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Inhibition of 5-lipoxygenase derived leukotrienes and hemiketals as a novel anti-inflammatory mechanism of urolithins

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Abstract

Scope—Urolithins, gut microbial metabolites derived from ellagic acid (EA), reach significant concentrations in the human colon. Urolithin-A (Uro-A) exerts anti-inflammatory activity in animal models of inflammatory bowel diseases (IBDs). We hypothesized that urolithins could modulate the biosynthesis of leukocyte-derived inflammatory eicosanoids from the 5-lipoxygenase (5-LOX), cyclooxygenase-2 (COX-2) and 5-LOX/COX-2 pathways, relevant in the onset and progression of IBDs, including 5-hydroxyeicosatetraenoic acids (5-HETEs), leukotriene-B_4 (LTB_4), prostaglandin E_2 (PGE_2) and hemiketals (HKE_2 and HKD_2).

Methods and results—Leukocytes, obtained from six healthy donors, were stimulated with lipopolysaccharide and calcium ionophore A23187. Urolithins, at concentrations found in the human colon (1−15 μM), decreased eicosanoid biosynthesis and COX-2 levels in the activated leukocytes. In contrast, EA and conjugated urolithins (glucuronides and sulfates) were inactive. Uro-A and isourolithin-A (IsoUro-A) reduced the formation of the 5-LOX/COX-2 products HKE_2 and HKD_2 through the COX-2 pathway (down-regulation of COX-2 and prostaglandin E_2), whereas urolithin C reduced 5-HETE and LTB_4 via inhibition of 5-LOX.

Conclusions—Our results show that physiologically relevant colonic urolithins target eicosanoid biosynthetic pathways. The effect on HKs and LTB_4 formation is unprecedented and expands the knowledge on anti-inflammatory mechanisms of urolithins against IBDs.

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Authors contribution

All authors designed experiments. A.G.S. and J.C.E. provided ellagic acid and urolithins. J.A.G.-B. performed all experiments. J.A.G.-B. wrote the manuscript with help from A.G.S., J.C.E. and C.S. All authors analyzed results and approved the final version of the manuscript.

Conflict of interest

Authors declare not having any financial or personal interest, nor having an association with any individuals or organizations that could have influenced inappropriately the submitted work.

Keywords

Urolithin; COX-2; 5-LOX; inflammatory bowel disease; eicosanoid

1. Introduction

The term inflammatory bowel disease (IBD) mainly refers to ulcerative colitis (UC) and Crohn's disease (CD), characterized by chronic inflammation of the gastrointestinal tract. After decades of investigation, the etiology of IBDs is still not fully understood [1]. An accepted model of IBD pathogenesis considers alteration of the host-microbiota interaction as a triggering factor. Microbial dysbiosis and gut barrier alteration allow the interaction of microbes with immune cells leading to dysregulated activation of leukocytes and enhanced synthesis of a plethora of molecules (i.e., cytokines and eicosanoids), resulting in constant leukocyte trafficking and massive accumulation of lymphocytes, neutrophils, and (or) macrophages [2]. This disturbs the ratio of pro-/anti-inflammatory molecules, which generates long-lasting inflammation and contributes to IBD complications such as ulcer formation, fibrosis, and cancer [3, 4].

5-Lipoxygenase (5-LOX) and cyclooxygenase-2 (COX-2) are two crucial enzymes in inflammatory disorders and cancer development through the formation of eicosanoids [5]. Biosynthesis of prostaglandins (PGs) is initiated with the oxygenation of arachidonic acid (AA) by COX-2 to form the endoperoxide PGH_2, which is the precursor of effector prostaglandins like PGE_2 and PGD_2. 5-Lipoxygenase (5-LOX) oxygenates AA forming 5*S*-hydroxyperoxyeicosatetraenoic acid (5*S*-HPETE), which is dehydrated to leukotriene (LT)-A_4 as the precursor to all LTs or reduced to 5*S*-HETE. 5*S*-HETE can serve as a substrate of COX-2, establishing a link between the 5-LOX and COX-2 pathways. The reaction of 5*S*-HETE with COX-2 generates an unstable di-endoperoxide (equivalent to PGH_2), which is subsequently transformed to two hemiketal (HK) eicosanoids, HKE_2 and HKD_2 [6–9] (Figure 1). PGs and LTs are well-investigated molecules in IBD. COX-2 expressing macrophages synthesize PGE_2, which regulates the immune response, induces epithelial cell proliferation, and increases vascular permeability [10]. LTB_4 is a potent chemoattractant agent that recruits neutrophils to the inflamed intestine, where it can promote epithelial injury through the production of reactive oxygen species [11]. Less is known about the biological activity of HKE_2 and HKD_2, which require the cross-over of 5-LOX- and COX-2 activities to be synthesized. These eicosanoids promote migration and tubulogenesis of endothelial cells [6] and inhibit platelet aggregation [12]. A common anti-inflammatory strategy involves the reduction of the biosynthesis of PGs and LTs via inhibition of the COX-2 and 5-LOX pathways. Aspirin is a paradigmatic inhibitor of PGs synthesis through blocking COX-2 activity [13], while drugs such as zileuton or montelukast target the 5-LOX pathway [14]. However, the side effects associated with these drugs [15, 16] indicate the importance of searching for alternative therapeutic and preventive strategies, including the use of natural products.

