Ellagic acid and its fermentative derivative urolithin A show reverse effects on the gp91-phox gene expression, resulting in opposite alterations in all-trans retinoic acid-induced superoxide generating activity of U937 cells

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**ABSTRACT**

Ellagitannins (esters composed of glucose and ellagic acid) are hydrolyzed to generate ellagic acid in gut followed by conversion of ellagic acid to urolithins such as urolithin A by intestinal bacteria. Since urolithins are absorbed by gut easier than ellagitannins and ellagic acid, and show various physiological activities (e.g. anti-cancer, anti-cardiovascular disease, anti-diabetes mellitus, anti-obesity and anti-Alzheimer disease activities), they are expected as excellent health-promoting phytochemicals. Here, using human monoblast U937 cells, we investigated the effect of ellagic acid and urolithin A on the superoxide anion \((O_2^-)\)-generating system of phagocytes, which is consisted of five specific protein factors (membrane proteins: p22-phox and gp91-phox, cytosolic proteins: p40-phox, p47-phox and p67-phox). Twenty micromolar of urolithin A enhanced the all-trans retinoic acid (ATRA)-induced \(O_2^-\)-generating activity (to ~70%). Semiquantitative RT-PCR showed that transcription level of gp91-phox was certainly decreased (to ~70%) in ATRA plus ellagic acid-treated cells, while that of gp91-phox was significantly increased (to ~160%) in ATRA plus urolithin A-treated cells. Chromatin immunoprecipitation assay suggested that urolithin A enhanced acetylations of Lys-9 residues of histone H3 within chromatin surrounding the promoter region of gp91-phox gene, but ellagic acid suppressed the acetylations. Immunoblotting also revealed that ATRA plus urolithin A-treatment up-regulated protein levels of p22-phox (to ~160%) and gp91-phox (to ~170%) although ATRA plus ellagic acid-treatment down-regulated protein levels of p22-phox (to ~70%) and gp91-phox (to ~60%). These results suggested that conversion of ellagic acid to urolithin A in gut may bring about reverse effects on the gp91-phox gene expression, resulting in opposite alterations in \(O_2^-\)-generating activity of intestinal macrophages.

**1. Introduction**

Ellagic acid (2,3,7,8-tetrahydroxy [1]-benzopyrano [5,4,3-cde] [1] benzopyran-5, 10-dione) exists in various vegetables and fruits (e.g. raspberry, strawberry, walnuts, pomegranate etc.) as ellagitannins that are esters composed of glucose and ellagic acid [1]. After hydrolysis reactions of ellagitannins, ellagic acid is generated. Furthermore, urolithins (dibenzo [b,d]pyran-6-one derivatives) such as urolithin A with anti-oxidant and anti-inflammatory effects are synthesized by the microbial conversion of ellagic acid and residual ellagitannins in human gut [1-9]. Urolithins are absorbed by gut easier than ellagitannins and ellagic acid, and exert various physiological activities: anti-cancer, anti-cardiovascular disease, anti-diabetes mellitus, anti-obesity, anti-Alzheimer disease and so on [2-8]. Even at physiological concentration, urolithins can show effects on various chronic degenerative diseases [2]. Therefore, urolithins and their related compounds are expected as health-promoting phytochemicals. However, molecular mechanisms of physiological functions of urolithins remain unclear.

