Fundamental Toxicological Sciences (Fundam. Toxicol. Sci.)
Vol.11, No.5, 243-249, 2024

The Japanese Society of Toxicology

Fundamental Toxicological Sciences

URL : http://www.fundtoxicolsci.org/index_e.html

Original Article

Puberulic acid displays remarkable cytotoxicity and strong inhibitory effect on the all-*trans* retinoic acid-induced superoxide-generating ability in U937 cells

Hidehiko Kikuchi[1], Kaori Harata[1], Takefumi Sagara[1], Harishkumar Madhyastha[2], Hitomi Mimuro[3] and Futoshi Kuribayashi[4]

[1]Department of Food and Nutrition, Shokei University Junior College, 2-6-78 Kuhonji, Chuo-ku, Kumamoto 862-8678, Japan
[2]Department of Cardiovascular Physiology, Faculty of Medicine, University of Miyazaki, 5200, Kihara, Kiyotake, Miyazaki 889-1692, Japan
[3]Division of Genome-wide Infectious Microbiology, Research Center for GLOBAL and LOCAL Infectious Diseases, Oita University, 1-1 Idaigaoka, Hasama, Yufu, Oita, 879-5593, Japan
[4]Department of Biochemistry, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama, 701-0192, Japan

(Received September 20, 2024; Accepted October 3, 2024)

ABSTRACT — A major health outbreak in March 2024 with renal impairments occurred by ingested food supplements made from beni-koji (red yeast rice) in Japan, which has been considered as major health issue. A troponoid compound puberulic acid (PA) that was produced by contaminated *Penicillium* spp. is attracting attention as a causative agent of this health disaster occurred by beni-koji (red yeast rice). Regarding toxicity, it was reported that PA showed weak cytotoxicity against fetal human lung fibroblast-like MRC-5 cells with an IC_{50} value of about 289 μM (Iwatsuki *et al*., 2011). However, understandings about the physiological effects of PA against human tissues and cells still remain poor and insufficiently studied. Therefore, in this study, we investigated the effect of PA on the viability and the all-*trans* retinoic acid (ATRA)-induced superoxide anion (O_2^-)-generating ability of human leukemia U937 cells. PA remarkably showed a strong cytotoxicity accompanied by apoptosis, which was enhanced by ATRA. Furthermore, PA dramatically down-regulated the ATRA-induced O_2^--generating activity in a dose-dependent manner. Quantitative RT-PCR and immunoblot analyses showed that PA significantly reduces the ATRA-induced O_2^--generating activity via down-regulating gene expression levels of gp91-phox, which is an essential factor for the O_2^--generating activity of leukocytes. These findings revealed that PA has not only the strong ATRA-enhancible cytotoxic effect but also the drastic reducing effect on the ATRA-induced O_2^--generating activity through down-regulating transcription of gp91-phox gene. We expect that our findings will contribute to resolve the large-scale health disaster caused by beni-koji (red yeast rice).

Key words: Puberulic acid, Superoxide, Cytotoxicity, All-*trans* retinoic acid, gp91-phox, Leukocyte

INTRODUCTION

In March 2024, a large-scale health disaster occurred by ingested health-food products containing beni-koji (red yeast rice) in Japan. It was suspected that any renal failure-inducible substances were involved in this large-scale health disaster caused by beni-koji (red yeast rice) because many patients had medical findings of renal