Urolithins, gut microbiota metabolites derived from ellagitannins (ETs) and ellagic acid (EA), are considered to be responsible for the health effects associated with the consumption

of ellagitannin-rich food products (pomegranate, walnuts or berries) [17]. Urolithins are absorbed from the intestinal lumen where they reach relevant concentrations [18] and undergo enterohepatic circulation [19] with extensive phase-II metabolism yielding mainly glucuronide and sulfate conjugates, which have been detected in the bloodstream (0.2–20 μM) and systemic tissues such as prostate or mammary tissue [20, 21]. *In vitro* and *in vivo* studies have reported that urolithins (Uro-A as the most active metabolite) exert anti-inflammatory effects through the preservation of the colonic architecture, attenuation of DSS-induced microbiota changes, inhibition of NF-κB and, COX-2 expression, and reduction of PGE_2 formation in intestinal tissues and cells [22–24]. Targeting the synthesis of soluble mediators by immune cells has emerged as an exciting approach in IBDs therapy [2, 4]. The 5-LOX/COX-2 pathway (and its HKE_2 and HKD_2 products) offers a new option to advance in the understanding of the molecular mechanisms underlying the effect of urolithins against IBDs. In this study, we have studied whether urolithins, including Uro-A, IsoUro-A, Uro-B, and Uro-C, and their most relevant phase-II conjugates (glucuronides and sulfates) modulate the formation of 5-LOX (5-HETE and LTB_4), COX-2 (PGE_2), and 5-LOX/COX-2 (HKE_2 and HKD_2) products in a human isolated leukocyte model. We have also investigated the effect of these metabolites on 5-LOX and COX-2 protein levels and the enzymatic activity of COX-2.

2. Material and Methods

2.1. Materials

Ellagic acid (EA), dimethylsulfoxide (DMSO), lipopolysaccharide (LPS) from Escherichia coli (0111:B4), and RIPA buffer were purchased from Sigma (St. Louis, MO, USA). Phosphatase and protease inhibitors were obtained from ROCHE (USA). Calcium ionophore A23187 and d_4-PGE_2 were obtained from Cayman Chemical (Ann Arbor, MI, USA). Urolithins (Uro) metabolites Uro-A, Uro-B, and isourolithin A (IsoUro-A), and the conjugates Uro-A glucuronide (Uro-A-glur), Uro-B glucuronide (Uro-B-glur), isourolithin-glucuronide (IsoUro-A-glur) and Uro-A sulfate (Uro-A-sulf) conjugates, were obtained from Villapharma Research S.L. (Parque Tecnológico de Fuente Alamo, Murcia, Spain) (Figure 2). Uro-C was obtained from Toronto Research Chemical (Toronto, ON, Canada).

2.2. Dosage Information

EA and urolithins were diluted in DMSO. The cells were treated with these compounds at concentrations ranging from 15 to 1 μM (≤0.5% DMSO, v/v). These concentrations are similar to those detected in vivo, and no toxic effects have been previously reported under the conditions of our study [17].

2.3 Leukocytes isolation and eicosanoids biosynthesis

A mixture of leukocytes, including neutrophils, lymphocytes, monocytes, eosinophils, and basophils were obtained from healthy donorś blood. The study was approved by the Vanderbilt University Medical Center Institutional Review Board (091243), and written informed consent was signed by the volunteers (n=6) before blood samples were obtained. Blood (45 mL) was collected in a syringe containing 6% dextran solution (10 mL) and sodium citrate (4.5 mL). The syringe was placed upright 60 min to separate red cells from

leukocytes. The upper layer rich in leukocytes was collected in a 50 mL tube, centrifuged (317×*g*; 15 min at 10 °C), and the cells obtained were washed with sterile PBS and centrifuged again (188×*g*; 10 min at 10 °C). Next, for red cells lysis, 9 mL deionized water was added to 2 mL of cell suspension for 30 s, and then the tonicity was immediately reestablished with 1 mL 10X PBS. The leukocytes were centrifuged again (543×*g*; 10 min at 10 °C) and diluted in Roswell Park Memorial Institute (RPMI) medium (2 g/L glucose, 0.1% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin) to a final concentration of 10^7 cells/mL. The cell solution, obtained from each volunteer, was distributed in a 24-well plate (1 mL per well) and pre-treated with 15 μM EA or urolithins and their conjugated metabolites, respectively, for 1 h. Next, the cells were treated with 10 μg/mL LPS for 5 h to stimulate COX-2 expression while cells were maintained in an incubator at 37°C and 5% CO_2. In the last 15 min of the LPS incubation, A23187 (5 μM) was added for 5-LOX activation. After the incubation, the cells were pelleted by centrifugation (545×*g* for 5 min), and the supernatant (1 mL) was mixed with an equal volume of 0.1% acetic acid (pH 3.5). As internal standard d_4-PGE_2 was added to the samples prior to extraction using Waters HLB cartridges (Waters, Milford, MA, USA). The samples were eluted in methanol (MeOH), evaporated, and AMPP-derivatized, as previously described [25]. For protein analysis, the pellet obtained was washed in cold PBS, followed by the addition of RIPA buffer supplemented with protease and phosphatase inhibitors. The sample was incubated on ice for 30 min, centrifuged at 13,300×*g* for 15 min, and the supernatant kept at −80 °C until analysis.

