through electrophoresis, before being electrophoresis transferred to nitrocellulose membranes described. Subsequently, the membranes were blocked with 5% skim milk for 0.5 h and incubated with primary antibodies overnight at 4°C. The next day, the membranes were reacted with peroxidase-conjugated secondary antibodies for 1 h, and the immunoreactive proteins were visualized using an ECL chemiluminescence detection kit (Thermo Scientific, Waltham, MA, USA).

2-5. RNA isolation and RT-PCR analysis

Total RNA was obtained by TRIzol (Invitrogen, Carlsbad, CA, USA) according

to the manufacturer's protocol provided. cDNA was synthesized from 2 μg of total RNA using the oligo(dT)$_{16}$ primer and amplified using a high-capacity cDNA synthesis kit with a thermal cycler (Bio-Rad, Hercules, CA, USA) equipped with a high-capacity cDNA synthesis kit (Bioneer, Daejeon, Korea). After PCR amplification using PCR premix (Bioneer), PCR amplification products were separated using 2% agarose gel by gel electrophoresis, stained with ethidium bromide (Sigma, St. Louis, MO, USA), and visualized in gel documentation system. (Fujifilm, Tokyo, Japan). The primer sequences were as follows: human NNMT Forward 5'-GTTTGGTTCTAGGCACTCTG-3', and Reverse 5'-GCAGGTTCTGGTCTGAGTAG-3'; human GAPDH Forward 5'-GAAGGTGA AGGTCGGAGTC-3, and Reverse 5'-GAAGATGGTGATGGGATTTC-3'. GAPDH was used as a reference gene for normalization. Quantitative RT-PCR for microRNA was performed adopting miRCURY LNA RT and miRCURT LNA SYBR Green PCR kit (Qiagen, Hiden, Germany), following the manufacturer's protocol. The primer sequences were as follows: hsa-miR-182-5p, 5'-UUUGGCAAUGGUAGAACUCACACU-3' and hsa-miR-383-5p, 5'-AGAUCAGAAGGUGAUUGUGGCU-3′. All results were normalized to U6 small RNA levels. The values of relative expression were calculated by $2^{\wedge-\delta\delta Ct}$.

2-6. Measurement of ROS production

HepG2 cells after t-BHP treatment were stained with 10mM DCFH-DA at 37°C for 30 min. ROS generation was determined by dichlorofluorescein fluorescence. The fluorescence intensity of the cells was measured at an excitation/emission wavelength of 485 nm/530 nm using a fluorescence microplate reader (Gemini, Molecular Devices, Sunnyvale, California).

2-7. MTT assay

To measure cell viability, HepG2 cells were plated in a 12-well plate at a density of 1 x 10^5 cells per well. Cells were treated with 100 μM MNA, 100 μM JB,

or 0.5 mM NR for 30 min and then treated 700 μM *t*-BHP for 5 h. After treatment, viable cells were stained with 0.2 mg/mL MTT and incubated for 10 min. Then, the medium was removed and all the formed formazan crystals in the wells were dissolved by adding 400 μL of dimethyl sulfoxide. The absorbance of each sample was measured at 550 nm using an enzyme-linked immunosorbent assay microplate reader (Spectra MAX, Molecular Device, Sunnyvale, CA). Cell viability was defined with respect to an untreated control group, *i.e.*, cell viability (% of control) = 100 × (absorbance of the treated sample) / (absorbance of the control group).

2-8. Transient transfection

NNMT overexpression plasmid was obtained from Origene (Rockville, MD, USA). Transfection was performed for 24 h with pCMV4 or NNMT using Lipofectamine^{TM} 2000 (Invitrogen; Thermo Fisher Scientific, Inc) according to the manufacturer's provided protocol. Then, the medium was replaced with MEM medium and treated with *t*-BHP for 12 h.

2-9. Immunofluorescence

Primary hepatocytes were seeded on cover slips fixed with 4% paraformaldehyde and permeabilized with Triton X-100. Next, the samples were immunostained overnight with the NNMT antibody, followed by incubation with Alexa Fluorâ 594 goat anti-rabbit IgG (IInvitrogen; Thermo Fisher Scientific, Inc). The samples were then covered with cover slips. Subsequently, the samples were detected using a fluorescence EVOS M5000 microscope (Olympus, Tokyo, Japan).

2-10. ATP Measurement

ATP levels were evaluated using the EnzyLight ADP/ATP Ratio Assay Kit

(BioAssay Systems, Hayward, CA, USA) referring as the manufacturer's guidelines. Briefly, cells grown in a 96-well plate were treated with the ATP reagent from the kit. Subsequently, the luminescence by ATP was evaluated utilizing a luminometer (Promega, Madison, WI).

2-11. Animal experiments

All experimental protocols for animal research were approved by the Chosun University Animal Care and Use Committee. Male ICR mice (six weeks old) were purchased from Oriental Bio (Sung-nam, Korea) and acclimated for 1 week. The mice (n = 8/group) were housed at 20 ± 2 ◦C under pathogenic-free air filtered at a 12-hour light/dark cycle and a relative humidity of 50 ± 5%, and food (Purina, Korea) and water were available. Acetaminophen, carbon tetrachloride, choline-deficient L-amino acid defined high fat diet, or high fat diet-fed mouse samples were adopted as previously used [19-22].

2-12. Statistical analysis

One-way ANOVA was used to assess significant differences between treatment groups. The Newman-Keuls test was used to assess the significance of the differences between the means of multiple groups. Results are expressed as mean ± standard Error (SE).

3. RESULTS

3-1. Downregulation of NNMT in oxidative stress-induced liver disease models

To examine the relationship between NNMT and oxidative stress, the expression of NNMT was envaluated in various ROS-mediated liver disease models (i.e. acetaminophen (APAP) or carbon tetrachloride (CCl_4)-treated mice model, choline deficiency L-amino acid-defined high-fat diet (CDA-HFD) or high-fat diet (HFD)-fed mice model) [19-22]. The level of NNMT was significantly decreased in liver homogenates from mice treated APAP or CCl_4 as compared with that observed in respective controls (Figs. 1A and 1B). The same change was seen in animals fed CDA-HFD or HFD (Figs. 1C and 1D). These results suggest that NNMT expression is downregulated in response to oxidative stress.