Correspondence: Hidehiko Kikuchi (E-mail: masakari@shokei-gakuen.ac.jp)

impairment. At the moment, puberulic acid (PA), a troponoid compound with seven-membered ring (see Fig. 1), has been considered to be a powerful candidate substance responsible for the health disaster caused by beni-koji (red yeast rice). PA is also a non-benzenoid aromatic compound which was identified as a metabolite of *Penicillium puberulum* Bainier, and its molecular formula is $C_8H_6O_6$ (Birkinshaw and Raistrick, 1932; Barger and Dorrer, 1934). Since beni-koji (red yeast rice) cannot produce PA, it is thought that the culture tanks of beni-koji (red yeast rice) were contaminated with some kinds of mold belonging to *Penicillium* spp. that produce PA. It was reported that PA showed weak cytotoxicity against fetal human lung fibroblast-like cell line MRC-5 cells with an IC_{50} value of about 289 μM (Iwatsuki *et al*., 2011). However, understandings about the physiological effects of PA still remain poor. We have been elucidating the effects of various phytochemicals using *in vitro* differentiation system of human monoblastic leukemia U937 cells. This system is useful for elucidating physiological effects of bioactive compounds on leukocyte function (Kikuchi *et al*., 2018, 2019, 2020, 2022). Therefore, in this paper, we examined the effects of PA on viability and the superoxide (O_2^-)-generating system using *in vitro* differentiation system of U937 cells.

Several differentiation inducers such as all-*trans* retinoic acid (ATRA) are able to promote differentiation of U937 cells to macrophage-like cells (Harris and Ralph, 1985). Although intact U937 cells produce an undetectable level of O_2^-, differentiated U937 cells can generate O_2^- in response to phorbol 12-myristate 13-acetate (PMA) (Kikuchi *et al*., 1994, 2011). The O_2^--generating system is essential for leukocytes-mediated bactericidal activity responsible for innate immune system, and is formed from six kinds of proteins like, membrane heterodimer protein cytochrome b_{558} (the small [p22-phox] and the large [gp91-phox] subunits), three specific cytosolic proteins (p40-phox, p47-phox and p67-phox), and ubiquitous small G-protein Rac (Dagher and Pick, 2007; Panday *et al*., 2015). Above all, the gp91-phox protein (large subunit of the cytochrome b_{558}) also called as NADPH oxidase 2 (Nox2) is considered as major protein of the O_2^--generating system in leukocytes.

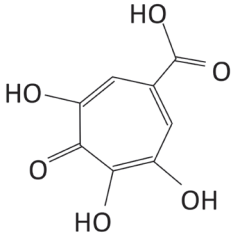

Fig. 1. Chemical structures of PA.

In this paper, we revealed not only that PA shows remarkable cytotoxicity against U937 cells but also that PA inhibits the ATRA-induced O_2^--generating activity of U937 cells via down-regulating gene expression of gp91-phox.

MATERIALS AND METHODS

Materials

PA (Nagara Science, Gifu, Japan), PMA, ATRA, luminol (Sigma, St Louis, MO, USA), ReverTra Ace qPCR RT Master Mix with gDNA remover (Toyobo, Osaka, Japan), RPMI-1640 culture medium (Thermo Fisher Scientific, Waltham, MA, USA), GeneAce SYBR qPCR mix α (Nippon Gene, Tokyo, Japan), Diogenes-luminol chemiluminescence probes (National Diagnostics, Atlanta, GA, USA), fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA), plasmocin (InvivoGen, San Diego, CA, USA), Trizol reagent (Invitrogen, Carlsbad, CA, USA), monoclonal anti-gp91-phox antibody (BD Biosciences, San Jose, CA, USA), monoclonal anti-p22-phox antibody (GeneTex, Irvine, CA, USA), monoclonal anti-Na^+/K^+-ATPase antibody (Abcam, Cambridge, UK), and horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin (Promega, Madison, WI, USA) were obtained from the companies indicated.

Cell culture, treatment with AAs in the presence of ATRA and measurement of cell viability

U937 (RCB0435), a human monoblastic leukemia cell line, was supplied by the RIKEN BRC (Saitama, Japan) through the National Bio-Resource Project of the MEXT, Japan. Cells were grown in RPMI-1640 culture medium containing 10% FBS and 5 μg/mL plasmocin as described previously (Kikuchi *et al*., 2019), and cells (1.0 x 10^6) in 5 mL of the culture medium were incubated with (5, 10, 15, 20 and 50 μM) or without PA in the presence or absence of 1 μM ATRA at 37°C for 48 hr. At 0, 24 and 48 hr, cell viability was determined by the trypan blue dye exclusion method using a hemocytometer under a microscope. (Kikuchi *et al*., 2020). DNA fragmentation analysis was performed as described previously (Kikuchi and Imajoh-Ohmi, 1995).