2.4 Cell culture conditions and treatments

Murine RAW264.7 macrophages were purchased from the American Type Culture Collection (ATCC, Rockville, USA) and cultured as recommended by ATCC. Cells were routinely grown in Dulbecco's Modified Eagle Medium (DMEM) medium (4.5 g/L glucose, 10% FBS, 2 mM L-glutamine, 1 mM Na-pyruvate, 25 mM HEPES, 100 U/mL penicillin, and 100 mg/mL streptomycin) until reaching 80–90% confluence. For the experiments, the cells were seeded at 300,000 cells/cm^2 in a 12-well plate and incubated under the same conditions described for leukocytes (see above) for 24 h. The medium was removed, the cells washed with PBS and incubated in DMEM containing 0.1% FBS for 4 h to minimize the effect of FBS on COX-2 expression [26]. Next, the cells were treated with Uro-A, IsoUro-A, and Uro-C at 15 μM for 1 h, followed by LPS treatment (10 μg/mL) for 5 h and 5 μM A23187 for the last 15 min. Control cells (0.5% DMSO) were run in parallel. Protein extraction was performed in RIPA buffer supplemented with protease and phosphatase inhibitors. The experiment was repeated 3 times. Passages 4–5 and population doubling level (PDL) between 8 and 9 were used for all the experiments.

2.5 Effect of urolithins on COX-2 activity

Human recombinant COX-2 was expressed in Sf9 insect cells as previously described [27] and incubated in 100 μL ammonium acetate (100 mM, pH 8.2; containing 2 μM hematin and 500 μM phenol) for 2 min at room temperature, followed by the addition of 15 μM Uro-A or IsoUro-A, and incubated for 15 additional min. The reaction was initiated by the addition of 3 μg arachidonic acid (AA), conducted for 15 min at room temperature, and stopped by adding 4.25 μL HCl (1N) together with 20 ng d_4-PGE_2 as an internal standard. Samples

were extracted using activated Waters 30 mg HLB cartridges, eluted in 500 mM MeOH, evaporated under a stream of N_2, diluted in 50 μL mobile phase, filtered using 0.22 μm Spin-X centrifuge tube filters, and analyzed in the negative ionization mode by LC-MS. Assays were done at least in triplicate for each treatment.

2.6 Western Blot analysis

The changes in 5-LOX and COX-2 protein content were evaluated by using the same protein amount of total lysates (20 μg protein/lane), which were quantified using the DC protein assay kit (Bio-Rad, Hercules, CA, USA). Proteins were separated by 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. After washing, the membranes were incubated with polyclonal 5-LOX (78 KDa, BD Bioscience, CA, USA) or COX-2 (72 KDa, Cayman Chemical, MI, USA) primary antibody at 1:1000 dilution, followed by anti-mouse IgG incubation at 1:3000 (Cell Signaling, MA, USA) and exposed to X-ray film. 5-LOX and COX-2 intensities were normalized to GAPDH. As COX-2 positive control, we included 2 μg cell lysate obtained from RAW264.7 macrophages treated with 100 ng/mL LPS for 5 h [28]. Western blot (WB) analyses were done at least in triplicate for each treatment.

2.7 LC-MS analysis

Eicosanoid separation and quntification in positive ion mode was carried out as previously described [25]. Eicosanoid formation in the COX-2 reaction with AA was analyzed in negative ion mode (underivatized samples) using chromatographic separation by a Zorbax Eclipse Plus C18 1.8-μm column (2.1 × 50 mm; Agilent Technologies). A linear gradient started with 100% of water:acetonitrile (95:5, v/v), reaching 100% of water:acetonitrile (5:95, v/v) at 5 min, and kept for 1 min. The initial conditions were re-established at 6.01 min and maintained for 1 min. The transitions recorded in the selected reaction monitoring (SRM) in negative and positive ion mode (AMPP-derivatized compounds) for LTB_4, 5-HETE, PGE_2, d_4-PGE_2, HKE_2, and HKD_2 were reported elsewhere [25]. The ion transition recorded for PGD_2 in negative ion mode was: *m/z* 351 to 271 (+15 eV).

2.8 Statistical analysis

Statistical differences were analyzed using Prism 5 (La Jolla, CA, USA). For normally distributed data, one-way ANOVA followed by Newman Keuls' *post-hoc* test was used. Results with p values <0.05 were considered statistically significant.