3-2. The effect of ROS on the expression of NNMT in hepatocytes.

The expression of NNMT against ROS was examined *in vitro* models. The NNMT level after treatment with *t*-BHP, a representative ROS, for different time periods in HepG2 cells (immortalized human hepatocyte-derived cells) (Fig. 2A). As shown in Fig. 2A, NNMT protein level was found to be gradually diminished after 3-12 h of *t*-BHP treatment. To further these results with the ROS-mediated NNMT downregulation, L02 cells as another hepatocyte-derived cell line and mouse primary hepatocytes were used. Decreased NNMT expression against *t*-BHP was also observed (Figs. 2B and 2C). Consistently, *t*-BHP treatment reduced the immunostaining level of NNMT in primary hepatocytes (Fig. 2D). Additionally, we investigated NNMT

expression after incubation with various concentrations of *t*-BHP in HepG2 cells (Fig. 2E). NNMT was found to markedly decreased by *t*-BHP treatment and reached at minimum at 100 μM of *t*-BHP. Our results demonstrate that oxidative stress induces reduction of NNMT expression.

3-3. Redox regulation of NNMT expression by ROS

To investigate the connection with ROS and NNMT expression, we evaluated the possibility of NNMT downregulation via ROS. We exposed HepG2 cells with antioxidants such as NAC, BHA, and DPI in combination with *t*-BHP (Fig. 3A). Antioxidants considerably attenuated the ROS-mediated NNMT reduction. Moreover, we determineed ROS generation in HepG2 cells after *t*-BHP incubation utilizing fluorescent microscopic images or fluorescence microplate reader (Fig. 3B). These findings illustrate that *t*-BHP-mediated NNMT downregulation occurred via ROS-dependent manner.

3-4. Decrease of NNMT expression is not governed by either transcriptional mechanism or protein degradation.

To reveal the molecular mechanism regulating NNMT reduction by *t*-BHP, we examined NNMT mRNA levels, and discovered that they were not changed in HepG2 cells (Fig. 4A). Hence, it is highly controlled that NNMT is post-transcriptionally regulated. Next, we investigated the NNMT expression level after treatment with chloroquine (a lysosomal inhibitor) or MG132 (a proteasomal inhibitor) alone or in combination with *t*-BHP (Fig. 4B and 4C). Unexpectedly, NNMT reduction by *t*-BHP was not affected under treatment of chloroquine or MG132. Hence, these results suggest that NNMT expression is not regulated by either transcriptional regulation or protein degradation.

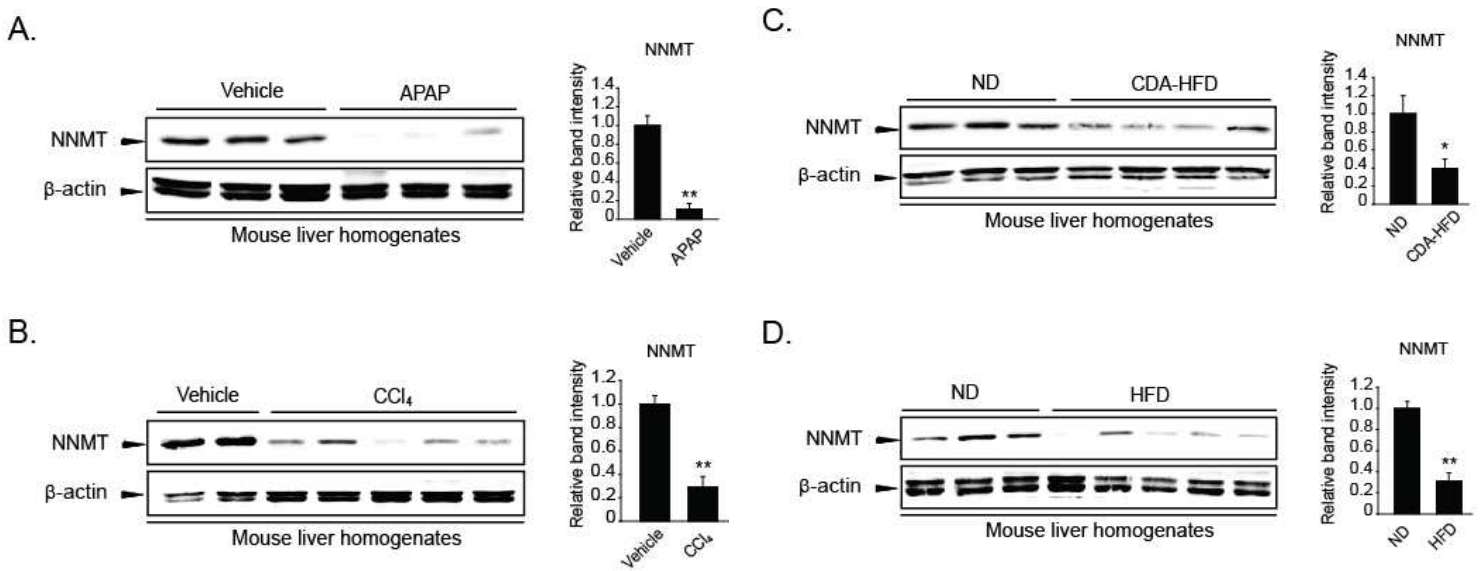

Fig. 1. NNMT expression in oxidative stress-mediated liver disease mice models. (A) Immunoblotting of NNMT in liver homogenates of acetaminophen (APAP)-treated mice liver tissue. (B) Immunoblotting of NNMT in liver homogenates of carbon tetrachloride (CCl_4)-injected mice liver tissue. (C) Immunoblotting of NNMT in liver homogenates of choline-deficient L-amino acid-defined high-fat diet (CDA-HFD) treated mice liver tissues. (D) Immunoblotting of NNMT in liver homogenates of high-fat diet (HFD) treated mice liver tissues. β-actin was used as loading control. The data represents the mean ± SE (n = 3, significant different versus respective controls, *p<0.05, **p<0.01).

