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Reversible induction of PARP1 degradation by p53-inducible cis-imidazoline compounds

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ABSTRACT

PARP1 is an important enzyme involved in various patho-physiological phenomena such as ischemia/reperfusion (I/R) injury, which occurs when blood flow is restored after cerebral infarction, myocardial infarction and transplantation of various organs. I/R-induced PARP1 over-activation is mediated by production of reactive oxygen species and is involved in NF-κB transactivation. For these reasons, PARP1 is an attractive target for strategies to protect against I/R injury. We previously reported that an MDM2 inhibitor Nutlin3a, a cis-imidazoline compound, induces PARP1 degradation in a p53 and proteasome-dependent manner. In this study, we evaluated the effect of Nutlin3a analogs, Nutlin3b and Caylin2, on PARP1 degradation. Like Nutlin3a, Caylin2, but not Nutlin3b, induced PARP1 degradation in both 3T3-L1 and 3T3-F442A. This result occurred almost in parallel with p53 accumulation. Furthermore Caylin2-induced PARP1 degradation was not observed in p53 deficient mouse embryonic fibroblasts or in the presence of the proteasome inhibitor MG132. These results suggest that Caylin2 induces PARP1 degradation by the same mechanism as Nutlin3a. Finally, we showed that Nutlin3a or Caylin2 treatment induces reversible PARP1 down-regulation without an inflammatory response. For protection against I/R injury, our results support the usability of the p53 inducible cis-imidazoline compounds, Nutlin3a and its analogs, as PARP1 inhibitors.

1. Introduction

Poly(ADP-ribosyl)ation, which is the post-translational protein modification, is involved in cell replication, DNA repair, cell death, and inflammation [1,2]. PARP1 is the most abundant PARP family member in cells, and is dramatically activated by DNA breaks. Therefore, massive DNA damage induces over-activation of PARP1, and then decreases ATP levels via over-consumption of cellular NAD^+, which is required for the ATP production in glycolysis and TCA cycle pathways. It has been also reported that PARP1 over-activation is involved in ischemia/reperfusion (I/R) injury, which occurs during the restoration of blood flow after cerebral infarction, myocardial infarction and organ transplantation [3–5]. For these reasons, PARP1 is an attractive target for protection against I/R injury [6].

We previously reported that Nutlin3a, an MDM2 ubiquitin ligase antagonist, induces p53 and proteasome-dependent PARP1 protein degradation [7]. It has been thought that Nutlin3a is a candidate for anti-tumor drugs, because MDM2 inhibition by Nutlin3a induces p53 stabilization, followed by p53-dependent apoptosis in tumor cells [8]. The discovery of Nutlin3a-induced PARP1 degradation prompted the use of Nutlin3a as a PARP1 inhibitor. Furthermore, considering that p53 has the potential to up-regulate anti-oxidant and anti-inflammatory genes [9–11], Nutlin3a may be a potent anti-I/R drug that has multiple points of action. However, the Nutlin3a pharmacophore that induce PARP1 protein degradation has not been identified. In the present study, to clarify whether Nutlin3a analogs were also able to induce PARP1 protein degradation in a manner similar to Nutlin3a, we examined the effect on PARP1 degradation by the commercially available Nutlin3a enantiomer, Nutlin3b [12,13], and by the Nutlin3a derivative, Caylin2 [14]. Furthermore, by using compounds possessing PARP1 degradation activity, we evaluated the reversibility of PARP1 degradation and the effect on anti-inflammatory IL6 gene expression.