3. Results

3.1 Urolithins decrease eicosanoids formed by the 5-LOX, COX-2 and 5-LOX/COX-2 pathways

Treatment of leukocytes with 15 μM Uro-A and IsoUro-A decreased formation of PGE_2 (Fig. 3A). Uro-C, at the same concentration, was the only compound tested to decrease formation of 5-HETE and LTB_4 in human leukocytes stimulated with LPS and A23187 (Fig. 3B,C). The two hemiketals, which require both 5-LOX and COX-2 for their formation, were inhibited by Uro-A, IsoUro-A, and Uro-C (Fig. 3D,E), consistent with the effects of these urolithins on the individual enzymes. Uro-B (15 μM), unexpectedly, inhibited formation of

HKE_2 although not of HKD_2. This could indicate an inhibitory effect on a – so far uncharacterized – enzyme that transforms the diendoperoxide intermediate specifically to HKE_2 or it may be due to variability in the non-enzymatic transformation of the diendoperoxide to the HKs. EA and the conjugated metabolites had no effect on eicosanoid formation. Representative LC-MS chromatograms illustrating the effect of Uro-A, IsoUro-A, and Uro-C on eicosanoids formation are shown in Supporting Information Figure S1. Quantitative analysis showed that Uro-A and IsoUro-A decreased biosynthesis of HKE_2 and HKD_2 by ~43% and ~55%, respectively, as well as PGE_2 by 46–55%. Uro-C decreased HKE_2 and HKD_2 by 40–60% and the 5-LOX products 5-HETE and LTB_4 by 73% and 65%, respectively, when compared to control activated leukocytes.

Based on these results, we selected Uro-A, IsoUro-A, and Uro-C for a dose-response analysis (15, 5, and 1 μM). In line with the initial screening, Uro-A and IsoUro-A decreased LPS-induced PGE_2 formation dose-dependently, although the effect was only statistically significant ($p<0.05$) at 15 μM (Figure 4A). Consistent with the previous analysis, no effect was observed on the formation of 5-LOX products (5-HETE and LTB_4) by Uro-A and IsoUro-A (Figure 4B and 4C), and likewise, Uro-C did not inhibit PGE_2 production (Figure 4A). Reduction of 5-HETE formation was observed in the samples treated with Uro-C at concentrations from 15 to 1 μM (~32–58%; $p<0.05$) (Figure 4B), but not at concentrations below 1 μM (data not shown). Uro-C exerted a dose-dependent inhibition on the biosynthesis of LTB_4, reaching 77% reduction at 15 μM ($p<0.05$) (Figure 4C).

Biosynthesis of the 5-LOX/COX-2 cross-over eicosanoids (HKE_2 and HKD_2) was dose-dependently decrease by Uro-A and IsoUro-A treatments, although this was statistically significant ($p<0.05$) only at 15 μM (Figure 5). Uro-C, at 15 μM, also exerted a significant attenuation on the formation of HKE_2 (54%; $p<0.05$) and HKD_2 (63%; $p<0.01$). At lower concentrations, 5 and 1 μM, Uro-C exerted a non-significant reduction of HKE_2 (35 and 38%, respectively). Unexpectedly, at 1μM, Uro-C exerted a significant reduction (46%; *$p<0.05$*) of HKD_2, whereas at 5 μM the decrease observed (40%) was not significant, what could be related to the higher variability observed (Figure 5B).

3.2 Effect of Uro-A and IsoUro-A on COX-2 activity

Inhibition of PGE_2 and HK formation by Uro-A and IsoUro-A in the leukocytes might be achieved via direct inhibition of the COX-2 enzyme or through downregulation of its expression. To test the former, we tested the effect of Uro-A and IsoUro-A on recombinant purified human COX-2 and analyzed enzymatic activity using LC-MS. In the absence of the urolithins, PGE_2 was the major eicosanoid formed while the concentration of PGD_2 was almost 20-fold lower. Uro-A and IsoUro-A failed to reduce formation of PGE_2 and PGD_2 by purified COX-2 (results not shown), suggesting inhibition in leukocytes was not due to a direct effect on the enzymatic activity of COX-2.

3.3 Effect of Uro-A, IsoUro-A, and Uro-C on COX-2 and 5-LOX expression levels

We next investigated whether the effect of Uro-A, IsoUro-A, and Uro-C on eicosanoid formation was mediated via downregulation of 5-LOX and COX-2 expression levels.

Downregulation of 5-LOX expression may explain the reduced 5-HETE and LTB_4 levels in Uro-C-treated leukocytes. WB analysis showed that Uro-C did not change the expression level of 5-LOX in the leukocytes, suggesting the effect is through inhibition of the 5-LOX enzyme (Figure 6).