Measurement of O_2^- generation

U937 cells (1.0 x 10^6) in 5 mL of the culture medium were incubated with (5 or 10 μM) or without PA

in the presence or absence of 1 μM ATRA at 37°C for 48 hr. Measurement of O_2^- generation of U937 cells was carried out using Lumat^3 LB9508 luminometer (Berthold Technologies, Bad Wildbad, Germany). Luminol and Diogenes-luminol chemiluminescence probes were used in order to enhance chemiluminescence as described previously (Kikuchi *et al*., 2018).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

U937 cells (1 x 10^6) in 5 mL of RPMI-1640 culture medium containing 10% FBS and 5 μg/mL plasmocin were incubated with or without 10 μM PA in the presence of 1 μM ATRA at 37°C for 48 hr. The cDNAs were synthesized with a first strand cDNA synthesis kit using total RNAs isolated from the cells using Trizol reagent. Next, RT-qPCR using GeneAce SYBR qPCR mix α was performed by the MyGo Mini S real-time PCR instrument (IT-IS Life Science, Cork, Ireland). PCR data obtained were analyzed in accordance with the attached manufactures' protocols. RT-qPCR data were normalized to GAPDH gene. Sequence data of PCR primers used in this study were listed in our previous report (Kikuchi *et al*., 2022).

Immunoblotting

U937 cells (1 x 10^6) in 5 mL of t RPMI-1640 culture medium containing 10% FBS and 5 μg/mL plasmocin were incubated with or without 10 μM PA in the presence of 1 μM ATRA at 37°C for 48 hr. After incubation, cells were disrupted in 100 μL of 50 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose, 2 mM EDTA and 1 mM PMSF, and centrifuged at 15,000 rpm for 10 min in a TMP-21 rotor (TOMY SEIKO, Tokyo, Japan). The precipitations (membrane fractions) were treated with 10% trichloroacetic acid. The protein samples obtained by TCA precipitation were centrifuged at 15,000 rpm for 5 min in a TMP-21 rotor, and dissolved in a solubilizing solution containing 9 M urea, 2% Triton X-100 and 5% 2-mercaptoethanol, and added a loading buffer [0.5 M Tris-HCl (pH 6.8) containing 5% SDS and 20% glycerol]. These protein samples of the membrane fraction were subjected to 12% SDS-PAGE followed by immunoblotting using anti-p22phox or anti-gp91phox antibodies as primary antibodies. Data analyses were performed using a luminescent image analyzer STAGE-5100 (AMZ System Science, Osaka, Japan) and the Quant-AMZ software (TotalLab., Newcastle upon Tyne, UK). Human Na^+/K^+-ATPase was used as a control protein (Kikuchi *et al*., 2019).

Statistical analysis

Quantitative data are presented as averages of three separate experiments. Error bars indicate standard deviation. Statistical differences were calculated with Student's *t* test.

RESULTS AND DISCUSSION

In this study, we investigated the influence of PA on cell viability and the ATRA-induced O_2^--generating activity of U937. As shown in Fig. 1, PA is a troponoid compound with seven-membered aromatic ring.