2. Materials and methods

2.1. Cell culture and drugs

Mouse fibroblast 3T3-L1 and 3T3-F442A cell lines were purchased from the RIKEN Bioresource Center (Japan) and the European Collection of Animal Cell Cultures (UK), respectively. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, low glucose) (WAKO, Japan) with 10% fetal calf serum

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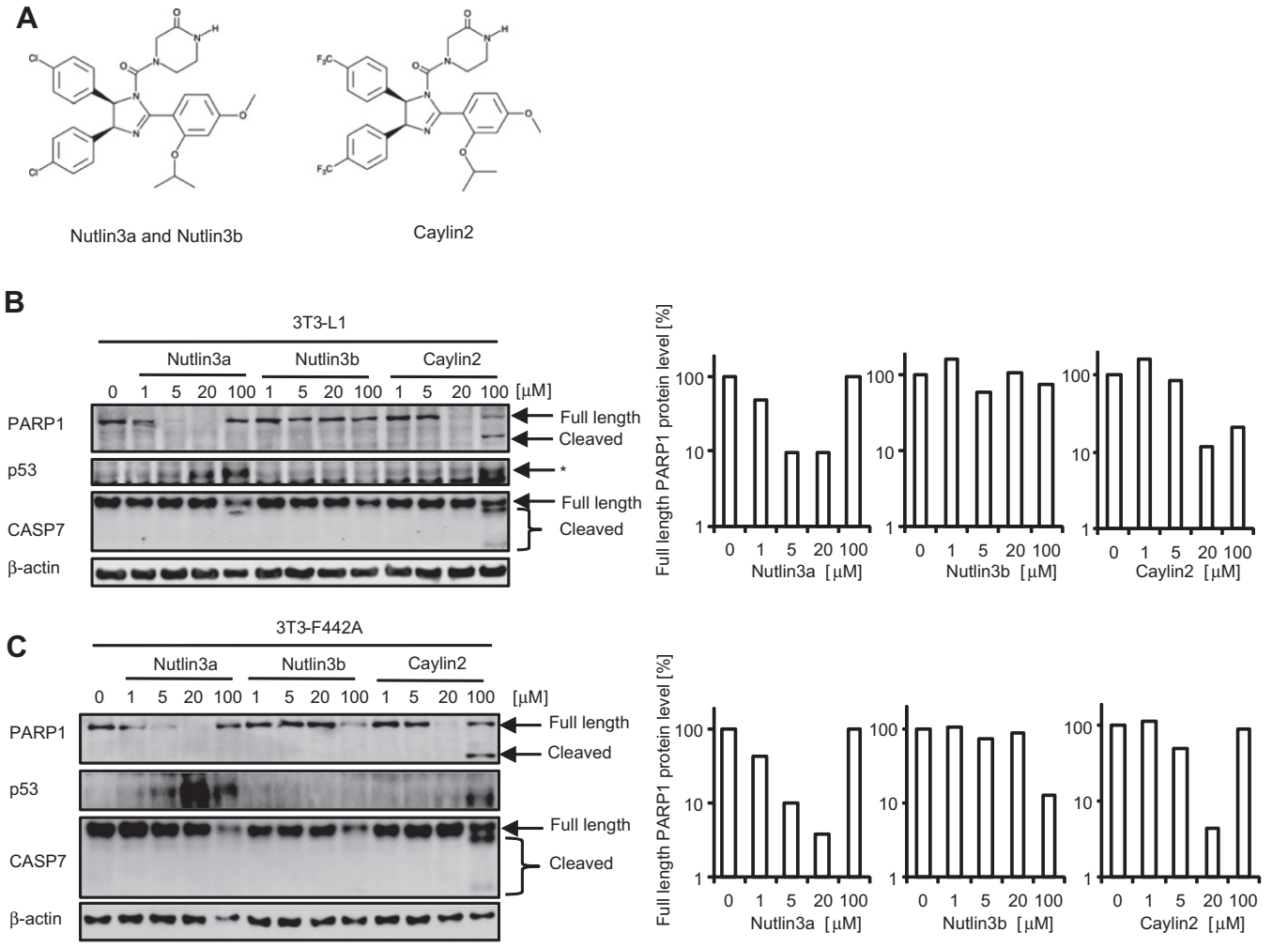

Fig. 1. Caylin2 but not Nutlin3b decreases in PARP1 protein levels in mouse fibroblasts. (A) Structures of Nutlin3a, Nutlin3b, and Caylin2. Mouse fibroblast 3T3-L1 (B) or 3T3-F442A (C) were treated with the indicated concentrations of Nutlin3a, Nutlin3b or Caylin2 for 8 h. The cell lysates were analyzed by Western blotting using the indicated antibodies (left pannel). Quantitative data are shown (right panel). In the p53 panel, the arrow and asterisk show the p53 and nonspecific bands, respectively. All experiments were performed at least three times, and representative data is shown.

