In vivo anti-inflammatory and antioxidant properties of ellagitannin metabolite urolithin A

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ABSTRACT

Urolithin A is a major metabolite produced by rats and humans after consumption of pomegranate juice or pure ellagitannin geraniin. In this study, we investigated the anti-inflammatory effect of urolithin A on carrageenan-induced paw edema in mice. The volume of paw edema was reduced at 1 h after oral administration of urolithin A. In addition, plasma in treated mice exhibited significant oxygen radical antioxidant capacity (ORAC) scores with high plasma levels of the unconjugated form at 1 h after oral administration of urolithin A. These results indicate strong associations among plasma urolithin A levels, the plasma ORAC scores, and anti-inflammatory effects and may help explain a mechanism by which ellagitannins confer protection against inflammatory diseases.

KEYWORDS: Ellagitannin; urolithin A; antioxidant activity; anti-inflammatory activity
Ellagitannins are natural antioxidants, which are found in many medicinal plants and foods such as pomegranates, raspberries, blackberries, and walnuts. Various biological studies of ellagitannins have demonstrated antioxidant, antiviral, antimutagenic, antimicrobial, anti-inflammatory, and antitumor activities and the absorption and metabolism of ellagitannins have recently been reported in animal and human studies. Consumption of ellagitannin-rich beverages, such as pomegranate juice, results in the production of ellagitannin metabolites, ellagic acid and 3,8-dihydroxy-6H-dibenzo[b,d]pyran-6-one (urolithin A) (Fig. 1). Furthermore, we have isolated and characterized seven urinary and gut microbial metabolites in rats including urolithin A after the ingestion of geraniin, which is a typical ellagitannin found in *Geranium thunbergii*. Urolithin A has been found to be the main metabolite in plasma after the administration of geraniin in rats and pomegranate juice in humans and it is the most potent antioxidant among major ellagitannin metabolites.

Free radical-mediated peroxidation of membrane lipids and oxidative damage of DNA are involved in a variety of pathological complications such as cancer, atherosclerosis, and neurodegenerative diseases. Because of their antioxidant activity, ellagitannins may play a vital role in protecting against these oxidative stress-mediated pathological conditions. We previously reported that urolithin A exhibited more potent antioxidant activity than intact ellagitannins, as indicated by oxygen radical absorbance capacity (ORAC) measurements, suggesting that urolithin A may be a key mediator of ellagitannin protection. In addition, because oxidative stress plays an important role in the pathogenesis of inflammation, the ability of antioxidants to scavenge reactive oxygen species (ROS) may also provide anti-inflammatory activity. Specifically, ellagic acid, an ellagitannin metabolite, has been shown to inhibit activated biomarkers of
inflammation, such as tumor necrosis factor-α and interleukin (IL)-1β. Recently, urolithin A has been shown to inhibit prostaglandin E2 production induced by IL-1β and attenuate the effect of colonic inflammation in a colitis rat model. In the present study, we investigated in vivo anti-inflammatory and antioxidant properties of the ellagitannin metabolite urolithin A in a carrageenan-induced paw edema model in mice and with an ORAC assay in order to clarify the possible role of ellagitannin metabolites as biological antioxidants after consumption of ellagitannins.

Carrageenan-induced inflammation is a useful model to evaluate the effect of potential anti-inflammatory agents after oral administration. Paw edema was induced in the right hind paw of ICR mice by the subcutaneous injection of 1% λ-carrageenan in physiological saline (50 μL). The inflammation level was quantified by the volume of paw edema. Urolithin A prepared by chemical synthesis in 0.5% carboxymethylcellulose suspension was orally administered to the mice at 1 or 6 h before carrageenan injection. The anti-inflammatory effects of urolithin A on carrageenan-induced edema in mice are summarized in Fig. 2. The volume of paw edema of mice treated with urolithin A at 1 h before carrageenan injection decreased to 35%, 26%, and 34% relative to the control group after 3, 6, and 24 h of inflammatory induction, respectively (Fig. 2A). The differences in mean values of the control group were statistically significant at p < 0.05; however, treatment with urolithin A at 6 h before inflammatory induction by carrageenan showed no effect (Fig. 2B). The edema induced by carrageenan injection is believed to be biphasic in nature. The initial phase, beginning 1 h after carrageenan administration, is due to the release of histamine and serotonin. The second phase, occurring 2 to 5 h after carrageenan ingestion, is induced by the release of bradykinin, proteases, prostaglandin, and lysozyme. Our data suggest
that treatment with urolithin A at 1 h before inflammatory induction is effective on both/phases of inflammation induced by carrageenan.