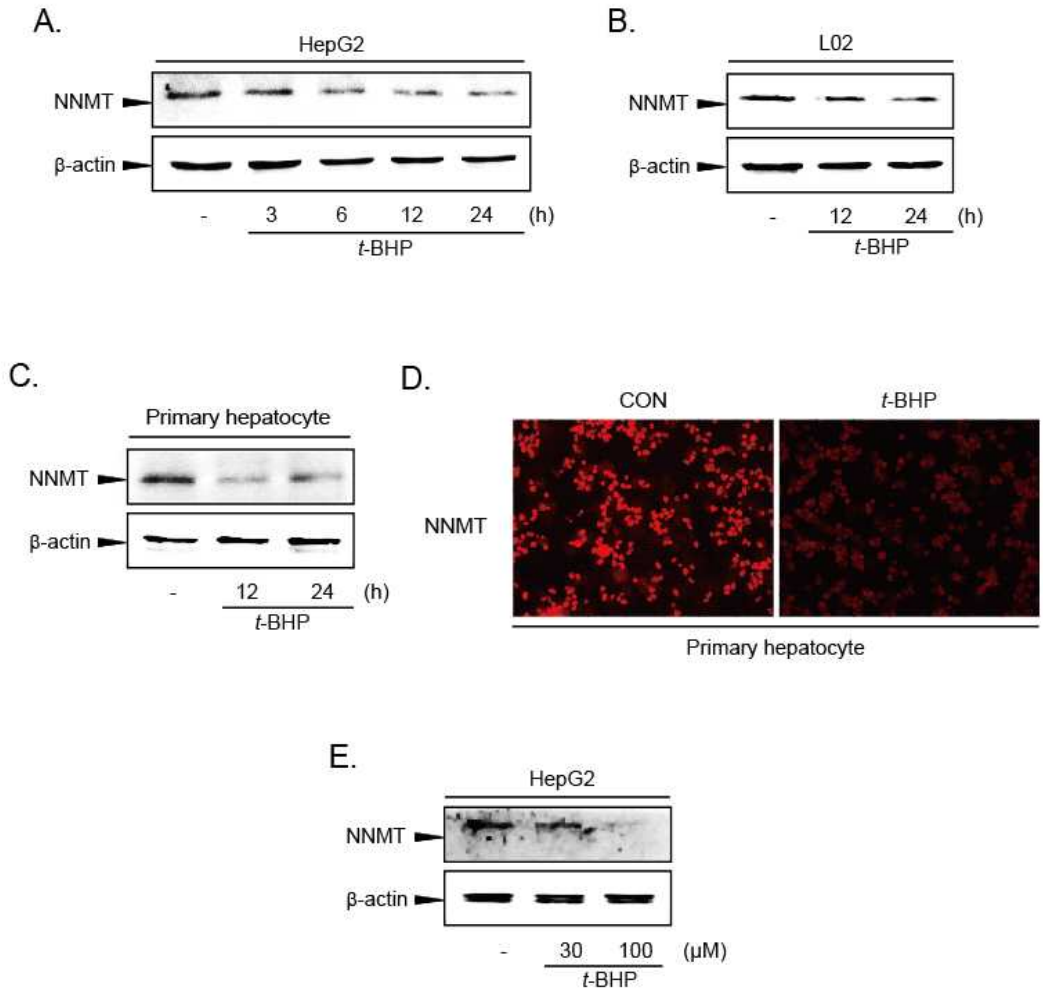

Fig. 2. The effect of ROS on NNMT expression in hepatocytes. (A) The time courses of NNMT expression in HepG2 cells treated with *t*-BHP (100 uM). Immunoblotting for NNMT was performed in cells treated with *t*-BHP (100 uM) for 3-24 h. (B and C) NNMT expression in L02 cells or primary hepatocytes treated with *t*-BHP (100 μM) for 12 and 24 h. (D) Immunostaining of NNMT in primary hepatocytes treated with *t*-BHP (100 μM) for 12 h. (E) The effect of concentration-dependent manner of *t*-BHP on NNMT expression in *t*-BHP (100 μM)-incubated HepG2 cells for 12 h.