and 1% penicillin/streptomycin (Sigma). p53+/+ or −/− MEFs were prepared as described previously [7]. The established MEFs were maintained in DMEM (high glucose) with 10% FCS, 0.1 mM 2-mercaptoethanol, and 1% penicillin/streptomycin. The proteasome inhibitor MG132 was purchased from WAKO (Japan). Nutlin3a, Nutlin3b, and Caylin2 were supplied by Cayman (USA).

2.2. Western blotting

Cell preparation and Western blotting were performed as described previously [7]. As primary antibodies, anti-PARP1 (clone C-2-10, WAKO, Japan), anti-p53 (clone Ab-1, Calbiochem, USA), anti-β actin (clone AC-15, SIGMA, USA), or anti-CASP7 (clone 1F3, MBL, Japan) antibodies were used. For secondary antibodies, horseradish peroxidase-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG or anti-rabbit IgG (Jackson Immunoresearch, USA) were used. The specific proteins were visualized with ImmunoStar LD reagent (WAKO, Japan) and LAS3000 (Fuji Film, Japan), and the data were analyzed using MultiGauge software (Fuji Film, Japan).

2.3. RNA purification and RT-PCR

RNA purification and RT-PCR were performed using RNAiso PLUS, FastPure RNA kit, PrimeScript Reverse Transcriptase and random hexamers (all from TaKaRa, Japan) as described previously [7]. The PCR was performed using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, USA) and primers for *TNFα* (forward, 5′-CCCTCACACTCAGATCATCTTCTC-3′; reverse, 5′-GCCTTGTCCCTTGAAGAGAACC-3′) *IL6* (forward, 5′-GCCTTCCCTACTTCACAAGTCC-3′; reverse, 5′-CAGAATTGCCATTGCACAAC-3′), or *TBP* (forward, 5′-CAGTACAGCAATCAACATCTCAGC-3′; reverse, 5′-CAAGTTTACAGCCAAGATTCACG-3′) as follows: initiation step, at 94 °C for 1 min; amplification step, at 94 °C for 1 min, at 60 °C for 15 s, at 68 °C for 15 s; termination step, 68 °C 15 s. PCR products were subjected to 1.8% agarose gel electrophoresis, stained with ethidium bromide, and visualized with LAS3000. The data was analyzed using MultiGauge software (Fuji Film, Japan).

3. Results

3.1. Caylin2. but not Nutlin3b induces a decrease in PARP1 protein levels in mouse fibroblast cell lines

Although we previously reported that Nutlin3a induces PARP1 protein degradation, we did not address whether Nutlin3a analogs also have the potential to induce PARP1 degradation [7]. Here, we investigated the inducibility of PARP1 degradation by two such analogs, Nutlin3b and Caylin2 in mouse fibroblast cell lines