Here, we investigated the effect of ellagic acid and urolithin A on the superoxide anion \((O_2^-)\)-generating system of phagocytes (e.g. neutrophil...
and macrophage) for killing the invading bacteria [10]. This system is consisted of five specific protein factors (membrane proteins p22-phox and gp91-phox, cytosolic proteins p40-phox, p47-phox and p67-phox), and small G-protein Rac [11]. Cytochrome c oxidase, a heme-binding membrane protein, is a heterodimer protein formed by p22-phox and gp91-phox proteins, and mediates the final steps of electron transfer to molecular oxygen resulting in the O$_2$ generation. In a response to infectious stimuli, phagocytes release O$_2$ outside of the cells or inside of phagosomes where various stronger reactive oxygen species are formed from O$_2$ step by step. These reactive oxygen species are used to kill the invading bacteria. U937, a human monoblastic leukemia cell line, has been used as an excellent model to study mechanisms of leukocyte differentiation [12-14]. Although U937 cells lack the O$_2$-generating activity, they are differentiated to macrophage-like cells and produce O$_2$ by various differentiation-inducing agents such as interferon-γ and all-trans retinoic acid (ATRA) [12-14]. Of course, gene expressions of the five specific protein factors of the O$_2$-generating system are remarkably up-regulated by ATRA during differentiation [15-18]. Moreover, various phytochemicals can affect gene expressions of the five specific protein factors in U937 cells. Our data in this study suggested that conversion of ellagic acid to urolithin A in gut may bring about reverse effects on the gp91-phox gene expression followed by opposite alterations in the O$_2$-generating activity of intestinal macrophages. 

2. Materials and methods

2.1. Materials

Ellagic acid, PMSF (FUJIFILM Wako, Osaka, Japan), urolithin A (Cayman Chemical, Ann Arbor, MI, USA), phorbol 12-myristate 13-acetate (PMA), ATRA, luminol (Sigma, St Louis, MO, USA), plasmocin (InvivoGene, CA, USA) and Diogenes (National Diagnostics, Atlanta, GA, USA) were purchased. Monoclonal anti-gp91-phox antibody, monoclonal anti-p47-phox antibody (BD Biosciences, San Jose, CA, USA), monoclonal anti-p67-phox antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p40-phox antibody (GeneTex, Irvine, CA, USA), monoclonal anti-β-actin antibody, 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. Monoclonal anti-human p22-phox antibody (449) was kindly provided by Dr. Roos and Dr. Verhoeven (Sanquin Research, and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, The Netherlands).

2.2. ATRA-induced monocytic differentiation of U937 cells

Human monoblastic leukemia cell line, U937 cells (RCB0435) were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan [16]. Cells were grown in RPMI-1640 culture medium containing 10% fetal bovine serum and 5 μg/mL plasmocin [16-18]. Cells (2.0 × 10$^6$) in 5 ml of the culture medium were incubated with 1 μM ATRA in the absence or presence (10 μM or 20 μM) of ellagic acid or urolithin A at 37 °C for 48 h.

2.3. Measurement of O$_2$ generation

Measurement of O$_2$ generation was carried out by Lumat LB9508 luminometer (Berthold Technologies, Bad Wildbad, Germany) using Diogenes-luminol chemiluminescence probes as described previously [16-18].

2.4. Semiquantitative RT-PCR

Cells (2.0 × 10$^6$) in 5 ml of the culture medium were incubated with 1 μM ATRA in the absence or presence of 20 μM ellagic acid or 20 μM urolithin A at 37 °C for 48 h. Semiquantitative RT-PCR was performed using specific sense and antisense primers of five human genes essential for the O$_2$-generation system (p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox) as described previously [14-18]. Human GAPDH gene was used as internal controls. Semiquantitative RT-PCR data were obtained using a luminescent image analyzer STAGE-5100 (AMZ System Science, Osaka, Japan), analyzed by Quant-AMZ software (TotalLab., Newcastle upon Tyne, UK) as described [16-18].

2.5. Chromatin immunoprecipitation (ChIP) assay

Cells (2.0 × 10$^6$) in 5 ml of the culture medium were incubated with...
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3.1. Urolithin A significantly enhances the ATRA-induced O$_2^-$generating activity
were calculated with Student experiments. Error bars indicate standard deviation. Statistical differences