Initially, in order to know the influences of PA on cell viability of U937 in the presence or absence of ATRA, the cells were treated with up to 50 μM of PA with or without 1 μM ATRA for 48 hr, and cell viability was measured at 0, 24 and 48 hr. The cell viability was about 98% at 0 hr, and ATRA showed no cytotoxicity at 1 μM concentration (data not shown). As shown in Fig. 2A, PA displayed remarkable negative effects on the viability of U937 cells at concentrations above 15 μM with or without ATRA at 24 hr and 48 hr, and cell viability was completely inhibited by 50 μM concentration of PA with or without ATRA at 48 hr. Figure 2A also showed that 1 μM ATRA significantly accelerated cell death caused by PA. In addition, internucleosomal fragmentation of DNA was markedly observed on an agarose electrophoretogram when U937 cells were cultured with 20 μM PA in the presence of 1μM ATRA at 24 hr (Fig. 2B), suggesting that PA caused apoptotic cell death against U937 cells. These data revealed that PA has no effect on viability of U937 cells up to 10 μM with or without 1 μM ATRA and 15, 20 or 50 μM PA shows strong cytotoxicity effects with apoptosis, and these cytotoxic effects of PA are significantly enhanced by 1 μM ATRA.

Second, to examine the influences of PA on the ATRA-induced O_2^--generating ability of U937 cells, the cells were treated with 5 μM or 10 μM of PA in the presence of 1 μM ATRA for 48 hr. As mentioned above, PA showed no effect on viability of U937 cells up to 10 μM in the presence of 1 μM ATRA (Fig. 2A). As shown in Fig. 3, PA remarkably reduced the ATRA-induced O_2^--generating ability of U937 cells (5 μM PA: to ~50% of the ATRA-treated cells, 10 μM PA: to ~29% of the ATRA-treated cells). These results demonstrated that PA has a strong inhibitory effect on the ATRA-induced O_2^--generating ability of U937 cells at non-cytotoxic concentrations (5 μM or 10 μM).

Third, to reveal the mechanism of inhibition of the ATRA-induced O_2^--generating activity by PA, RT-qPCR method was performed for analyzing the effects of PA on