We next tested the effect of Uro-A and IsoUro-A on COX-2 expression levels in stimulated leukocytes. As expected, LPS treatment increased COX-2 levels in leukocytes, whereas in the presence of Uro-A or IsoUro-A (15 μM), this effect was attenuated (Figure 7A and B). A positive control (RAW264.7 cells protein extract) and molecular weight markers were included to ensure identification of the correct protein band representing COX-2. Uro-A and IsoUro-A (15 μM) had a similar inhibitory effect on COX-2 expression in LPS-treated RAW264.7 macrophages. (Figure 7C and D).

4. Discussion

Uro-A was first reported by our group 10 years ago as the molecule responsible for the anti-inflammatory effects associated with the consumption of ETs-rich foodstuff at the intestinal level [24]. Since then, the investigations have focused on elucidating the biological activity of urolithins (i.e., Uro-A, Uro-B, Uro-C, etc.) and EA, as well as the underlying molecular mechanisms [22, 23, 29]. A common point in these studies was the investigation of the anti-inflammatory effects of urolithins. However, the possible effect on some crucial inflammatory mediators produced by immune cells, such as eicosanoids, has been scarcely studied. Here we show that urolithins, at concentrations detected in human colonic tissue and lumen [18], might exert anti-inflammatory effects via modulation of 5-LOX and COX-2 metabolite formation in primary human leukocytes. Primary human leukocytes (monocytes, granulocytes and lymphocytes) is an interesting cellular model used in previous *in vitro* studies of intestinal inflammation [30]. We report effects of urolithins on traditional eicosanoids like COX-2 derived PGE_2 and 5-LOX derived 5-HETE and LTB_4 but also on the more recently discovered hemiketal eicosanoids, namely HKD_2 and HKE_2, formed by consecutive transformation of AA by 5-LOX and COX-2 [6].

The COX-2 metabolite PGE_2 is a key mediator of intestinal inflammation. PGE_2 promotes immune tolerance, epithelial proliferation and healing, as well as inhibition of apoptosis in intestinal epithelial cells [3]. However, a high level of PGE_2 in animal models of colitis exacerbates the intestinal damage [31]. These studies indicate that the modulation of intestinal PGE_2 level is an essential factor determining whether it is a disease-suppressive or pro-inflammatory molecule. Down-regulation of PGE_2 in colitis animal models after consumption of pomegranate- and Uro-A-enriched diet was found to ameliorate intestinal inflammation [24], and has been associated with the effect of urolithins on intestinal cells under inflammatory conditions [22, 23]. In this study, similar to Uro-A, we found that its isomer IsoUro-A down-regulated PGE_2 biosynthesis in activated leukocytes. These results suggest that the main colonic urolithins (Uro-A and IsoUro-A) could target PGE_2 biosynthesis *in vivo* interacting with different cell types under inflammatory conditions.

5-LOX plays a unique role in IBD through the formation of leukotrienes. Massive accumulation of 5-LOX expressing neutrophils and marked high levels of LTB_4 are

hallmarks of IBD reported in mucosa from IBD patients [32] and colitis animal models [33]. In this regard, inhibition of LTB_4 formation by natural products could be an important target for the treatment of IBD. We found that Uro-C exerted a dose-dependent reduction of LTB_4 biosynthesis by stimulated leukocytes. This finding brings an additional mechanism to those reported by Piwowarski *et al.*, who described lower levels of IL-8, ROS, and myeloperoxidase in stimulated neutrophils treated with Uro-C [34]. In addition to LT, 5-HETE is a major product of 5-LOX activity in leukocytes. In line with its effect on LTB_4, Uro-C also interfered with the 5-LOX pathway reducing the formation of 5-HETE. The significance of 5-HETE formation in IBD and its potential role are unclear. 5-HETE may have limited activity by itself but may serve as a precursor to biologically active molecules [35, 36].

5*S*-HETE is an efficient substrate for COX-2 [7, 8]. The reaction of 5*S*-HETE with COX-2 generates an unstable di-endoperoxide, an intermediate that rearranges into the biologically active hemiketal eicosanoids, HKE_2, and HKD_2 [9]. Their effect on angiogenesis, including the promotion of endothelial cell migration and tubulogenesis [6], could have importance in IBD since the gut microvascular endothelium is a critical element in the onset and perpetuation of inflammation [37]. Thus, inhibition of HKE_2 and HKD_2 biosynthesis might be an attractive therapeutic antiangiogenic strategy by targeting neovascularization in IBD. Our results showed that free urolithins (Uro-A, IsoUro-A, and Uro-C), at the concentrations investigated, reduced the formation of HKE_2 and HKD_2. These effects were consistent with the effect of Uro-A and IsoUro-A on PGE_2 (COX-2 product), and Uro-C on 5-HETE and LTB_4 (5-LOX products). Uro-A and IsoUro-A reduced the formation of HKE_2 and HKD_2 (and PGE_2) via down-regulation of COX-2 expression. The effect of Uro-A is in agreement with previous *in vitro* and animal studies that reported an anti-inflammatory effect via reduction of PGE_2 levels together with a downregulation of COX-2 in colonic myofibroblasts and DSS-colitis animal model in F344 rats [22, 24]. Our data show the potential anti-inflammatory effect of the Uro-A isomer, IsoUro-A, that is specific for individuals belonging to the so-called “urolithin metabotype (UM)-B” (human subjects who produce Uro-A, IsoUro-A, and Uro-B as final urolithins) [38].

