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Research Article

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Efficacy of PARP1 inhibitor nutlin3a on liver injury induced by Ischemia-reperfusion

Daisuke Kawahara¹, Kosho Yamanouchi¹, Naoyuki Okita², Tamotsu Kuroki¹, Yoshikazu Higami² and Susumu Eguchi¹

¹Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki, 852-8501, Japan

²Laboratory of Molecular Pathology and Metabolic Disease, Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda-shi, Chiba 278-0022, Japan

ABSTRACT

Nutlin 3a was known to have effects of stabilization of p53 and degradation of Poly (ADP-ribose) polymerase 1 (PARP1) in cell-specific manner. This implied that the reagent potentially would alleviate hepatic ischemia reperfusion (I/R) injury. First, to evaluate an efficacy of nutlin3a in normal liver, we administered it to male C57Bl/6 mice intraperitoneally at doses of 0 (vehicle only), 5, 20, and 100 mg/kg of body-weight. The animals were killed at 4, 8, and 12 hours after administration and proteins of p53 and PARP1 in the liver were evaluated. As results, no changes were observed in proteins of p53 and PARP1 in both the normal and I/R liver by administration of nutlin3a. Next, we assessed the effects of nutlin3a on the liver after I/R. Twenty-four hours after administration of nutlin3a, the mice suffered from hepatic ischemia, and then were sacrificed at 8 and 24 hours after reperfusion. Nutlin3a did not only show the effect of alleviation of hepatic I/R injury, but also seemed to have cytotoxicity to hepatocyte in dose-dependent manner. In conclusion, a protective effect of nutlin3a on hepatic I/R was not evident in our model. To elucidate definitive effects of nutlin3a in the liver, in vitro study on cell-selectiveness would be needed.

Keywords: PARP1, liver, ischemia-reperfusion

ARTICLE INFO

CONTENTS

1. Introduction	195
2. Experimental.	195
3. Results and Discussion.	196
4. Conclusion.	197
5. References	197

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*Corresponding Author

Kosho Yamanouchi,
Department of Surgery, Nagasaki University
Graduate School of Biomedical Sciences,
1-7-1 Sakamoto, Nagasaki, 852-8501, Japan.
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1. Introduction

Hepatic ischemia-reperfusion (I/R) frequently occurs following liver resection or transplantation, and can result in hepatic injury and dysfunction [1, 2]. Hepatic I/R injury likely involves inflammation and reactive oxygen species (ROS), which directly damage hepatocytes [3]. Poly (ADP-ribose) polymerase 1 (PARP1) is a major enzyme catalyzing poly (ADP-ribose) ation, which is involved in replication, DNA repair, and cell death [4, 5]. DNA breaks lead to PARP1 activation and then poly (ADP-ribose) ation is catalyzed on substrate proteins in DNA damage regions, which is required for efficient recruitment of DNA repair factors to the loci [6]. In contrast, over-activation of PARP1 consumes cellular NAD⁺ and ATP, resulting in breakdown of energy metabolism and cellular necrosis [7, 8]. I/R induces PARP1 over-activation by production of reactive oxygen species [9]. PARP1 should be an attractive target of not only cancer chemotherapy but also protection from I/R injury and PARP1 inhibitors have been evaluated in animal experiments and clinical trials [10].

A tumor-suppressor p53 has versatile physiological roles including forming a cell cycle checkpoint and functioning in DNA repair, apoptosis, and energy metabolism [11]. Because p53 upregulates anti-oxidant and anti-inflammatory genes, stabilization of p53 would lead to protecting cells from I/R-induced damages. The biological activity of p53 is regulated by murine double minute-2 (MDM2), an E3 ligase, which is known to inactivate p53 via ubiquitination and proteasomal degradation. [12, 13]

The small molecular compound nutlin3a is an MDM2 inhibitor and is potent to stabilize p53 [14]. Nutlin3a was associated with tubular cell healing during acute kidney injury induced by I/R in mice [14]. On the other hand, nutlin3a also induces the proteasomal degradation of PARP1 in mouse fibroblast cell lines [15]. Thus, nutlin3a has potentially protective effects on I/R injury, via multiple mechanisms, i.e., p53 stabilization and PARP1 degradation. In this preliminary study, we evaluated efficacies of nutlin 3a on expression or activation of these 2 kinds of proteins and hepatic warm I/R injury in mice.

