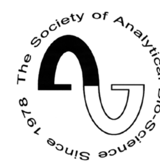
〈Brief Note〉



Effects of doxorubicin-induced cell senescence on the expression of renalase in Caco-2 cell line.

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Summary Aging is a biological process that occurs in all forms of life and causes deterioration of tissue functions. Its effects have been investigated in a range of tissues as well as in the intestinal tract. One of the causes of age-related functional deterioration is DNA damage due to oxidative stress. Therefore, it is crucial to comprehend the defense mechanisms against oxidative stress and related molecules in cellular senescence. Renalase (RNLS) is a monoamine oxidase that has attracted much attention recently owing to its antioxidant and anti-inflammatory effects. We hypothesized that RNLS might function as an antioxidant in intestinal cellular senescence and examined its expression changes using the Caco-2 cell line. Caco-2 cells were treated with 25, 50, and 100 μmol/L doxorubicin (Dox) for 24 hours, and cell viability, thiobarbituric acid reactive substances (TBARS) level, and protein expressions were determined. Cell viability was significantly decreased, while TBARS was increased at all concentrations of Dox; senescence markers were increased at 50 and 100 μmol/L of Dox; RNLS and interleukin (IL) -6 were significantly increased at 50 μmol/L doxorubicin, whereas gene expression of glutathione peroxidase (Gpx) 2—an antioxidant enzyme—was decreased. This suggests that RNLS expression was induced as a protective response in cellular senescence when the antioxidant function is impaired by increased oxidative stress and induced inflammation.

Key words: Renalase, Senescence, Aging, Caco-2, Oxidative stress

1. Introduction

As the world's aging population keeps expanding, there is a pressing need to advance research on aging. Aging is a biological process that naturally occurs in all living organisms. Age-related research on the brain includes Alzheimer's disease and dementia[1,2], while

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research on muscle aging includes dynapenia and sarcopenia[3]. Similarly, the gastrointestinal tract, which interacts with various organs, is subjected to extensive age-related studies. For example, it has been reported that aging may cause a functional decline in intestinal stem cells that are involved in the repair of intestinal tissue[4]. The DNA damage caused by oxidative stress is widely recognized to play a role in cellular senescence[5,6]. Oxidative stress-induced DNA damage activates an innate immune response as senescence-associated secretory phenotype (SASP)[7], which in turn spreads cellular senescence to the surrounding cells by paracrine action[8]. Therefore, understanding the intracellular mechanisms and related molecules that counteract oxidative stress caused by aging may thus be critical for maintaining a healthy intestinal tract.

Renalase (RNLS) is an enzyme discovered by Xu et al.[9] in 2005. It degrades circulating catecholamines and has attracted much attention for its role in blood pressure regulation[10]. However, many cytokine-like functions such as cytoprotection[11], antioxidant function[12], and anti-inflammatory effect[13] have been reported in recent years. Our group has also reported that RNLS expression increased in the intestinal tract of mice subjected to oxidative stress, and it showed antioxidant effects in cell experiments[14]. Therefore, we hypothesized that RNLS might change expression level as a defense mechanism against oxidative stress in the intestinal tract during cellular senescence. To validate this hypothesis, we developed a senescence model using Caco-2 cells and examined the changes in RNLS expression during senescence.

2. Materials and Methods

Cell culture

Caco-2 cells (18H036, European Collection of Authenticated Cell Cultures (ECACC), UK) were cultured in Dulbecco's modified Eagle medium (Thermo Fisher Scientific, MA, USA), supplemented with 10% fetal bovine serum and 1% MEM non-essential amino acids solution (Nacalai Tesque, Kyoto, Japan) and incubated at 37°C, 5% CO_2. Cells were seeded in 12-well plates at a density of 1.0×10^5 cells. After incubation, 25, 50, and 100 µmol/L doxorubicin (Dox) (Cayman Chemical, MI, USA) were added to a serum-free medium and incubated for 24 hours. Dox has been reported to induce cellular senescence by causing oxidative stress[15-16]. Dimethyl sulfoxide (DMSO) was added to the control group and the samples were used for subsequent analysis.