Peripheral inflammatory responses have been mechanistically linked to enhanced
production of ROS, such as superoxide anion, peroxynitrite anion, hydroxyl radical, and
hydrogen peroxide radical, at the inflamed site. Systematic comparison studies on the
antioxidant and anti-inflammatory effects of phytochemicals have recently been
performed. Natural antioxidants such as polyphenols may protect against
oxidant-mediated inflammation and tissue damage by their ability to scavenge free
radicals. The antioxidant capacity of urolithin A proved more potent than that of the
intact ellagitannins, such as geraniin and corilagin, as measured by the ORAC assay, so that urolithin A is predicted to directly contribute to suppression of
carrageenan-induced inflammation after oral administration. The ORAC method is
based on the inhibition of peroxyl radical-induced oxidation and has the advantage of
utilizing a biologically relevant radical source.

We investigated the association between the plasma ORAC scores and plasma
levels after oral administration of urolithin A in mice. Mouse plasma samples collected
at 1 h and 6 h after administration were employed for the ORAC assay and estimation
of plasma urolithin A levels. The ORAC scores were increased to 142% in plasma of
mice at 1 h after administration compared to those of control plasma samples obtained
before administration (Fig. 3). The scores were reduced to 118% of the control scores at
6 h.

Plasma levels of urolithin A analyzed by the HPLC-ESI-MS/MS method are
shown in Table 1. Total urolithin A levels reached 3.9 μM at 1 h after ingestion and
decreased to 1.3 μM at 6 h. On the other hand, the related metabolite,
3-hydroxy-6H-dibenzo[b,d]pyran-6-one (urolithin B) (Fig. 1), which may be a gut microbial metabolite derived from urolithin A in mammals,\textsuperscript{31} could not be detected in any plasma samples. We recently demonstrated that urolithin A plasma levels in rats reached a maximum of 0.45 $\mu$M at 6 h after ingestion of 5 mg/head of ellagitannin geraniin.\textsuperscript{16} Furthermore, Seeram \textit{et al.} reported that plasma levels of urolithin A in humans reached 0.04 $\mu$M and 0.11 $\mu$M at 0.5 h and 6 h, respectively, after consumption of pomegranate juice (180 mL containing ellagitannin punicalagin 318 mg).\textsuperscript{13} Both studies revealed that plasma levels of the main metabolite, urolithin A, reached maximum values 6 h after consumption of pure ellagitannin or pomegranate juice. In this study, we were the first to demonstrate that urolithin A was rapidly absorbed and had good bioavailability after oral administration.