1 μM ATRA in the absence or presence of 20 μM ellagic acid or 20 μM urolithin A at 37°C for 48 h. ChIP assay was carried out using a ChIP assay kit (Merck, Darmstadt, Germany) according to the instruction manual as described previously [16,17]. To confirm the presence of human g991-phox promoter sequences containing critical cσ-element (and also Hox/Pbx consensus-like cσ-element) and inverted PU.1 binding site [14,19-21], the immunoprecipitated DNA samples were analyzed by PCR technique. PCRs were performed at 96°C for 20 s, 55°C for 30 s and 72°C for 30 s, for 36–40 cycles, using forward primer (5′-TCAGTTGACCAATGATTATTAGCCAATT-3′) and reverse primer (5′-CATGGCTCTTCCTCAATGACCAAAT-3′). PCRs were stopped before reaching the plateau. PCR products were analyzed using a luminescent image analyzer STAGE-5100 with Quant-AMZ software as described [16,17].

2.6. Immunoblotting

Immunoblotting was performed as described previously [14-18]. In brief, cells (2.0 × 10$^6$) in 5 ml of the culture medium were incubated with 1 μM ATRA in the absence or presence of 20 μM ellagic acid or 20 μM urolithin A at 37°C for 48 h. Cells (5 × 10$^6$) were sonicated in 100 μl of 50 mM Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose, 2 mM EDTA and 1 mM PMSF. Disrupted cells were divided into cytosolic fractions and membrane fractions by centrifugation. These protein samples were subjected to SDS-PAGE followed by immunoblotting. Data analyses were carried out using a luminescent image analyzer STAGE-5100. Human β-actin (for cytosolic fractions) and Na$^+$/K$^+$-ATPase (for membrane fractions) were used as controls [17,18].

2.7. 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.

3. Results and discussion

3.1. Urolithin A significantly enhances the ATRA-induced O$_2^-$generating activity while ellagic acid inhibits the ATRA-induced O$_2^-$generating activity

As well known phenomenon, ellagic acid is converted to urolithin A by intestinal bacteria and schematic is represented in Fig. 1A. To investigate the physiological functions of ellagic acid and urolithin A in monocytic differentiation, first, we examined the influences of both of them, on the ATRA-induced O$_2^-$generating activity in U937 cells (Fig. 1B). As with our previous reports [15-18], ATRA-treated U937 cells showed remarkable O$_2^-$generating activity by PMA treatment while untreated U937 cells only generated a negligible level of O$_2^-$ by PMA treatment. Although 10 μM of ellagic acid showed no effect on the ATRA-induced O$_2^-$generating activity, 20 μM of ellagic acid significantly down-regulated the activity (to ~70%). In contrast, very interestingly, urolithin A could enhance the O$_2^-$generating activity in a dose-dependent manner (10 μM: to ~150%, 20 μM: to ~175%). On the other hand, both ellagic acid and urolithin A showed little effect on the O$_2^-$generating activity in the absence of ATRA (Supplementary Fig. 1). These results revealed that ellagic acid and urolithin A would play opposite roles in the regulation of the ATRA-induced O$_2^-$generating activity in U937 cells.

3.2. Opposite effects of ellagic acid and urolithin A on transcription levels of the gp91-phox genes essential for the O$_2^-$generating system during ATRA-induced monocytic differentiation

As shown in Fig. 1B, urolithin A significantly up-regulated the ATRA-induced O$_2^-$generating activity while ellagic acid definitely down-regulated the activity in U937 cells. These findings suggested that ellagic acid and urolithin A may affect transcription of five genes of the essential components (p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox) for the O$_2^-$generation in the presence of ATRA. To confirm the effects of ellagic acid and urolithin A on transcription of these five genes in the presence of ATRA, we carried out semiquantitative RT-PCR and compared transcription levels of these five genes in ATRA plus ellagic acid- and urolithin A-treated cells with those in ATRA-treated cells (Fig. 2). Total RNAs were prepared from ATRA-treated, ATRA plus ellagic acid-treated and ATRA plus urolithin A-treated U937 cells. Quantitative data of semiquantitative RT-PCR were indicated as per the data of ATRA-treated cells.