Cell viability assay

Cell viability was measured by resazurin assay as described in the previous studies[17]. Resazurin powder (TOKYO CHEMICAL INDUSTRY, Tokyo, Japan) was dissolved in sterile distilled water at a final concentration of 0.1% and sterilized by filtration. 24 h after the Dox stimulation, 1 mL of media containing 0.004% resazurin was added to each well, and cells were incubated at 37°C for 3 h. Then, 100 µL of medium from each well was transferred to a 96-well black plate. The fluorescence intensity of the medium was measured at Ex.569 nm/Em.590 nm.

Thiobarbituric acid reactive substances (TBARS) assay

The TBRAS assay was used to measure the oxidative stress as described previously[18]. Briefly, the cell lysates were prepared using NP-40 cell lysate buffer (1% NP-40, 50 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L EDTA, including proteinase inhibitor cocktail [Wako, Osaka, Japand]). Subsequently, protein concentrations were adjusted to 1 g/L. Then, 100 µL of adjusted sample was added to 900 µL master mix (0.2% [w/v] sodium dodecyl sulfate, 0.25% [w/v] thiobarbituric acid, butylated hydroxytoluene solution, and acetic acid buffer [0.5% {v/v} acetic acid, 0.07% {w/v} sodium acetate, pH 3.5]), stored at 4°C for 1 h followed by heating the mixture at 100°C for 1 h. The mixture were extracted with 1.0 mL of 1-butanol–pyridine (15:1, v/v) solution. The fluorescence intensity of the extract was measured at Ex.532 nm/Em.585 nm.

Quantitative polymerase chain reaction (qPCR)

Total RNA extraction methods and qPCR methods were performed as per our previous study[14].

Table 1 Lists of antibodies

Antibody name	Company and code	Dilution
Renalase	Proteintech Group, 15003-1-AP	1:2000
p53	Santacruz Biotechnology, sc-6243	1:1000
p27	Santacruz Biotechnology, sc-528	1:1000
p21	Santacruz Biotechnology, sc-817	1:500
p16	Santacruz Biotechnology, sc-56330	1:500
p-p65(Ser536)	ABclonal Technology, AP0475	1:1000
p65	ABclonal Technology, A6667	1:1000
IL-6	Santacruz Biotechnology, sc-32296	1:500
Cleaved-Caspase3	CST, #9661S	1:1000
GAPDH	Santacruz Biotechnology, sc-365062	1:1000

The acidic ribosomal phosphoprotein (Rplp0) gene was used as the housekeeping gene. The cycle threshold (Ct) value of the glutathione peroxidase 2 (Gpx2) was normalized by the Ct value of the housekeeping gene (ΔΔCt method). The relative expression of the target gene was calculated by dividing the test samples with that of the control (DMSO). The primer sequences of the genes are as follows: Rplp0 (Forward 5'-TCTACAACCCTGAAGTGCTTGAT-3', Reverse 5'-CAATCTGCAGACAGACACTGG-3') and Gpx2 (Forward 5'-AGTCTCAAGTATGTCCGT-3', Reverse 5'-CCTTTATTGGTCTCTTCT-3').

Immunoblotting

Total proteins were extracted, and immunoblotting was performed according to the method described in our previous study[14]. Primary antibodies listed in Table 1 were incubated with the blotted membranes. The horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology, #7074, US) and anti-mouse IgG (Cell Signaling Technology, #7076, US) were used as secondary antibodies, and signals were detected using the chemiluminescence reagent (EzWestLumi One or EzWestLumi plus, ATTO, Tokyo, Japan). Blots were analyzed using a chemiluminescence imaging system (FUSION FX7. EDGE, Vilber Lourmat, Marne-la-Vallee, France).

Statistical analysis

Data have been represented as mean ± standard deviation. Comparison between DMSO and Dox treatments was subjected to one-way analysis of variance followed by Dunnett's post hoc test to evaluate significance. Statistical analyses were performed using GraphPad Prism 8.4.3 for Mac (GraphPad Software, San Diego, CA, USA). A value of $p < 0.05$ was considered significant.

3. Results

Dox-induced oxidative stress and upregulation of senescence markers

The biological aging process increases oxidative stress. In addition, the expression of senescence-specific markers that affect the cell cycle were also increased in cells. To examine whether Dox treatment induces oxidative stress as well as cellular senescence, TBARS as a marker of oxidative stress and p16, p21, p27, and p53 as markers of senescence were measured.

Each concentration of Dox reduced the cell survival rate and increased the TBARS levels compared to DMSO (Fig. 1A, B). 50 and 100 μmol/L of Dox upregulated the expression of p16, p21, and p53. Also, 50 μmol/L of Dox upregulated the expression of p27 (Fig. 1C).