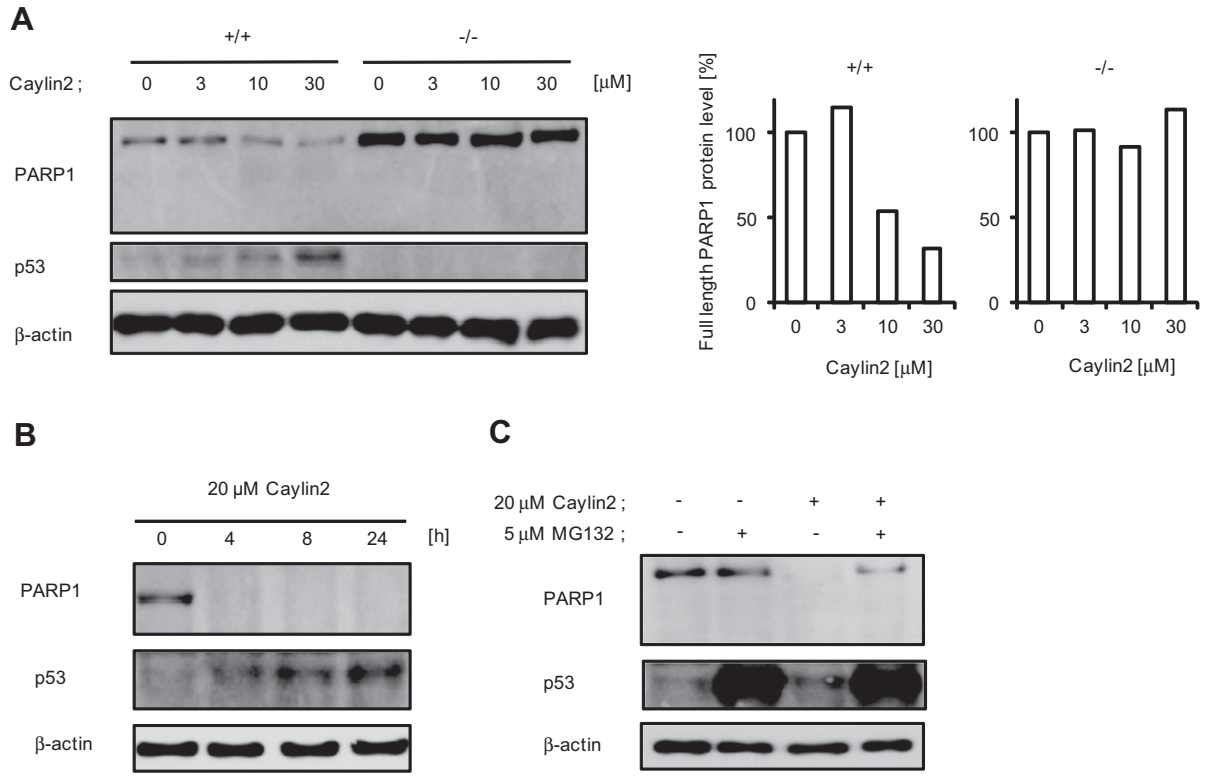

Fig. 2. Caylin2-induced PARP1 degradation is p53 status and proteasome-dependent. (A) p53+/+ and p53−/− MEFs were treated with the indicated concentrations of Caylin2 for 8 h. Cell lysates were analyzed by Western blotting using the indicated antibodies (left panel). Quantitative data are shown (right panel). Each 2 to 3 clones of p53+/+ and p53−/− MEFs were analyzed and representative data are shown. (B) 3T3-L1 cells were treated with 20 μM Caylin2 for the indicated times. The proteins were subjected to Western blotting. (C) 3T3-L1 cells were treated with 20 μM Caylin2 in the presence or absence of 5 μM MG132 proteasome inhibitor (MG) for 8 h, and cell lysates were then subjected to Western blotting using the indicated antibodies.

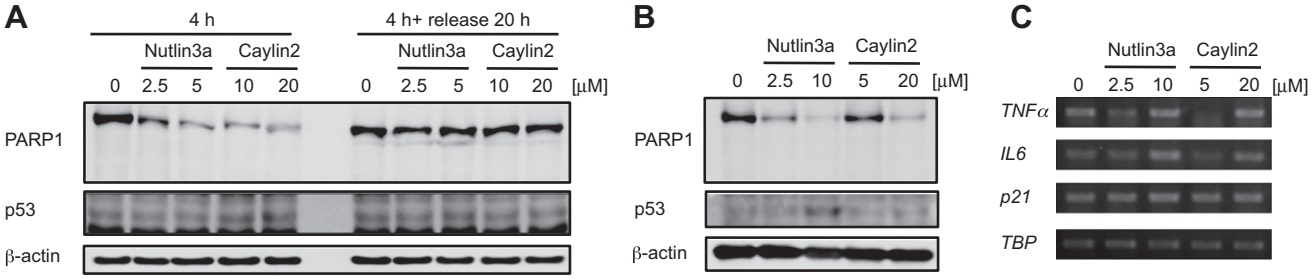

Fig. 3. Nutlin3a or Caylin2 treatment induces reversible PARP1 down-regulation without an inflammatory response. (A) 3T3-L1 cells were treated with Nutlin3a (2.5 or 5 μM) or Caylin2 (10 or 20 μM) for 4 h. After these treatments, cells were also cultured in normal growth medium without treatment for a further 20 h. The cell lysates were analyzed by Western blotting using the indicated antibodies. (B, C) 3T3-L1 cells were treated with 2.5 μM Nutlin3a or 5 μM Caylin2 for 4 h. The protein and RNA expression were analyzed by Western blotting (B) or RT-PCR (C).