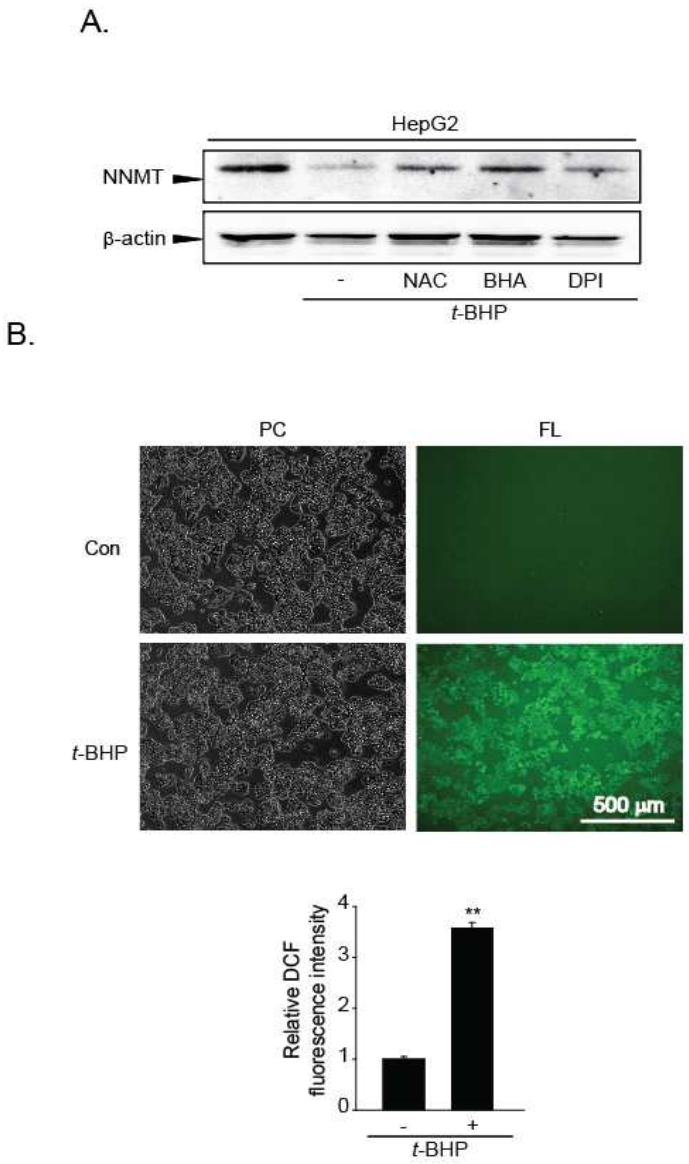

Fig. 3. Redox regulation of NNMT expression by *t*-BHP-induced oxidative stress. (A) HepG2 cells were stimulationed to *t*-BHP (100 μM) for 12 h after pretreatment with NAC (2 mM), BHA (100 μM) and DPI (1 μM) for 30 min. NNMT protein levels in lysates were measured by immunoblotting. (B) Intracellular fluorescence intensities were measured using DCFH-DA analysis. Data represent means ± SE of three replicates (n = 3, significant as compared with vehicle-treated cells, $^{**}p<0.01$).

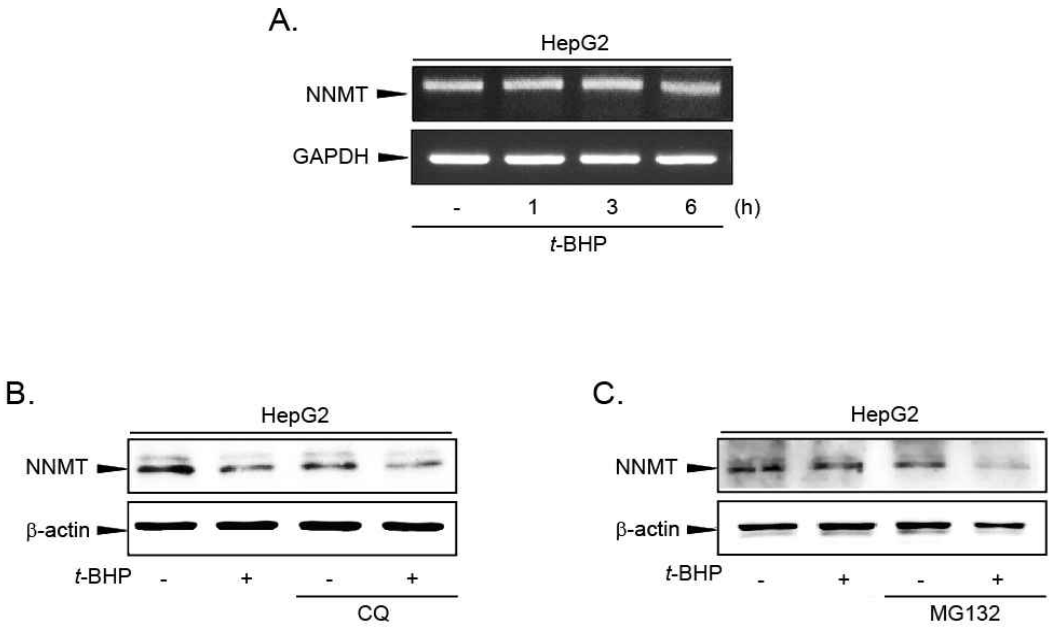

Fig. 4. *t*-BHP-mediated NNMT downregulation by neither post-translational mechanism nor protein degradation. (A) PCR analysis. HepG2 cells were exposed to *t*-BHP (100 μM) for 1-12 h. The transcription levels of NNMT were determined by RT-PCR using GAPDH as an internal control. (B) The effect of lysosome inhibitors on NNMT expression by *t*-BHP. HepG2 cells were incubated for 12 h with chloroquine (CQ) in the presence or absence of *t*-BHP, and then NNMT expression in cell lysates was evaluated by immunoblotting. (C) The effect of proteasome inhibitors on NNMT expression by *t*-BHP. HepG2 cells were cultured for 12 h with or without MG132 in the presence or absence of *t*-BHP, and then NNMT expression in cell lysates was evaluated by immunoblotting.

3-5. Identification of microRNA-182-5p as a possible regulator of NNMT.

miRNAs are major cellular controller of post-transcriptional mechanism, which bind to the 3′ ends of mRNA via base-pairing with complementary sequences, inhibiting the translation efficiency and/or reduce mRNA level [23]. We searched for predicted miRNAs targeting 3′-untranslated region (UTR) of NNMT utilizing the TargetScan 8.0 algorithm (http://www.targetscan.org). miR-182-5p and miR-383-5p were predicted as candidate miRNAs regulating the NNMT expression (Fig. 5A). Among them, miR-182-5p was predicted as the highest affinity for NNMT. Additionally, it was examined the pairing between miR-182-5p and 3'-UTR of NNMT, and found complete complementarity (Fig. 5B). The pairing sequence present in the 3'-UTR of NNMT mRNAs were well-conserved in human and mice. When we examined miR-182-5p level in *t*-BHP-exposed HepG2 cells, miR-182-5p levels were effectively increased in *t*-BHP-treated HepG2 cells (Fig. 5C). However, miR-383-5p, another probable miRNA for 3'-UTR of NNMT, was not detected. These results suggest the possibility that miR-182-5p dysregulation resulted in NNMT downregulation in ROS-exposed hepatocytes.