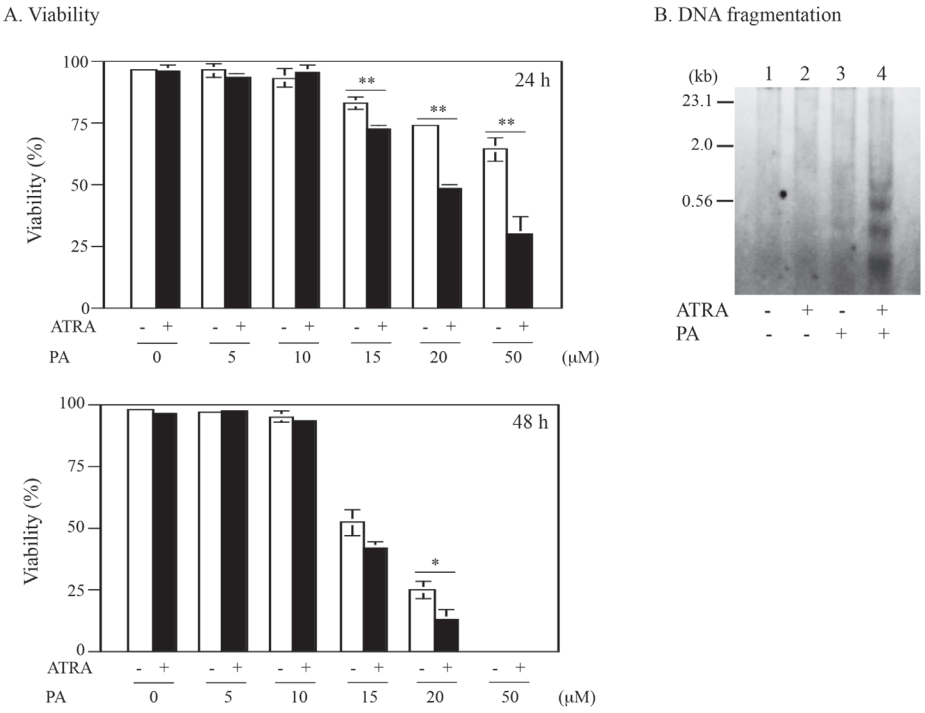

Fig. 2. Cytotoxicity of PA against U937 cells. (A) Viability. Cells (1.0 x 10^6) in 5 mL of culture medium were incubated without or with 5 μM, 10 μM, 15 μM, 20 μM or 50 μM PA in the absence (open bars) or presence (closed bars) of 1 μM ATRA for 48 hr. Viable cells were counted at 0, 25 and 48 hr by the trypan blue dye exclusion method. Data represent the averages of three separate experiments. Statistical differences were calculated by Student's *t* test. *, $p < 0.05$, **, $p < 0.01$. (B) DNA fragmentation. Cells (1.0 x 10^6) in 5 mL of culture medium were incubated without or with 20 μM PA in the absence or presence of 1 μM ATRA for 24 hr. DNAs extracted from the cells were analyzed by 2.0% agarose gel electrophoresis. The sizes of the l-DNA digested with *Hin*dIII are indicated in base pairs.

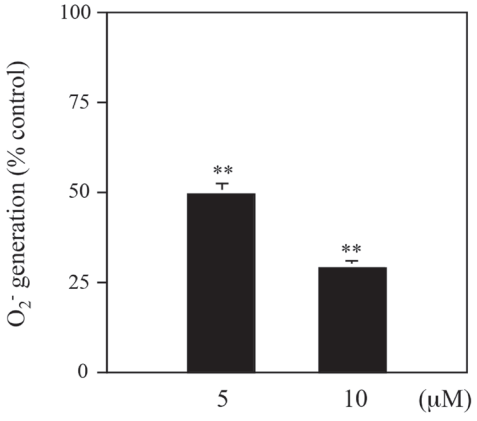

Fig. 3. Effects of PA on the ATRA-induced O_2^--generating activity of U937 cells. After cultivation without or with 5 or 10 μM PA in the presence of 1 μM ATRA for 48 hr, cells (1 x 10^5 cells/mL) were stimulated with 200 ng/mL PMA at 37°C. PMA-induced chemiluminescence was measured at 10 min after stimulation using a Lumat^3 LB9508 luminometer as described previously (Kikuchi *et al*., 2018). Quantitative data are indicated as percentages of control value obtained from ATRA-treated U937 cells, and presented as the average of three separate experiments. Error bars indicate standard deviation. Statistical differences were calculated by Student's *t* test. **, $p < 0.01$ compared with the data of ATRA-treated U937 cells.

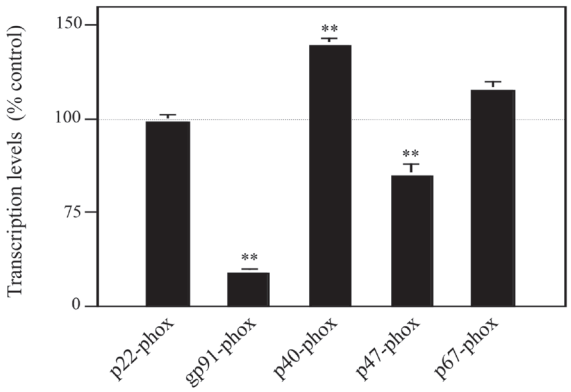

Fig. 4. Effects of PA on the transcription levels of the O_2^--generating system-related factors. The transcription levels of p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox were determined by RT-qPCR using total RNA extracted from ATRA-treated, ATRA plus 10 μM PA-treated U937 cells as described in "MATERIALS AND METHODS". All qPCR data of p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox genes were normalized to qPCR data of GAPDH gene. Quantitative data are indicated as percentages of control value obtained from ATRA-treated U937 cells, and presented as the average of three separate experiments. Error bars indicate standard deviation. Statistical differences were calculated using Student's *t* test. **, $p < 0.01$ compared with the data of ATRA-treated U937 cells.