The effect of Uro-C on formation of HKs as well as 5-HETE and LTB_4 most likely occurred through modulation of the 5-LOX activity, not the expression level of the enzyme. Uro-C decreased HK formation together with the 5-LOX products while sparing the level of the COX-2 metabolite PGE_2. This result is comparable to that exerted on cancer cell models [39] and leukocytes [25]by the specific 5-LOX competitive inhibitor AA861, which blocked 5-LOX metabolites and HK biosynthesis without affecting PGE_2 levels. This possible mechanism needs confirmation in future studies by incubation of the purified 5-LOX enzyme with Uro-C and specific inhibitors.

The structural differences between Uro-A, IsoUro-A, and Uro-C may help understand (at least partially) their different effects observed. Previous studies have described two hypotheses regarding the structure-activity of urolithins: i) their activity is related to the -OH group at C8 [40–42] or ii) to the number of -OH groups present in the molecule [43]. The effect of Uro-A on COX-2 pathway aligns with the first hypothesis, while the activity showed by Uro-C on 5-LOX pathway is in agreement with the second. Notably, these

hypotheses fail to explain the effect of IsoUro-A on COX-2 pathway, what opens the door to investigate in future studies the structure-activity relationship of this molecule.

The intestine is not an isolated system, and IBD is frequently related to extra-intestinal manifestations, including colitis-associated musculoskeletal manifestations and hypertrophic osteoarthropathy [4, 44, 45]. Based on the anti-inflammatory activity reported for circulating phase-II metabolites of urolithins [46], we tested whether conjugated metabolites could modulate the formation of COX-2, 5-LOX, and 5-LOX/COX-2 metabolites. However, the treatment with Uro-A 3-glur, Uro-A 3-sulf, IsoUro-A 3-glur, and Uro-B 3-glur exerted no effect on eicosanoid production. This lack of anti-inflammatory activity was consistent with previous data observed for other activities of urolithins such as antiproliferative, vasorelaxant, and (or) estrogenic, suggesting that phase-II conjugation of urolithins dampens their biological activity *in vivo* and *in vitro* [47–49]. Although free urolithins are hardly detected in the bloodstream, a recent *in vivo* study has shown deconjugation of Uro-A glucuronide to Uro-A in a systemic LPS-induced inflammation rat model, suggesting presence of free Uro-A in microenvironments subjected to inflammatory stimuli [50]. Nevertheless, the capacity of leukocytes to hydrolyze urolithins has not been investigated, justifying future studies on the metabolism of urolithins by human leukocytes.

Clinical management of IBD using single 5-LOX or COX-2 inhibitors (i.e., analgesic and anti-inflammatory drugs) is inefficient [51–53]. An explanation for this failure resides on the possible shunting of the common substrate AA between 5-LOX and COX-2 when one pathway is inhibited [14]. Simultaneous inhibition of both enzymes has been proposed as an effective treatment in the prevention of intestinal inflammation and cancer [39, 54]. The dissimilar effects of Uro-A and IsoUro-A compared to Uro-C on 5-LOX and COX-2 offer an interesting strategy for dual inhibition of both enzymes. This could be relevant in human subjects belonging to the UM-A metabotype (80% Uro-A and 20% Uro-C), and UM-B (50% IsoUro-A, 20% Uro-B, 20% Uro-A, 10% Uro-C) compared to UM-0 (non-producers of urolithins) [18, 48, 49]. To determine whether these metabotypes are relevant in IBD carefully designed *in vitro* and *in vivo* studies are required.