Dox-induced upregulation of RNLS expression and alteration of Gpx expression.

Antioxidant function of the intestinal tract was declined with cellular senescence, and RNLS may be altered in expression as a defense mechanism against this. Therefore, protein expression level of RNLS and gene expression level of Gpx2- a intes-

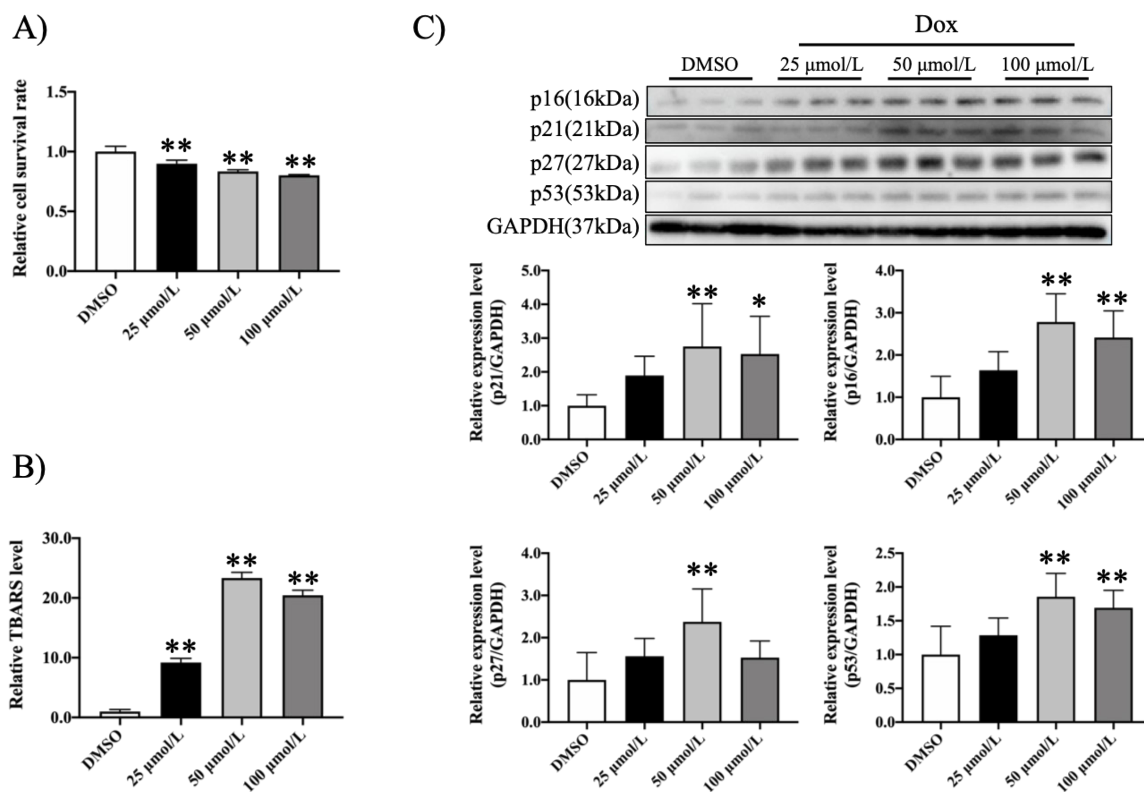

Fig. 1. Dox-induced the oxidative stress and upregulated the senescence markers in Caco-2 cell line. A) Relative cell survival rate. B) Relative TBARS level. C) Expression levels of aging markers. Cell survival rate was analyzed by resazurin assay. TBARS level was analyzed by TBARS assay. Protein expression level of senescence markers were analyzed by immunoblotting. Caco-2 cells were stimulated with various concentrations of Dox for 24 h. The asterisks indicate significant decrease (Fig1-A) and increase (Fig1-B, C) as compared to DMSO (*p < 0.05, **p < 0.01). Data are shown as mean ± SD (n = 6 in each group).