the transcription levels of the five genes (p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox) that are essential for the leukocyte O_2^--generating system. After 48 hr cultivation with or without 10 μM PA in the presence of 1 μM ATRA, total RNAs were isolated from the 1 μM ATRA-treated and the 1 μM ATRA plus 10 μM PA-treated U937 cells. Quantitative data of RT-qPCR were indicated as percentages of control values obtained from the ATRA-treated U937 cells (Fig. 4). Ten micromolar of PA showed no effect on the transcription levels of p22-phox and p67-phox genes in the presence of 1 μM ATRA. On the other hand, the transcription level of p40-phox gene was slightly enhanced in the 1 μM ATRA plus 10 μM PA-treated U937 cells (to ~140% of the ATRA-treated cells). Moreover, interestingly, the transcription level of gp91-phox gene was drastically down-regulated in the 1 μM ATRA plus 10 μM PA-treated U937 cells (to ~17% of the ATRA-treated cells), and that of p47-phox gene was certainly suppressed in the1 μM ATRA plus 10 μM PA-treated U937 cells (to ~69% of the ATRA-treated cells). These results suggested that PA inhibits the ATRA-induced O_2^--generating activity of U937 cells mainly through down-regulating the transcription of gp91-phox gene because our previous studies clarified that the expression level of gp91-phox protein is the most essential limiting factor of the O_2^--generating ability in U937 cells among the five essential protein factors (p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox) (Kikuchi *et al*., 1994, 2019, 2022). Besides, to reveal the effect of 10 μM PA on the amounts of cytochrome b_{558} composed of p22-phox and gp91-phox proteins during cultivation in the presence of 1 μM ATRA, immunoblotting was carried out using antibodies specific for p22-phox or gp91-phox proteins as primary antibodies. Typical profiles of immunoblotting for p22-phox and gp91-phox proteins in the 1 μM ATRA-treated and the 1 μM ATRA plus 10 μM PA-treated U937 cells are shown in Fig. 5A. Quantitative data of immunoblotting were indicated as percentages of control values obtained from the 1 μM ATRA-treated U937 cells (Fig. 5B). As expected, the protein levels of both p22-phox (to ~44% of the 1 μM ATRA-treated cells) and gp91-phox (to ~20% of the 1 μM ATRA-treated cell) were significantly reduced in the 1 μM ATRA plus 10 μM PA-treated U937 cells. As is well known, both p22-phox and gp91-phox proteins are cooperatively associated resulting in assembly of cytochrome b_{558} heterodimer in the plasma membrane (Dagher and Pick, 2007; Panday *et al*., 2015). Moreover, our previous studies revealed that the amounts of p22-phox protein mostly depend on those of gp91-phox in U937 cells regardless of the transcription level of p22-phox (Kikuchi *et al*., 1994, 2019, 2022). As a result, the amount of complete form of cytochrome b_{558} protein in the 1 μM ATRA plus 10 μM PA-treated U937 cells should be about 20% of that in the 1 μM ATRA-treated U937 cells reflecting the