2. Experimental

Animals

Male C57Bl/6 mice (Japan SLC, Inc., Hamamatsu, Japan) weighing 22 - 25 g were used. The animals were housed in a temperature-controlled environment with a 12-hr light/dark cycle and had free access to standard rat chow and water. All experiments were performed according to the Guidelines for Animal Experimentation at Nagasaki University.

Surgical procedure

Hepatic warm I-R was induced as previously reported [16]. Briefly, animals were anesthetized with isoflurane inhalation for induction and maintenance. A midline International Journal of Chemistry and Pharmaceutical Sciences

laparotomy was made and the inflow vessels, including the hepatic arteries and the portal veins supplying the left and median lobes of the liver, were occluded with small vascular clamps. After 45 minutes of ischemia, the vessels were reperfused and the abdomen was then closed.

Experimental design

First, we evaluated the effects of nutlin3a on the expression of p53 and PARP1 proteins in the normal liver. Nutlin3a was dissolved in 50% DMSO/ 50% phosphate buffered saline and administered intraperitoneally at doses of 0 (vehicle only), 5, 20, and 100 mg/kg of body-weight in 6 mice respectively. Two animals in each group were killed at 4, 8, and 12 hours after administration, liver samples were taken, and immediately immersed in liquid nitrogen. Second, we addressed to evaluate the effect of nutlin3a on hepatic I-R injury. After 24 hours of intraperitoneal administration of nutlin3a at the doses mentioned above in 40 mice, animals suffered hepatic I-R. Blood and liver samples were collected and the animals were killed at 8 and 24 hours after reperfusion. Blood samples were drawn from the inferior vena cava. The left lobes of the liver immediately immersed in liquid nitrogen. The serum and liver samples were stored at -80 °C until analysis. The median lobes of the liver were sliced at a thickness of 5 mm and fixed with buffered formalin for histological examination.

Histological examination

Formalin-fixed specimens were embedded in paraffin. Sections of 5µm thickness were stained with hematoxylin and eosin and examined histologically.

Biochemical analysis

Serum levels of glutamic-pyruvic transaminase (GPT) levels were determined by enzymatic methods at SRL, Inc. (Tokyo, Japan).

Western blot studies

We carried out Western blot to see the effects of nutlin3a on expression of PARP1, p53, and apoptosis related protein, and lipid peroxidation. Ten micrograms of liver lysates were loaded on an SDS/glycine/5%-12% polyacrylamide gradient gel. Following electrophoresis, proteins were transferred onto a cellulose nitrate membrane (PALL Corporation, NY). Membranes were blocked with 2.5% Skim milk (WAKO Pure Chemical Industries, Ltd., Osaka, Japan) /0.25% BSA in TTBS (25mM Tris-HCl pH7.4, 140mM NaCl, 2.5mM KCl, 0.1% Tween 20) followed by incubation with a antimouse p53 antibody (Merck, NJ) or a antirabbit PARP1 antibody (Cell Signaling Technology, Inc., MA) or a antirabbit Caspase-7 antibody (Cell Signaling Technology) or a antimouse 4-HNE antibody (Japan Institute for the Control of Aging, NIKKEN SEIL Co., LTD, Shizuoka, Japan). After washing with TTBS, membranes were incubated with horseradish peroxidase-conjugated F(ab')₂ fragment of goat anti-mouse IgG, Light Chain Specific or horseradish peroxidase -conjugated F(ab')₂ fragment of goat anti-rabbit IgG or horseradish peroxidase- conjugated F(ab')₂ fragment of

goat anti-mouse IgG (Jackson Immunological Research Laboratories, Inc., PA) and developed using Western blotting detection reagents (Immuno Star LD, WAKO).

Statistical Analysis

Results are expressed as a mean±standard deviation (SD). The one-way ANOVA was used for continuous variables among three groups. A p-value of less than 0.05 was considered statistically significant.

3. Results and Discussion

Results:

Effects of nutlin3a in the normal liver

We evaluated the expression of p53 and PARP1 proteins by nutlin3a administration in the normal liver. Nutlin3a did not show the affect of either p53 activation or PARP1 degradation at any time point (Figure 1).