As shown in Fig. 1B, urolithin A significantly up-regulated the ATRA-induced O$_2^-$generating activity while ellagic acid definitely down-regulated the activity in U937 cells. These findings suggested that ellagic acid and urolithin A may affect transcription of five genes of the essential components (p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox) for the O$_2^-$generation in the presence of ATRA. To confirm the effects of ellagic acid and urolithin A on transcription of these five genes in the presence of ATRA, we carried out semiquantitative RT-PCR and compared transcription levels of these five genes in ATRA plus ellagic acid or urolithin A-treated cells with those in ATRA-treated cells (Fig. 2). Total RNAs were prepared from ATRA-treated, ATRA plus ellagic acid-treated and ATRA plus urolithin A-treated U937 cells. Quantitative data of semiquantitative RT-PCR were indicated as percentages of control values (100%) obtained from ATRA-treated U937 cells and represent the averages of three separate experiments. Error bars indicate standard deviation. **, P < 0.01 compared with the data of ATRA-treated cells.

3.2. Opposite effects of ellagic acid and urolithin A on transcription levels of the gp91-phox genes essential for the O$_2^-$generating system during ATRA-induced monocytic differentiation

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3.3. Urolithin A enhanced acetylations of Lys-9 residues of histone H3 within chromatin surrounding the promoter region of gp91-phox gene, but ellagic acid suppressed these acetylations

As mentioned above, our findings suggested that ellagic acid suppresses transcription of gp91-phox and urolithin A participate in acetylations of histone H3 within chromatin surrounding the promoter region of gp91-phox gene, we carried out ChIP assay using anti-acetylated Lys-9 and Lys-14 residues of histone H3 (H3K9 and H3K14) antibodies (Fig. 3). These two Lys residues of histone H3 are the typical acetylation sites that are involved in transcriptional activation [22-24]. As shown in our previous reports, ATRA remarkably accelerated acetylation levels of H3K9 residues within chromatin surrounding the promoter region of gp91-phox gene, but not H3K14 residues [16,17]. On the other hand, acetylation levels of H3K14 residues were enhanced by resveratrol and butein in the presence of ATRA [16,17]. In this study, we compared ATRA plus ellagic acid- or urolithin A-treated U937 cells with ATRA-treated U937 cells. Typical electrophoresis patterns are shown in Fig. 3A. As expected, ellagic acid caused remarkable down-regulation of acetylation levels of H3K9 residues within chromatin surrounding the promoter region of gp91-phox gene (to ~60% of control value obtained from ATRA-treated U937 cells) while urolithin A brought about significant up-regulation of them (to ~150% of control value obtained from ATRA-treated U937 cells) (Fig. 3B). In contrast, both ellagic acid and urolithin A did not affect acetylation levels of H3K14 residues within chromatin surrounding the promoter region of gp91-phox gene (Fig. 3B). These data revealed that ellagic acid down-regulates the ATRA-induced O2−-generating activity via suppressing acetylations of H3K9 residues within chromatin around the promoter regions of gp91-phox gene while urolithin A up-regulates the ATRA-induced O2−-generating activity through enhancing them. In other words, ellagic acid and urolithin A may regulate the ATRA-induced O2−-generating activity in opposite epigenetic manners. Further studies are required for exploring what kinds of histone acetylation-related enzymes participate in acetylations of H3K9 residues within chromatin around the promoter regions of gp91-phox gene, and how these two phytochemicals are involved in the histone acetylations.