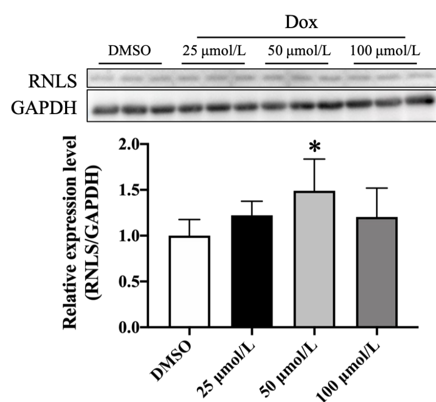

Fig. 2. Dox upregulated the protein expression level of RNLS in Caco-2 cell line. Protein expression level of RNLS was analyzed by immunoblotting. Caco-2 cells were stimulated with various concentrations of Dox for 24 h. The asterisks indicate significant increase as compared to DMSO (*p < 0.05). Data are shown as mean ± SD (n = 6 in each group).

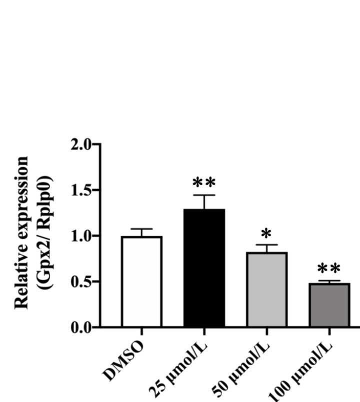

Fig. 3. Dox changed the gene expression level of Gpx2 in Caco-2 cell line. Gene expression level of Gpx2 was analyzed by qPCR. Caco-2 cells were stimulated with various concentrations of Dox for 24 h. The asterisks indicate significant increase (25 μmol/L) and decrease (50 and 100 μmol/L) as compared to DMSO (*p < 0.05 and **p < 0.01). Data are shown as mean ± SD (n = 6 in each group).

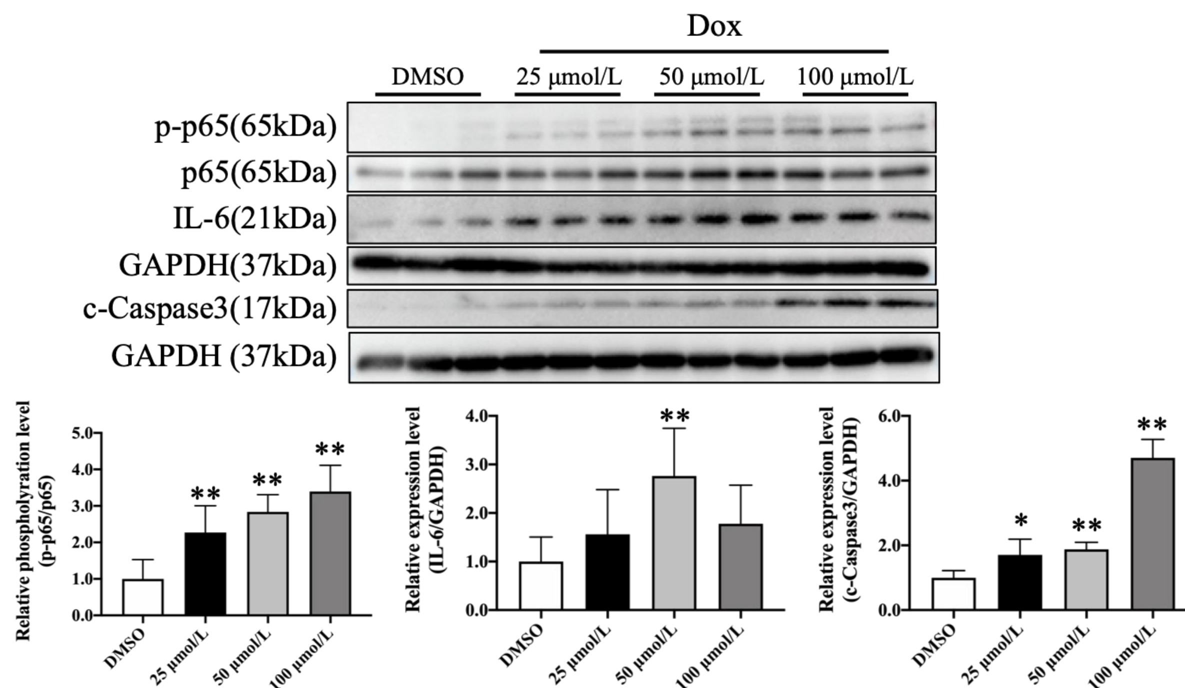

Fig. 4. Dox upregulated the expression level of inflammation and apoptosis markers in Caco-2 cell line. Protein expression level of inflammation and apoptosis markers were analyzed by immunoblotting. Caco-2 cells were stimulated with various concentrations of Dox for 24 h. The asterisks indicate significant increase as compared to DMSO (*$p < 0.05$, **$p < 0.01$). Data are shown as mean ± SD (n = 6 in each group).

tinal type Gpx were measured.