Overall, this study describes, for the first time, the capacity of colonic urolithins (Uro-A, IsoUro-A, and Uro-C) to modulate well-defined branches of eicosanoid biosynthesis (i.e., 5-LOX and COX-2) as well as their cross-over in human leukocytes. We are aware that this model overlooks the interactions between immune and intestinal cells. However, it allows studying biosynthesis in a mixture of cells that can be found in the inflamed mucosa. Besides, this is the best model reported to study HK biosynthesis to date. Uro-A, IsoUro-A, and Uro-C showed the capacity to interfere with the 5-LOX/COX-2 pathway, reducing the formation of the two hemiketal eicosanoids HKE_2 and HKD_2 in a dose-dependent manner. This effect of urolithins (or any other phenolic compound) on the 5-LOX/COX-2 pathway is unprecedented. HK eicosanoids are novel mediators of inflammation that could be physiologically relevant in IBD (their specific role is yet to be determined), and will undoubtedly broaden our understanding of the mechanisms by which urolithins exert their anti-inflammatory effects, at the intestinal level, associated with the consumption of ETs-rich foodstuff.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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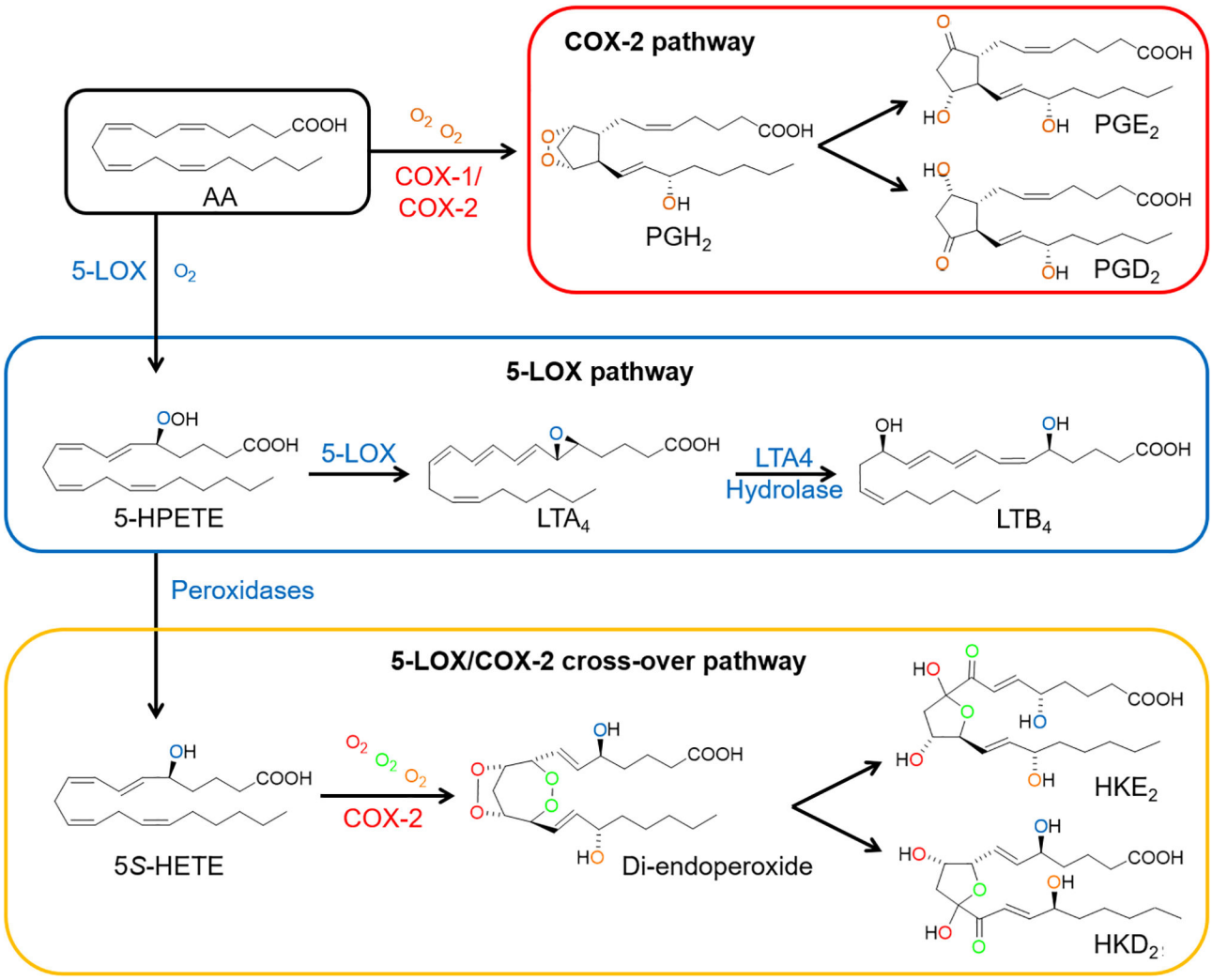

Figure 1.

Key reactions in the biosynthesis of eicosanoids by 5-lipoxygenase (5-LOX) and cyclooxygenase-2 (COX-2). Arachidonic acid (AA) is the common substrate for the synthesis of PGs (COX-2 pathway) and LTs (5-LOX pathway). In the 5-LOX/COX-2 crossover pathway, both enzymes act sequentially to synthesize HKE_2 and HKD_2 as final products.

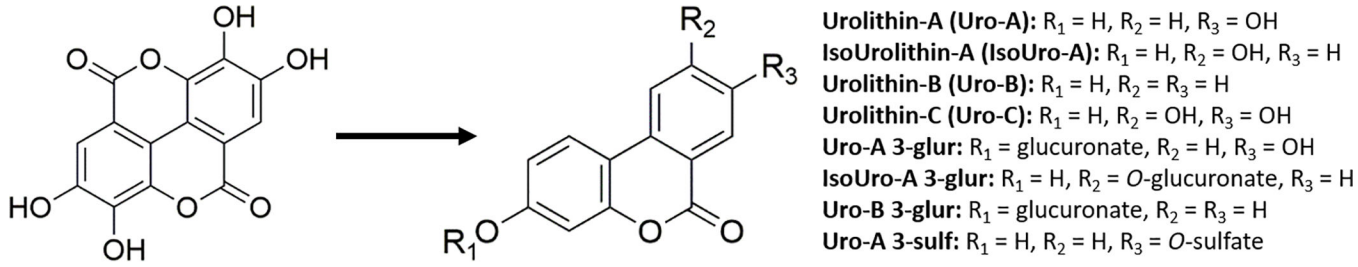

Figure 2.
Chemical structures of ellagic acid and its free and conjugated urolithin metabolites