3-6. Impact of *t*-BHP-induced NNMT reduction on cell viability and ROS levels.

Based on the report that excessive ROS leads to cell death, it was necessary to determine the functional role of NNMT in cell viability against oxidative stress in hepatocytes. It was observed fluorescence intensities with DCFH-DA to detect ROS in HepG2 cells (Fig. 6A). Unexpectedly, NNMT activation using NR and NNMT overexpression did not suppress *t*-BHP-mediated ROS production. NNMT inhibition using two chemical inhibitors (JB or MNA) was not affected ROS generation, either.

Sequentially, the effect of NNMT on cell survival was examined (Fig. 6B). When MTT assay in HepG2 cells was performed, NR treatment and ectopic expression of NNMT failed to reverse *t*-BHP-induced cytotoxicity. NNMT inhibitors exhibited same patterns of cell viability as those seen for NNMT activation. Therefore, these evidences suggest that NNMT does not seem to possess hepatocytoprotective effect.

3-7. Involvment of SIRTs in NNMT downregulation-mediated cellular metabolism.

NNMT is associated with NAD^+, a crucial metabolic cofactor for energy homeostasis and cellular metabolism [24, 25]. It was investigated the role of NNMT in ATP production. It was found that *t*-BHP decreased ATP, which was further intensified by cotreatment with *t*-BHP and MNA or JB. In contrast, *t*-BHP-mediated ATP depletion was notably reversed by treatment with NR (Fig. 7A). Based on the fact that SIRTs have been proposed as nutrient-sensing regulators that directly related to cellular redox state and homeostasis of energy metabolism in connection with NAD^+ [26], it explored the connection with SIRTs against oxidative stress. Among the various isoforms of SIRTs, SIRT-1, SIRT-3, and SIRT-5 are considered key NAD^+ sensors [27-31]. To test possible connection of SIRTs and NNMT-involved oxidative stress, it was introduced NNMT into HepG2 cells and check SIRT-1, SIRT-3, and SIRT-5 expressions (Fig. 7B). Interestingly, *t*-BHP treatment of pCMV4-transfected HepG2 cells slightly increased SIRT-1, SIRT-3, and SIRT-5 levels, which were further incremented by NNMT overexpression. Among them, SIRT-5 showed the greatest changes by ectopic expression of NNMT. These results suggest that NNMT reduction in hepatocytes might facilitate cellular metabolism via SIRT-dependent pathway.

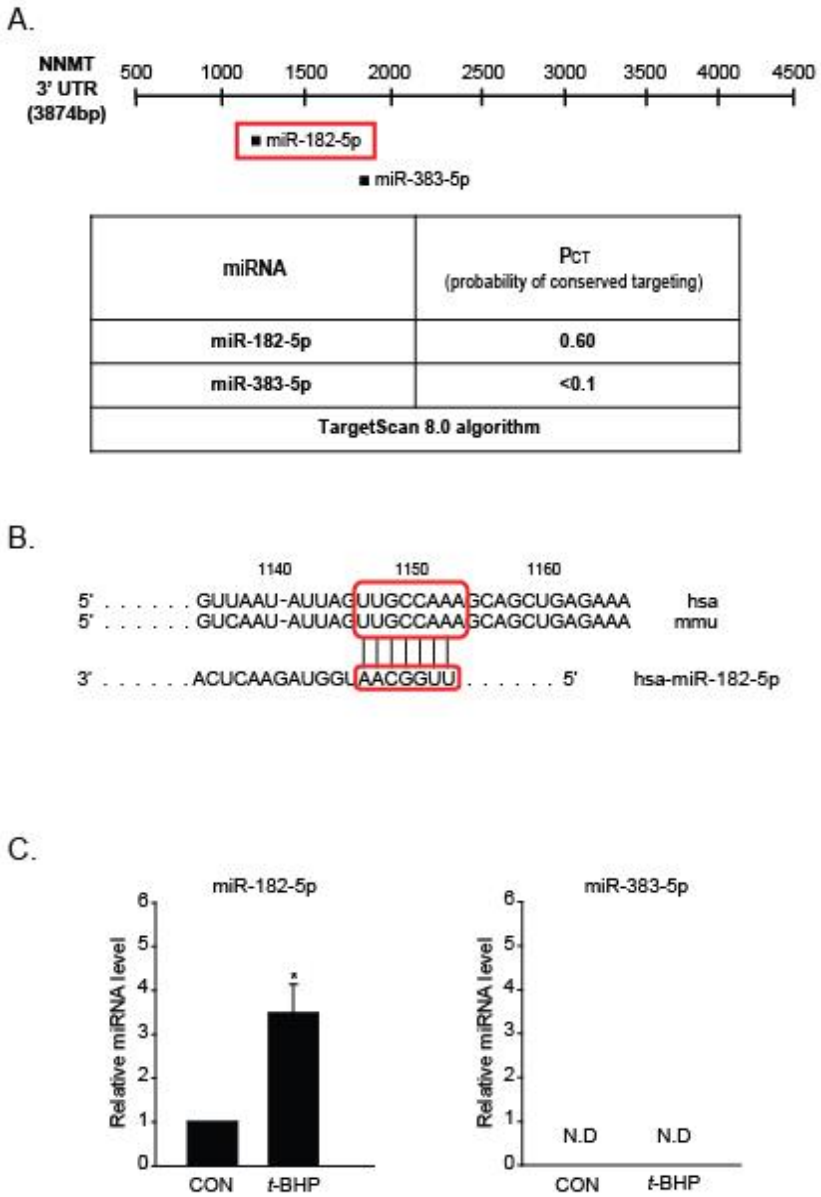

Fig. 5. Increased miR-182-5p expression upon *t*-BHP treatment. (A) The location of the predicted miRNA binding sites within the 3'-UTR of NNMT mRNA. (B) Alignments of miR-182-5p binding to the 3'UTRs of NNMT mRNAs. (C) Real-time RT-PCR analysis for miR-182-5p in HepG2 cells. HepG2 cells were incubated for 6 h with *t*-BHP (100μ M). Data represent means ± S.E. of three replicates (n = 3, significant as compared with vehicle-treated cells, **p* < 0.05; N.D., not detected; CON., Control).