The expression level of RNLS was increased by 50 μmol/L of Dox (Fig. 2). Gpx2 was increased at 25 μmol/L of Dox but decreased at 50 and 100 μmol/L compared to DMSO (Fig. 3).

Dox upregulated the expression of inflammation and apoptosis markers

Aging causes inflammation, which in turn causes further aging. Finally, it leads to cell death, so inflammation and cell death markers were measured.

Cleaved-Caspase3 and Phosphorylation of p65 were increased by each concentration of Dox. Similarly, IL-6 was increased by 50 μmol/L of Dox. (Fig. 4).

4. Discussion

We investigated the effects of Dox-induced cell senescence on the expression level of RNLS in the Caco-2 cell line. We adopted the Dox-induced cell senescence model because prior research reported that Dox caused oxidative stress and DNA damage, resulting in cells becoming senescent[15,16].

First, we examined the effects of different concentrations of Dox on cell viability and oxidative stress marker TBARS. At various concentrations of Dox, we observed a decrease in cell viability and an increase in TBARS levels (Fig. 1A, B). The findings in our study support previous research because Dox induces aging by causing oxidative stress[15,16]. Furthermore, at 50 and 100 μmol/L of Dox, the cellular senescence markers, p16, p21, p27, and p53, were elevated (Fig. 1C), and since these markers are cell cycle inhibitor[19], the protein expression is increased during cellular senescence. Therefore, these results suggest that Dox above 50 μmol/L induce cellular senescence. Next, we examined the expression of RNLS and found that its expression was elevated upon the addition of 50 μmol/L Dox (Fig. 2), representing for the first time that RNLS expression gets upregulated during cellular senescence. Three factors may induce RNLS expression: oxidative stress, decreased antioxidant capacity, and inflammation. In fact, at 50 μmol/L Dox, gene

expression level of Gpx2 was downregulated (Fig. 3), whereas RNLS expression was upregulated. Gpx2 is a gastrointestinal-type Gpx abundant in the intestinal tract and protects cells from oxidative stress via its antioxidant effects[20]. A decrease in Gpx2 expression, increase in TBARS, and RNLS expression are all consistent with our recent report[14], implying that RNLS may be replacing the role of Gpx2 as antioxidant capacity declines. Futhermore IL-6, a pro-inflammatory cytokine, was upregulated coupled with increased phosphorylation of p65, as was the expression of RNLS (Fig. 4). Besides the antioxidant function of RNLS, it also has an anti-inflammatory effect, as reported previously[13]. Therefore, it is considered that RNLS expression increased along with the expression of IL-6. However, at 100 μmol/L, the expression of RNLS and IL-6 was decreased and could be due to the excessive cell death that occurred. In support of this, the expression of Cleaved-Caspase3 was markedly increased at 100 μmol/L (Fig. 4). Therefore, 50 μmol/L Dox is the upper limit when focusing on cellular senescence.

This study had several research limitations. First, this study could only determine the effect of Dox on RNLS expression and not whether the increased RNLS expression was due to its antioxidant, anti-inflammatory, or some other unknown function. In the future, it will be necessary to use different tools such as RNLS overexpression, knockout cells, and renalase peptide (RP-220)[11] as reported in previous studies to understand the mechanistic function. Second, the present study fails to mention the mechanism of RNLS regulation during cellular senescence. Although a previous study reported that p65 is a transcription factor involved during increased oxidative stress[14], the expression of p65 and RNLS did not coincide in some aspects in this study. It is also possible that known transcription factors alone may not explain the regulation of RNLS in our previous study with Caco-2 cells[21]. Therefore, the regulatory mechanism using reporter assay and chromatin immunoprecipitation must also be considered.

In conclusion, our study found that RNLS expression is upregulated during Dox-induced cellular senescence. This upregulation is likely to have antioxidant and anti-inflammatory effects, implying that RNLS may contribute to intestinal health during aging.

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Conflict of interest

All authors declare that there is no conflict of interest regarding the publication of this article.

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