Effects of nutlin3a on the hepatic ischemic-reperfusion injury:

Because all the animals except for one administered at a dose of 100 mg/kg of nutlin3a died before 8 hours of reperfusion, we compared the results from the animals administered 0, 5, and 20 mg/kg of nutlin3a. The serum ALT was elevated significantly at 8 hours after reperfusion and the values were not different dependent on the doses of nutlin3a (Figure 2). The values of ALT at 24 hours tended to be getting higher dependent of doses of nutlin3a ($p=0.07$). 168, 396, 1436 IU/L each for 0, 5, 20mg of nutlin3a). Although pathological examination showed sinusoidal dilatation, structural derangement, and cytoplasmic vacuolization around the central vein at 8 hours after reperfusion in the liver of animals with vehicle administration (Figure 3b), these changes were reduced with 5 mg of nutlin3a administration (Figure 3c). Larger amount of nutlin3a (20 mg/kg), however, seemed to deteriorate the hepatic injury (Figure 3d).

In Western blotting, nutlin3a did not affect the expression of either p53 or PARP1 at 8 and 24 hours after reperfusion (Figure 4). Although cleaved forms of Caspase-7 were identified faintly at 8 hours after reperfusion, whose intensity was identical among the groups, they disappeared at 24 hours. Expression of 4-HNE was obvious at both 8 and 24 hours after reperfusion with nutlin3a administration compared with vehicle only.

Discussion:

Nutlin3a did not induce degradation of PARP1 and activate p53 in either normal or I/R liver in vivo. One possible reason of the results is cell-selective effect of nutlin3a, which was demonstrated to be in in vitro experiments, i.e., positive in 3T3-F442A (mice fibroblast) and A549 (human lung cancer) and negative in U2OS (human osteosarcoma) and HepG2 (human hepatoma) [15]. We should carry out the experiment on effect of nutlin3a on hepatocytes in vitro to elucidate this hypothesis. We administered nutlin3a intraperitoneally at 24 hours before hepatic I/R, following the protocol reported by Mulay, et al. [14], where they demonstrated the effects of ntlin3a on acute kidney injury induced by I/R. Nutlin3a was, however, known to be catalyzed and almost disappeared within 24 hours of oral administration and PARP1 degraded by nutlin3a

recovered quickly [17]. So in our experiment, even if PARP1 was degraded in hepatocyte, it could recover to basal level by our observed time-points.

Cleaved Caspase-7, which was a activated form, was seen faintly after 8 hours of reperfusion, but not after 24 hours. Caspase-7-specific activities and liver cell apoptosis was reported to increase in ischemic hepatic lobes at 3 and 6 hours after hepatic I/R in rats [18, 19]. These findings might indicate that activation of p53 or degradation of PARP1 occurred before 8 hours of reperfusion, leading to only faint apoptotic signal observed after 8 hours in our experiment.

Nutlin3a itself seemed to enhance oxidative stress in the liver after I/R, as shown by expression of 4-HNE. Serum ALT tended to get higher with increasing doses of given nutlin3a after 24 hours of reperfusion. Moreover, although pathological damage seemed to alleviate after 8 hours of reperfusion by administration of nutlin3a at a dose of 5mg/kg, it was deteriorated with high dosage (20mg/kg) of nutlin3a. These results implied that nutlin3a itself had toxicity to the liver in a dose-dependent manner and we have to inject this reagent to normal animals and evaluate liver injury to elucidate the premise.