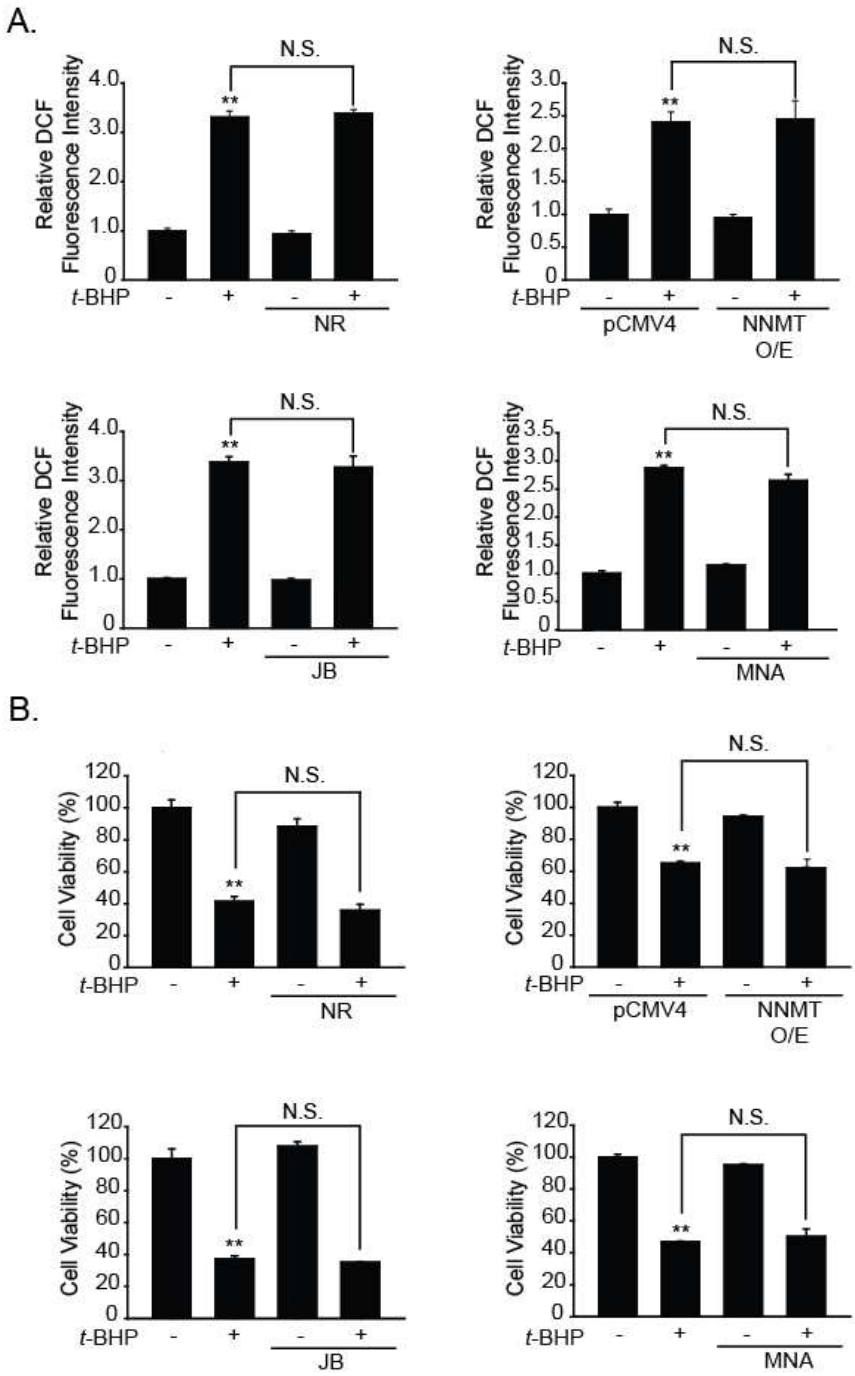

Fig. 6. The role of NNMT against cell viability and ROS levels.

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(A) The role of NNMT on ROS generation. HepG2 cells were pretreated with nicotinamide riboside (NR, 0.5 mM), JBSNF000088 (JB, 100 μM), or 1-methylnicotinamide (MNA, 100 μM) for 30 min and then treated with *t*-BHP (800 μM) for 1 h. The cells were transfected with plasmid encoding the pCMV4 or NNMT construct for 12 h. and then cells were treated with *t*-BHP (800 μM) for 1 h. Subsequently, the fluorescence intensity was measured using DCFH-DA. Data represent means ± SE of three replicates (n = 3, significant as compared with vehicle-treated cells; $^{**}p < 0.01$, significant as compared with *t*-BHP-treated cells; N.S. not significant). (B) The role of NNMT on cell viability against ROS. HepG2 cells were pretreated with NR (0.5 mM), JB (100 μM), or MNA (100 μM), for 30 min and then treated with *t*-BHP (700 μM) for 5 h. The cells were transfected with plasmid encoding the control (pCMV4) or NNMT construct for 12 h. After cells were exposed to *t*-BHP (700 μM) for 5 h, the cell viability was evaluated by MTT assay. Data represent means ± SE of three replicates (n = 3, significant as compared with vehicle-treated cells, $^{**}p < 0.01$; significant as compared with *t*-BHP-treated cells, N.S., not significant).