(Fig. 1). Nutlin3b is an inactive enantiomer of Nutlin3a, whereas Caylin2 is a Nutlin3a derivative in which trifluoromethyl groups are substituted for chlorine on the 2 phenyl rings (Fig. 1A) [8,12]. As shown in Fig. 1B and C, for both cell lines, 1–20 μM Nutlin3a treatment markedly decreased PARP1 protein levels in a dose dependent manner, whereas 100 μM Nutlin3a treatment had no effect, as per our previous report. p53 accumulation was dose dependent, increasing with the concentration range. Additionally, after 100 μM Nutlin3a-treated, both cell lines were detached from the culture dish and appeared to die without significant CASP7 activation. This observation was consistent with our previous data [7]. Nutlin3b treatment did not markedly alter p53 protein levels in either cell line. In 3T3-L1 cells, Nutlin3b treatment did not affect PARP1 protein level (Fig. 1B). On the other hand, in 3T3-F442A cells, only 100 μM Nutlin3b treatment decreased the PARP1 protein level (Fig. 1C). Similar to the Nutlin3a treatment, 100 μM Nutlin3b-treated cells seemed to die without significant CASP7 activation. Interestingly, Caylin2 treatment showed a signature profile of PARP1 protein in both cell lines. 20 μM Caylin2 treatment induced a significant decrease in PARP1 protein and 100 μM Caylin2 treatment induced PARP1 cleavage, which is considered as an apoptotic hallmark as well as activation of apoptotic caspases such as CASP2, 3, 6, 7, 9, and 10 [15–17]. Indeed, a trypan blue exclusion assay showed that Caylin2-treated cells were viable at 20 μM and dead at 100 μM (Supplemental Fig. 1).

3.2. PARP1 down-regulation by Caylin2 treatment is p53 and proteasome-dependent

Since we previously reported that Nutlin3a-induced PARP1 degradation occurs in a p53 and proteasome dependent manner, we sought to confirm using the same methods as our previous report whether Caylin2-induced PARP1 degradation is inhibited by p53 depletion or proteasome inhibition. As shown in Fig. 2A, p53 WT MEFs, but not p53 KO MEFs, displayed decreasing PARP1 protein levels in a Caylin2 dose dependent manner. Furthermore, as shown in Fig. 2B, Caylin2-induced PARP1 degradation was inhibited by co-treatment with the proteasome inhibitor MG132. These results indicate that Caylin2, like Nutlin3a, induces PARP1 degradation in a p53 and proteasome-dependent manner.