3.4. Opposite effects of ellagic acid and urolithin A on protein levels of cytochrome b558 composed of p22-phox and gp91-phox proteins during ATRA-induced monocytic differentiation

To investigate the effects of ellagic acid and urolithin A on amounts of five proteins (p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox proteins) essential for the O2−-generating system in phagocytes, immunoblotting was performed for these proteins using antibody specific for each protein. Quantitative data obtained from ATRA plus ellagic acid- or urolithin A-treated U937 cells were indicated as percentages of control values (100%) obtained from ATRA-treated U937 cells (Fig. 4). Both ellagic acid and urolithin A showed insignificant effect on protein level of three cytosolic factors (p40-phox, p47-phox and p67-phox proteins) as well as amounts of their mRNA (see Fig. 2). On the other hand, ATRA plus ellagic acid-treatment down-regulated protein levels of p22-phox (to...
These findings suggested that protein levels of cytochrome gp91-phox protein limits the O$_2^-$ generating activity to ~160% and gp91-phox (to ~170%) (Fig. 4B). Expression of ATRA plus urolithin A-treatment up-regulated protein levels of p22-phox (to ~70%) and gp91-phox (to ~60%) (Fig. 4A). In contrast, as expected, it is thought that the amount of p22-phox protein also was increased according to enhancement of gp91-phox protein since cytochrome b$_{558}$ is a heterodimer protein composed of gp91-phox and p22-phox proteins. These findings suggested that protein levels of cytochrome b$_{558}$ would reflect the changes of amount of gp91-phox mRNA caused by ellagic acid or urolithin A in the presence of ATRA, resulting in their opposite influences on the ATRA-induced O$_2^-$-generating activity (see Fig. 1B).

3.5. Concluding remarks

Both ellagic acid and urolithin A influenced protein level of only cytochrome b$_{558}$ composed of p22-phox and gp91-phox among the four essential proteins (cytochrome b$_{558}$, p40-phox, p47-phox and p67-phox) for the O$_2^-$-generating system. Although resveratrol enhanced the ATRA-induced O$_2^-$-generating activity via increase of cytochrome b$_{558}$ protein in U937 cells, it also caused the accumulation of p47-phox protein [16]. The accumulation mechanisms of p47-phox protein caused by resveratrol are unresolved yet. In addition, both ellagic acid and urolithin A showed little cytotoxicity up to 20 μM (Supplementary Fig. 2). Therefore, ellagic acid and urolithin A may be used as modifiers having slight side effects for cytochrome b$_{558}$ functions. Interferon-γ, one of trans activators for gp91-phox gene improves the splicing efficiency of the gp91-phox gene primary transcripts in a particular group of X-linked chronic granulomatous disease patients [25,26]. For example, combination of interferon-γ and urolithin A may rescue a certain kind of X-linked chronic granulomatous disease patients with a mutation in gp91-phox gene from infectious diseases.

Moreover, in this study, we demonstrated using U937 cells that urolithin A epigenetically enhances the ATRA-induced O$_2^-$-generating activity via activating gp91-phox transcription while ellagic acid epigenetically suppresses the ATRA-induced O$_2^-$-generating activity via inhibiting gp91-phox transcription. As is well known, U937 cells have been used as an in vitro model for studying differentiation mechanisms and functions of macrophages [12]. Macrophages also exist in intestine and play important roles to maintain the intestinal homeostasis including intestinal immunity [27,28]. In addition, interestingly, urolithin A is generated from ellagic acid by intestinal bacteria [1-9]. As is well known, a large number of bacteria live in human gut. It is believed that they act as key factors of health and disease through participating in various metabolic pathways in human gut [29]. Taken together, these findings showed the possibility that conversion of ellagic acid to urolithin A by intestinal bacteria may affect the intestinal homeostasis via controlling intestinal macrophage functions. Unfortunately, we have no data how much concentration of urolithin A affects human intestinal macrophages in human gut under physiological conditions. Although localization of urolithin A is especially difficult to be elucidated in human gut, these constraints will be resolved in the future.

Author statement

Hidehiko Kikuchi: Data curation, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. Koari Harata: Data curation, Writing – review & editing. Harishkumar Madhyastha: Writing – review & editing. Futoshi Kuribayashi: Writing – review & editing.

Declaration of competing interest

The authors declare the following interests/personal relationships which may be considered as potential competing interests: This work was supported in part by JSPS KAKENHI Grant Number 19K02329 (to H. K.) and 18K07804 (to F. K.).

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Appendix A. Supplementary data

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