Most of the polyphenolic compounds present in the blood circulatory system exist in conjugated forms, such as glucuronide and sulfate, so that biological activity of some conjugates are believed to be reduced compared to free form. In a tandem mass spectrometry, the bonds of glucuronides and sulfates are easily cleaved in the collision cell to generate product ions of $[\text{M-H-176}]^-$ and $[\text{M-H-80}]^-$ respectively, which correspond to the fragments resulting from the deprotonated molecule. The neutral loss scan is a powerful tool for identifying the existence of conjugated forms in biofluids. For detection of urolithin A conjugates in mouse plasma, neutral loss scans were performed for glucuronide and sulfates (Fig. 4). The peak due to glucuronide was observed at 2.5 min in the neutral loss of 176 dalton scan data (Fig. 4A) and the mass spectrum of the peak at 2.5 min showed the ion peak at $m/z$ 403, corresponding to urolithin A monoglucuronide (Fig. 4B). The peak corresponding to sulfates could not be detected in any plasma samples.
Plasma levels of free urolithin A were estimated by treatment with and without β-glucuronidase, and it was determined that urolithin A was present as free form in 77.2% and 65.7% of the plasma samples at 1 h and 6 h after administration, respectively (Table 1). Lysosomal enzymes, including β-glucuronidase, are released from inflammatory cells such as neutrophils and macrophages at the inflammatory site. Some flavonoid glycosides have been reported to be deconjugated into aglycone by β-glucuronidase released from neutrophils after the induction of inflammation.\textsuperscript{32-33} Urolithin A glucuronide in plasma may also serve to reduce the inflammation after deconjugation at the inflamed site. Our findings indicate a strong association among plasma urolithin A levels, the plasma ORAC scores, and anti-inflammatory effects in the carrageenan-induced paw edema mice model. Thus, the potent antioxidant capacity of urolithin A in mouse plasma may contribute to the anti-inflammatory response at the affected sites after oral administration.

In this study, we investigated anti-inflammatory activity in the carrageenan-induced paw edema mice model and \textit{in vivo} antioxidant activity of urolithin A. Our data indicate that urolithin A has profound anti-inflammatory effects, which are associated with the significant ORAC scores and high plasma levels of free urolithin A at 1 h after oral administration. These findings suggest that urolithin A as an antioxidative metabolite of ellagitannins may contribute to the prevention of inflammatory diseases after oral administration and could help explain the protective effects of ellagitannin consumed from natural sources.
ACKNOWLEDGMENTS

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References and notes

2004, 43, 205.


20. Six-to-eight-old female ICR mice, weighing 20–32 g, were obtained from Japan SLC (Shizuoka, Japan). The mice were kept at a controlled temperature of 24 °C under a 12 h light/dark cycle. Each mouse was placed in a cage (Natsume Seisakusho, Tokyo, Japan) with MF standard diet (Oriental Yeast, Tokyo, Japan) and water *ad libitum*, but fasted for 24 h before the experiment. Urolithin A (300 mg/kg) was orally administered to mice in the form of suspension in 0.5% carboxymethylcellulose (CMC). The mice in the control group were administered with 0.5% CMC. Urolithin A solution was administered at 1 or 6 h before injection with 50 μL 1% λ-carrageenan dissolved in physiological saline to the right hind paw. After carrageenan injection, the hind paw volume was measured at 3, 6, and 24 h. Volume of the edema was immediately measured after 3, 6, and 24 h of carrageenan injection with a plethysmometer (TK-101; Unicom, Tokyo, Japan). The percentage protection was calculated in comparison to the control group. Data are reported as means ± SEM. The experimental protocol was
approved by the animal research control committee of Okayama University.


29. The blood samples were collected at 1 h and 6 h after oral administration of urolithin A to mice at 300 mg/kg by abdominal aorta puncture in a heparin-coated syringe under diethyl ether anesthesia. Each blood sample was centrifuged at 7830 × g for 10 min at 4 °C, to obtain plasma samples for plasma ORAC assay and HPLC-ECI-MS-MS analysis. The collected plasma sample (70 μL) was deproteinized with acetone/water/acetic acid (140 μL; 70:29.5:0.5, v/v) and was subsequently centrifuged at 10,000 × g for 10 min at 4 °C. Fluorescein and trolox were dissolved in phosphate buffer (75 mM). The plasma sample, blank (phosphate buffer), or trolox solution (20 μL; 125, 250, 500, and 1000 μM) were added to the wells of a 96-well plate. After adding 200 μL of fluorescein solution (94.4 nM) to each well, the plate was preincubated for 10 sec at 37 °C.
2,2′-Azobis(2-amidinopropane)dihydrochloride (AAPH) (75 μL; 307 mM) in phosphate buffer solution at 37 °C was added. Fluorescence was recorded every 2 min for 90 min at excitation and emission wavelengths of 485 and 528 nm, respectively, using Powerscan HT (DS Pharma Biomedical, Osaka, Japan). Data are reported as means ± SEM.