3.3. Nutlin3a or Caylin2 treatment induces reversible PARP1 down-regulation without an inflammatory response

Since PARP1 plays roles in the maintenance of cellular homeostasis through various signal transduction pathways [1,2], reversible down-regulation of the PARP1 protein level is important to protect tissues from I/R injury. Therefore we investigated the reversibility of Nutlin3a- or Caylin2-induced PARP1 degradation. 3T3-L1 cells were treated with Nutlin3a (2.5 or 5 μM) or Caylin2 (10 or 20 μM) for 4 h, and then cultured for 20 h. After 4 h of Nutlin3a or Caylin2 treatment (transient treatment), PARP1 protein levels decreased, although p53 protein levels were not markedly altered (Fig. 3A). After release from those treatments (+20 h), PARP1 protein levels were recovered (Fig. 3A). These results show that Nutlin3a or Caylin2-induced PARP1 degradation is reversible. As it has been reported that Nutlin3a-induced p53 activation leads to up-regulation of inflammatory cytokines [18], we also investigated the influence on inflammation by the transient Nutlin3a or Caylin2 treatment (Fig. 3B). 3T3-L1 cells were treated with the indicated doses of Nutlin3a or Caylin2 for 4 h, and then analyzed the TNFα and IL6 inflammatory genes by RT-PCR. Under these conditions, Nutlin3a or Caylin2 treatment induced PARP1 degradation in a dose dependent manner. Interestingly, we observed different inflammatory responses under these condition (Fig. 3C). The higher dose treatments of Nutlin3a or Caylin2 significantly induced IL6 mRNA expression. However, these doses had little effect or only slightly induced TNFα mRNA expression. On the other hand, the lower dose treatments of Nutlin3a or Caylin2, which were capable of inducing PARP1 degradation, inhibited TNFα mRNA expression and did not affect or only slightly inhibited IL6 mRNA expression. Taken together with Fig. 3B and C, these results indicate that the lower dose treatment of Nutlin3a or Caylin2 has the potential to induce PARP1 degradation without inducing an inflammatory response.

4. Discussion

In this study, we examined the effect of treatment by Nutlin3a analogs on PARP1 protein levels. We demonstrated that Caylin2 induces PARP1 degradation in a similar manner to Nutlin3a. Taken together with our previous study, these results indicate that p53-inducible cis-imidazoline compounds have the potential to induce PARP1 degradation. In the context of using Nutlin3a, Caylin2 and related derivatives as "PARP1 degradation inducers" for I/R injury therapy, a major advantage of this study is that it has demonstrated that Nutlin3a- or Caylin2-induced PARP1 degradation is reversible (Fig. 3A). I/R injury is the tissue damage that occurs during the ischemic and reperfusion period, and as such commonly occurs as a result of ischemic infarction and its treatment or during organ transplantation. In the injured tissues, PARP1 is over-activated by reactive oxygen-mediated DNA damage, resulting in decreases in ATP levels via over-consumption of cellular NAD^+ [1,2]. Therefore, PARP1 inhibition has protective effects on I/R injury. Furthermore, PARP1 itself plays roles in the maintenance of cellular homeostasis through its involvement in the regulation of various signal transduction pathways [1,2]. Taken together, transient PARP1 degradation is valuable in regard to both protection from I/R injury and to allowing for a quick recovery from the harmful effects of PARP1 inhibition. There have been some previous reports of IL6 regulation by p53 or PARP1. p53 has been reported to repress not only IL6 but also the promoter activity of NF-κB, a transcriptional factor of various inflammatory genes including IL6 [10,11]. Additionally, PARP1 activation inhibits the DNA-binding activity of NF-κB [19]. In this study, we showed that Nutlin3a or Caylin2 causes differential effects on inflammatory responses depending on the magnitude of the doses used (Fig. 3C). Our results suggest that the choice of appropriate doses and timing of treatments would be critical to obtain only the beneficial effects on PARP1 degradation when using Nutlin3a or Caylin2 for protection from I/R injury.

Recently, it was reported that inflammasome activation of cardiac fibroblasts is essential for myocardial I/R [20]. So far, our work has revealed that the PARP1 degradation pathway functions efficiently in fibroblast cell lines [7]. These findings support the possibility of practical use of this PARP1 degradation pathway. Further research will require several lines of investigation. Firstly, it will be interesting to identifiy the stereocenter that specifically induces PARP1 degradation. The chiral separation of Nutlin3 (Nutlin3a and Nutlin3b) has been achieved, although the absolute stereocenter has not been known [12,13]. In Caylin2 the chiral separation has not been achieved. We predict that Caylin2a (Caylin2 of Nutlin3a type), but not Caylin2b (Caylin2 of Nutlin3b type), may be the potential to induce PARP1 degradation and are performing further analyses now. Secondly, it will be important to explore PARP1 degradation inducers that different structures than the cis-imidazoline compounds such as Nutlin3a or Caylin2. Thus, elucidation of the mechanism of reversible PARP1 degradation induction is important for the optimization of compounds which induce this phenomenon, resulting in the establishment of selective chemotherapeutic strategies against I/R injury.

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