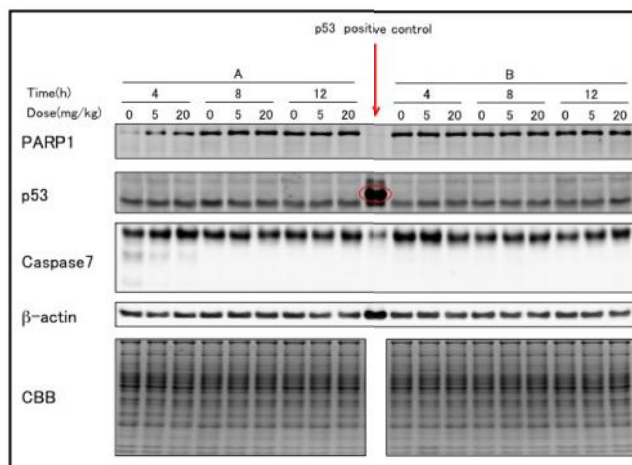


Figure 1

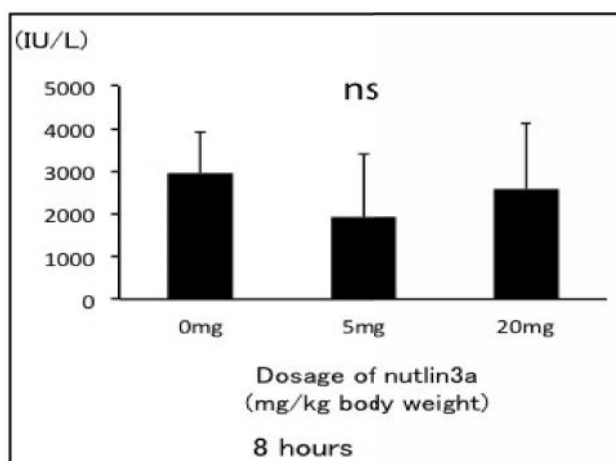


Figure 2

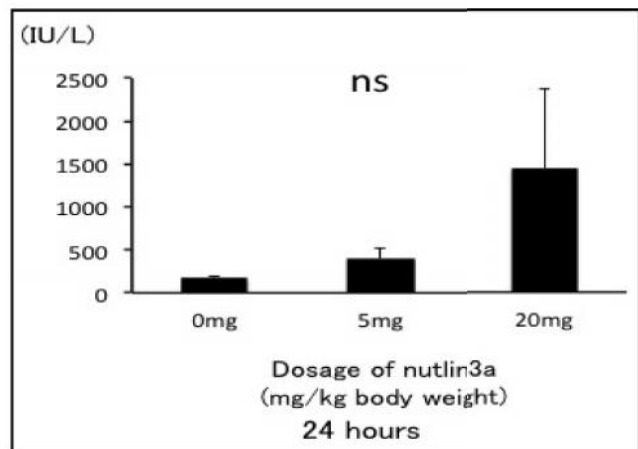


Figure 3

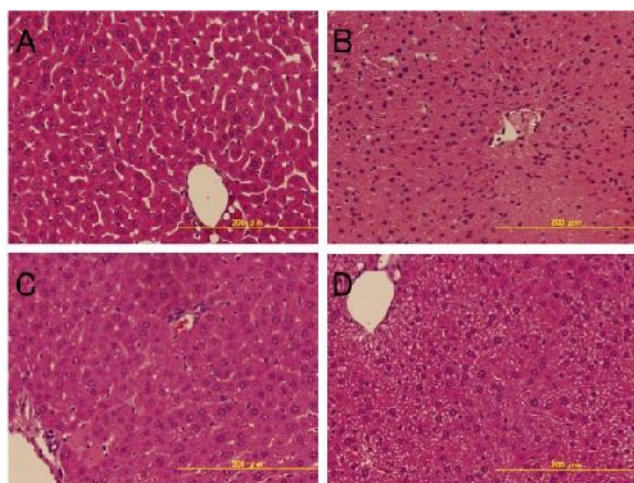


Figure 4

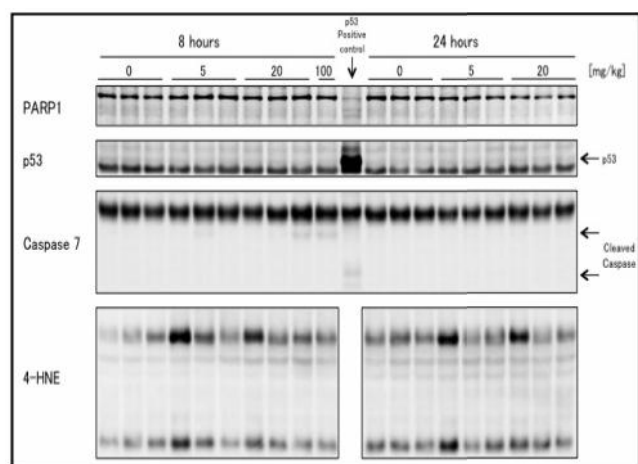


Figure 5

4. Conclusion

In summary, we could not demonstrate the effect of nutlin3a on expressions of either p53 or PARP1 in the liver at time-points at which we investigated. Moreover, nutlin3a itself seemed to have cytotoxicity. To elucidate conclusive effects of nutlin3a in the liver, shorter interval between administration and evaluation, and less dosage of the reagent would be study points in the future.

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