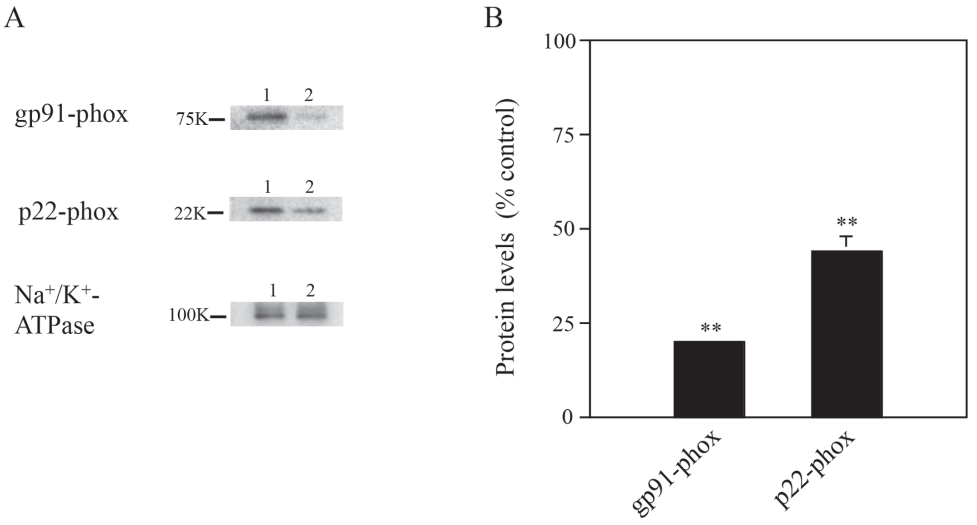

Fig. 5. Effects of PA on the protein levels of cytochrome b_{558} composed of p22-phox and gp91-phox proteins. (A) Typical profiles of immunoblotting. Protein levels of p22-phox and gp91-phox were determined by immunoblotting using membrane fractions prepared from ATRA-treated (lane 1) and ATRA plus 10 μM PA-treated (lane 2) U937 cells. Human Na^+/K^+-ATPase was used as a control. (B) Quantitative data of immunoblotting. Data of ATRA plus 10 μM PA-treated U937 cells are indicated as percentages of control value obtained from ATRA-treated U937 cells, and presented as the average of three separate experiments. Error bars indicate standard deviation. Statistical differences were calculated using Student's *t* test. **, $p < 0.01$ compared with the data of ATRA-treated U937 cells.

transcription level of gp91-phox, resulting the remarkable suppression of ATRA-induced O_2^--generating activity (10 μM PA: to ~29% of the ATRA-treated cells) in proportion to the amount of complete form of cytochrome b_{558}. Taken together, our data in this study demonstrated that PA drastically reduced the transcription levels of gp91-phox gene, resulting in significant decrease of complete form of cytochrome b_{558} protein followed by remarkable down-regulation of the ATRA-induced O_2^--generating activity in U937 cells.

Finally, in this communication, we showed the inhibitory effect of PA on the ATRA-induced O_2^--generating activity via reducing transcription level of gp91-phox, suggesting that PA can affect the expression of particular genes. As mentioned above, PA has been considered to be a powerful candidate compound responsible for the health disasters such as renal failure caused by beni-koji (red yeast rice). Because cytochrome b_{558} is also expressed in kidney (Sedeek *et al.*, 2013), PA may be involve in the health disasters including renal failure caused by beni-koji (red yeast rice) via suppressing the gp91-phox-mediated O_2^--generating activity in kidney. Of course, there is a possibility that PA causes the renal failure through modifying the expression of various genes responsible for any kidney function. Although the relationship between inhibitory effect on the O_2^--generating activity of PA (and cytotoxicity) and renal failure caused by beni-koji (red yeast rice) should be considered as unknown till now, we think that cytotoxicity and O_2^--generation-reducing activity of PA against U937 cells cannot be ignored in elucidating the mechanism of renal failure caused by beni-koji (red yeast rice).

In summary, our data also revealed not only that PA showed strong cytotoxicity against U937 cells enhanced by ATRA but also that PA has the remarkable inhibitory effect on the ATRA-induced O_2^--generating activity in U937 cells in a dose-dependent manner. In other words, it is possible that PA shows the physiological effects on various cells and tissues including the kidney. We hope that our findings in this paper will contribute to the elucidation of the mechanisms of a large-scale health disaster caused by beni-koji (red yeast rice) including renal failure.

ACKNOWLEDGMENTS

We thank R. Madhyastha for editorial reading of the manuscript. This work was supported in part by JSPS KAKENHI [Grant Number: JP22K02173 (to H. K.)] and the Research Center for GLOBAL and LOCAL Infectious

Diseases, Oita University [Grant Number: 2022B06 (to H. K.)].

Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Barger, G. and Dorrer, O. (1934): Chemical properties of puberulic acid, $C_8H_6O_6$, and a yellow acid, $C_8H_4O_6$. Biochem. J., **28**, 11-15.