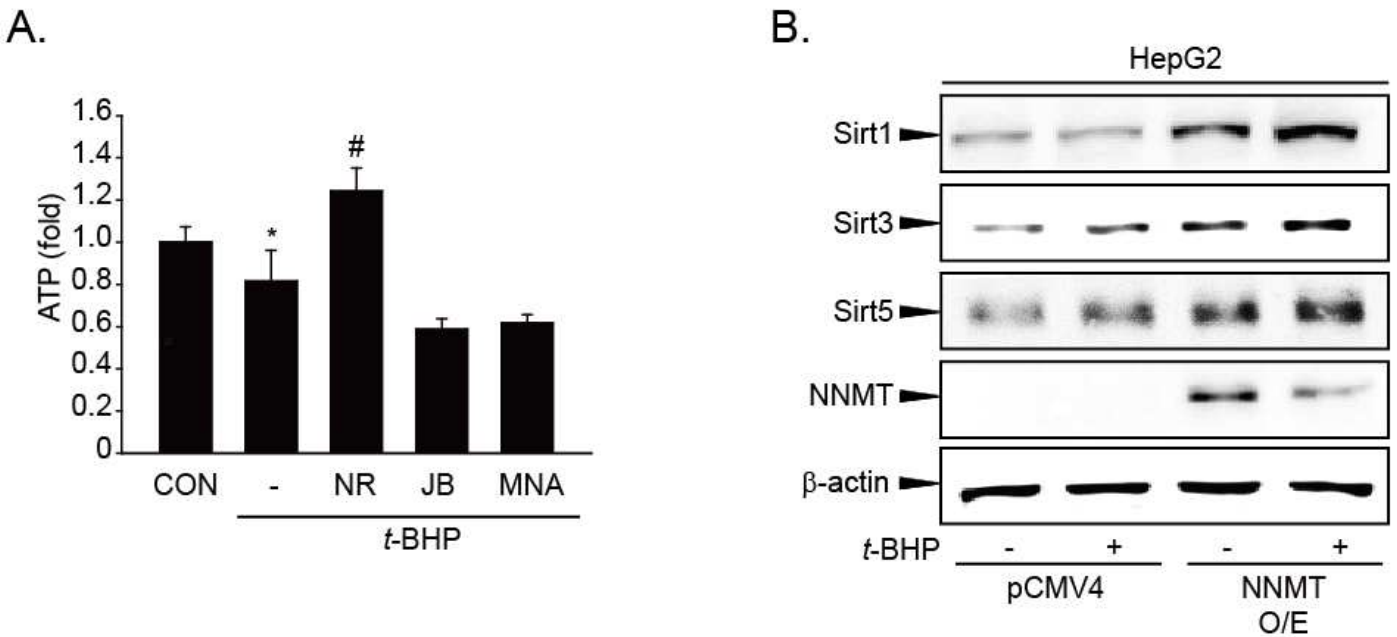

Fig. 7. Association of SIRTs with NNMT (A) ATP Measurement. HepG2 cells were treated with *t*-BHP (100 μM) and continuously exposed to NR (0.5 mM), JB (100 μM), or MNA (100 μM) for 18 h. Data represent means ± SE of three replicates (n = 3, significant as compared with vehicle-treated cells, $^{*}p < 0.05$; significant as compared with *t*-BHP-treated cells, $^{\#}p < 0.05$). (B) The effect of NNMT on ROS-mediated SIRTs induction. The cells were transfected with pCMV4 or NNMT construct for 12 h. After cells were exposed to *t*-BHP (100 μM) for 12 h, SIRT-1, SIRT-3, and SIRT-5 were detected by immunoblotting.

4. DISCUSSION

Redox state and energy metabolism are strongly connected. ROS control the flux of cellular metabolism, which is associated with cellular NAD^+ concentration [32]. NNMT is emerging as a promising metabolic regulator a methyltransferase that catalyzing nicotinamide adopting S-adenosylmethionine, directly linking cell methylation balance and NAD^+ metabolism [13, 33]. The fact that NNMT is the most abundantly expressed in metabolically active tissue such as the liver and adipose tissue supports this notion. As the liver is the organ most potently possessing NNMT [34], NNMT may play a crucial role in liver pathophysiology. The fact that hepatocytes are crucially responsible for maintaining the redox homeostasis in liver upon oxidative stress during acute or chronic liver injury suggest the necessity of study the role of NNMT under oxidative stress in hepatocytes. Although NNMT has been studied to regulate energy metabolism in several liver pathophysiologic conditions, its expression as well as its role under oxidative stress in hepatocytes remains to be explored. In the current study, it was discovered that NNMT was downregulated in different ROS-mediated liver injury mouse models. Consistently, NNMT reduction in *t*-BHP-treated HepG2 and murine primary hepatocytes was observed. These results were fortified by data from recovery of NNMT expression by antioxidant compounds. To the best of our knowledge, this is the first study of the NNMT regulation against oxidative stress in hepatocytes.

The regulatory mechanism of NNMT level has been elucidated; NNMT gene expression is controlled by several signaling pathway and transcription factors such as hepatocyte nuclear factor-1β and STAT3 [35, 36]. Despite the known mechanism of NNMT expression, results of this study illustrated that NNMT expression was not dependent on transcriptional regulation. Furthermore, it was attempted to examine the possibility of involvement in posttranscriptional mechanism for NNMT reduction. Unfortunately, protein degradative mechanism was not found to have a relationship with downregulation of NNMT.

MicroRNAs, a type of small non-coding RNAs around 22 nucleotides long, are

another posttranscriptional mechanism that regulate gene expression by binding specific sequence within target mRNA. They are involved in normal cellular processes including cell differentiation and proliferation as well as progression of liver diseases with multiple targets [37]. Additionally, hepatic oxidative stress is also governed by several miRNAs; regulation of miR-155, miR-223, and miR-214 are responsible the redox homeostasis in liver diseases [38-43]. It was validated miR-182-5p was elevated upon oxidative stress, which might be a novel regulator of NNMT. Despite the known effect of miR-182-5p on oxidative stress, the role of miR-182-5p in NNMT regulation has never been investigated. Further studies revealing NNMT as a direct target of miR-182-5p are necessary to strengthen our hypothesis, and are currently being perused in our laboratory. Despite the finding on increase of miR-182-5p under ROS exposure, a contradictory report has suggested that miR-182-5p level was decreased and exhibited protective effect in atherosclerosis [44]. This discrepancy in data might be derived from cell variation and/or difference in stimuli and growth conditions.