30. The collected plasma (200 μL) was incubated with or without β-glucuronidase (40 μL, 2000 units, G7896, Sigma, CA, USA) for 4 h at 37 °C. The plasma samples were deproteinized with methanol/hydrochloric acid (600 μL; 95:5, v/v). The mixture was centrifuged at 10,000 × g for 10 min at 4 °C, and the resulting supernatant was evaporated to dryness. The residue was dissolved in acetonitrile/water/formic acid (200 μL; 50:50:0.1, v/v) and filtered (PTFE membrane, 0.45 μM; Milipore, Bedford, MA, USA), followed by injection (10 μL) into the HPLC-ESI-MS-MS system. HPLC-ESI-MS-MS analysis was performed on a Shimadzu LC system (LC-20AD delivery pump, SIL-20AC autosampler, CTO-20AC column oven and CBM-20A system controller; Shimadzu, Kyoto, Japan) coupled to a triple quadrupole mass spectrometer (API-4000; Applied Biosystems, Creemore, ON, Canada). The chromatographic column was a Hydrosphere C18 column (50 × 2 mm i.d., particle size 3 μm; YMC, Kyoto, Japan) maintained at 40 °C, and the mobile phase consisted of acetonitrile/water/formic acid (95:5:0.1, v/v) (solvent A) and acetonitrile/water/formic acid (20:80:0.1, v/v) (solvent B). A gradient was applied as follows: the proportion of solvent B in the eluent increased from 0 % to 25 % (t = 1 min), remained at 25 % (t = 3 min), increased from 25 % to 100 % (t = 10 min), and decreased back to 0 % (10.1 min) until the next injection (t = 15 min).
The gradient with a flow rate of 0.5 ml/min was directed into the mass spectrometer.


Figure Legends

Figure 1. Chemical structures of urolithins A and B.

Figure 2. Anti-inflammatory effects of urolithin A on paw edema induced by carrageenan in mice at 1 (A) and 6 (B) h after oral administration. Data are expressed as means of the difference between the final and initial volumes ± SEM (n = 10). Mean value was significantly different from control: *p < 0.05.

Figure 3. Plasma Oxygen Radical Absorbance Capacity (ORAC) scores after urolithin A intake by mice. Data are expressed as means ± SEM (n = 7–10). Mean value was significantly different from the value at 0 h: **p < 0.01.

Figure 4. Neutral loss scan data of (A) 176 dalton for glucuronide form of urolithin A obtained using HPLC-ESI-MS/MS method and (B) mass spectrum of the peak at 2.5 min containing urolithin A monoglucuronide at m/z 403.
Table 1

Plasma levels of total or free urolithin A treated with or without β-glucuronidase\textsuperscript{a}

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Total urolithin A (μM)</th>
<th>Free urolithin A (μM)</th>
<th>Percentage of free urolithin A (%)\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.88 ± 0.25</td>
<td>2.85 ± 0.32</td>
<td>77.2 ± 10.9</td>
</tr>
<tr>
<td>6</td>
<td>1.27 ± 0.06</td>
<td>0.83 ± 0.05</td>
<td>65.7 ± 4.4</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Data are expressed as means ± SEM (n = 5–10)

\textsuperscript{b} Free urolithin A / total urolithin A × 100
Urolithin A: R = OH
Urolithin B: R = H
Figure 2

(A) Control

(B) Control
Figure 3

Percent of plasma ORAC before administration (%)

Time after oral administration of urolithin A (h)
Figure 4

Neutral loss of 176 dalton

\[ \text{t}_R: 2.5 \text{ min} \]

\[ 403 \]

\[ \text{[Urolithin A monoglucuronide-H]}^- \]
We investigated anti-inflammatory activity of a major ellagitannin metabolite urolithin A on carrageenan-induced paw edema in mice and antioxidant activity of urolithin A in mouse plasma after the oral administration by the ORAC assay.