Birkinshaw, J.H. and Raistrick, H. (1932): Studies in the biochemistry of micro-organisms: puberulic acid $C_8H_6O_6$ and an acid $C_8H_4O_6$, new products of the metabolism of glucose by Penicillium puberulum Bainier and Penicillium aurantio-virens Biourge. With an appendix on certain dihydroxybenzenedicarboxylic acids. Biochem. J., **26**, 441-453.

Dagher, M.C. and Pick, E. (2007): Opening the black box: lessons from cell-free systems on the phagocyte NADPH-oxidase. Biochimie, **89**, 1123-1132.

Harris, P. and Ralph, P. (1985): Human leukemic models of myelomonocytic development: a review of the HL-60 and U937 cell lines. J. Leukoc. Biol., **37**, 407-422.

Iwatsuki, M., Takada, S., Mori, M., Ishiyama, A., Namatame, M., Nishihara-Tsukashima, A., Nonaka, K., Masuma, R., Otoguro, K., Shiomi, K. and Omura, S. (2011): *In vitro* and *in vivo* antimalarial activity of puberulic acid and its new analogs, viticolins A-C, produced by *Penicillium* sp. FKI-4410. J. Antibiot. (Tokyo), **64**, 183-188.

Kikuchi, H., Fujinawa, T., Kuribayashi, F., Nakanishi, A., Imajoh-Ohmi, S., Goto, M. and Kanegasaki, S. (1994): Induction of essential components of the superoxide generating system in human monoblastic leukemia U937 cells. J. Biochem., **116**, 742-746.

Kikuchi, H. and Imajoh-Ohmi, S. (1995): Activation and possible involvement of calpain, a calcium-activated cysteine protease, in down-regulation of apoptosis of human monoblast U937 cells. Cell Death Differ., **2**, 195-199.

Kikuchi, H., Kuribayashi, F., Kiwaki, N., Takami, Y. and Nakayama, T. (2011): GCN5 regulates the superoxide-generating system in leukocytes via controlling gp91-phox gene expression. J. Immunol., **186**, 3015-3022.

Kikuchi, H., Mimuro, H. and Kuribayashi, F. (2018): Resveratrol strongly enhances the retinoic acid-induced superoxide generating activity via up-regulation of gp91-phox gene expression in U937 cells. Biochem. Biophys. Res. Commun., **495**, 1195-1200.

Kikuchi, H., Mimuro, H., Madhyastha, H. and Kuribayashi, F. (2019): Chalcone skeleton promotes transcription of gp91-phox gene but inhibits expression of gp91-phox protein, and hydroxyl groups in hydroxychalcones participate in the stable expression of gp91-phox protein. Microbiol. Immunol., **63**, 438-443.

Kikuchi, H., Harata, K., Kawai, C., Madhyastha, H., Yamauchi, A. and Kuribayashi, F. (2020): Retinoic acid dramatically enhances cytotoxicity of equol against human monoblastic U937 cells, but not against human peripheral neutrophils. Fundam. Toxicol. Sci., **7**, 201-206.

Kikuchi, H., Harata, K., Akiyoshi, S., Sagara, T., Madhyastha, H., Mimuro, H. and Kuribayashi, F. (2022): Bifunctional activity of tangeretin (5,6,7,8,4′-pentamethoxyflavone) in suppression of cell growth and gene expression of the superoxide-generating system-related proteins in U937 cells. Fundam. Toxicol. Sci., **9**, 151-157.

Panday, A., Sahoo, M.K., Osorio, D. and Batra, S. (2015): NADPH oxidases: an overview from structure to innate immunity-associated pathologies. Cell. Mol. Immunol., **12**, 5-23.

Sedeek, M., Nasrallah, R., Touyz, R.M. and Hébert, R.L. (2013): NADPH oxidases, reactive oxygen species, and the kidney: friend and foe. J. Am. Soc. Nephrol., **24**, 1512-1518.