It has been reported that NNMT is associated with cellular function of cell proliferation, tumor migration and invasion [45-47]. This was derived by the PI3K-mediated signaling pathway, which is a prime signaling for cell survival [45, 48]. Moreover, it has also been studied that NNMT was associated with response to ROS [49]. Despite the reports on the roles of NNMT in cell survival and redox homeostasis, our findings showed that NNMT was not involved in either cell viability or ROS generation under oxidative stress. On the other hand, NNMT was participated in cellular metabolism using NAD^+. Therefore, NNMT might act as a signaling modulator related to control the oxidative stress-mediated energy metabolism in hepatocytes. Here, it was reported that NNMT has an ability to regulate energy states, which was reinforced by changes in cellular ATP levels upon NNMT modulation.

Metabolic actions of NNMT are mediated by MNAM via stabilizing SIRT-1, a NAD^+-consuming deacetylase [13]. In this study, SIRT-1, SIRT-3 and SIRT-5, which were classified as more susceptible SIRTs to NAD^+ levels than other isoforms [20], were changed by NNMT. The detailed molecular basis associated with NNMT regulation of SIRT-1, SIRT-3, and SIRT-5 remains elusive. Additional research is necessary to determine the roles of SIRT isoforms in NNMT-mediated energy

metabolism under oxidative stress.

In conclusion, it was revealed that decreased NNMT upon oxidative stress might be elicited by miR-182-5p, which affects hepatocyte energy metabolism via SIRTs. These findings provide novel sights into the NNMT-related signaling pathway and may be of assistance to understand the pathophysiological role of NNMT in the liver.

5. 적요

산화적 스트레스에 의한
니코틴아마이드 N-메틸전이효소의 발현 조절

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주요 대사 기관으로 알려진 간은 생체의 항상성을 유지하여 다양한 생리학적 과정을 조절한다. 이러한 간의 기능 변화는 활성산소종(Reactive Oxygen Species, ROS)에 의한 산화적 스트레스 증가 및 항산화 기능 감소와 관련되어 있다. 소량의 활성산소종은 주로 세포 내 신호 분자로 기능하여 세포의 증식, 분화와 같은 생리적 현상을 조절하는 반면, 세포 내 활성산소종 생성과 이를 제거하는 항산화 기능 사이의 불균형으로 인해 유발되는 활성산소종의 과도한 축적은 산화적 스트레스를 발생시키고 이는 여러 화학물질 및 생체 분자와 상호작용하여 미토콘드리아 기능 장애 유발을 통해 세포 대사에 문제를 야기하고 이를 통해 다양한 병리적 상태를 유발한다. 산화적 스트레스로 인해 매개되는 여러가지 간 질환이 발병한다는 사실은 널리 알려져 있지만, 이러한 산화적 스트레스 매개성 간 질환을 유발하는 원인분자 및 관련 메커니즘에 대한 연구는 부족한 실정이다. 산화적 스트레스 제어 시스템은 NADH/NAD^+ 비율 의존적으로 작동하게 되어, 세포 내의 NAD^+의 양이 산화적 스트레스 제어에 중요하다. 이러한 NAD^+ 양을 조절하는 분자 중, 간세포에서 높게 발현되는 니코틴아미드 N-메틸전이효소 (nicotinamide N-methyltransferase, NNMT)는 다양한 세포 기능 및 에너지 항상성 유지에 기여하고, 여러 간질환 모델에서의 그 역할이 연구된 바 있다. 그러나 간세포에서 생성된 과량의 활성산소종에 의해 유발되는 간질환 모델에서의 NNMT의 발현 변화

여부와 역할, 그 조절 기전에 대해서는 보고된 바 없다. 따라서 본 연구는 산화적 스트레스 상황에서 NNMT의 발현 변화 여부와 조절 기전, 나아가 이의 기능에 관해 규명하고자 하였다. 산화적 스트레스가 동반된 여러 마우스 간질환 모델에서 NNMT의 발현이 감소되어있음을 발견하였다. 또한, 인위적으로 활성산소종을 발생시키는 *ter*-butylperoxide (*t*-BHP)를 간세포주인 HepG2에 처리하였을 때 NNMT 발현은 감소되었으며, 이러한 결과는 또다른 간세포주인 LO2세포와 마우스 1차 간세포에서 동일하게 관찰되었다. 또한, NNMT의 발현 감소는 항산화제 처리에 의해 반전되었다. 이와 더불어 NNMT의 발현 변화는 전사적 조절 및 단백질 분해단계에서 조절되지 않음이 관찰되었으며, 특정 microRNA에 의해 변화될 가능성을 제시하였다. NNMT 발현과 활성 조절 시 과량의 활성산소종에 의한 세포 사멸에는 영향을 주지 못하였지만 세포 내 ATP 양을 변화시킴을 발견하였다. 이러한 결과를 바탕으로 하여 에너지 항상성 및 대사에 중요한 역할을 하며 NAD^+를 활용하는 SIRT과의 관련성을 조사하였을 때, NNMT 과발현에 의해 SIRT의 발현 변화가 유도됨을 관찰하였다. 결과적으로 산화적 스트레스에 반응하여 유도된 NNMT 발현 감소가 SIRT를 매개로 하여 간세포 대사에 영향을 미칠 수 있는 가능성 및 산화적 스트레스 동반 간질환의 새로운 치료 타겟으로써 NNMT를 본 연구 결과를 통해 제시한다.

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