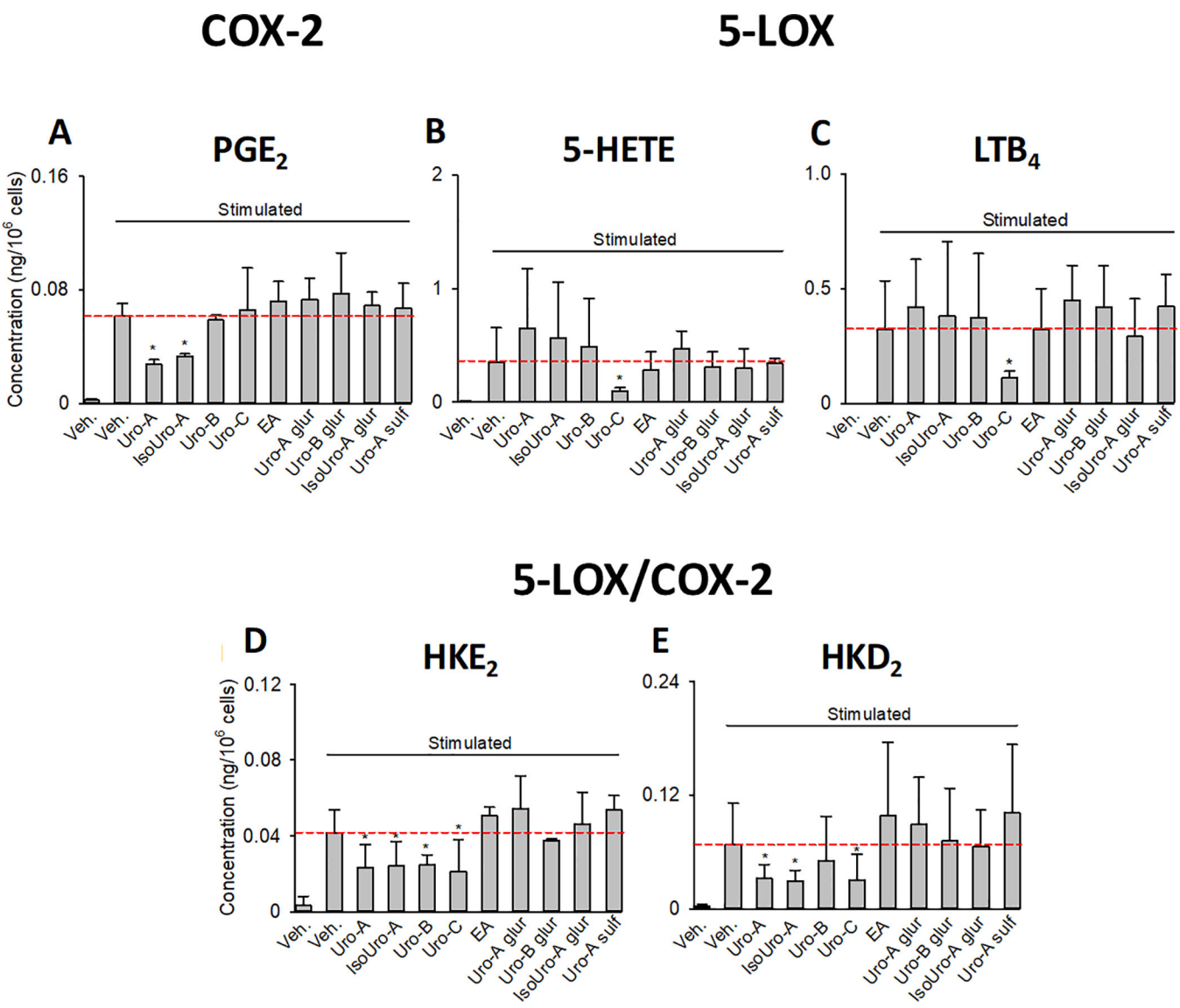

Figure 3.

Effect of EA and urolithins (conjugated and free forms) on eicosanoid biosynthesis in stimulated human leukocytes. The bar charts show the level of PGE_2 (A), 5-HETE (B), LTB_4 (C), HKE_2 (D), and HKD_2 (E) quantified using LC-MS/MS analysis after AMPP-derivatization. The dashed red horizontal line indicates the concentration quantified in stimulated leukocytes. The results are shown as average ± standard deviation (SD) of six volunteers. Each treatment was analyzed in duplicate.

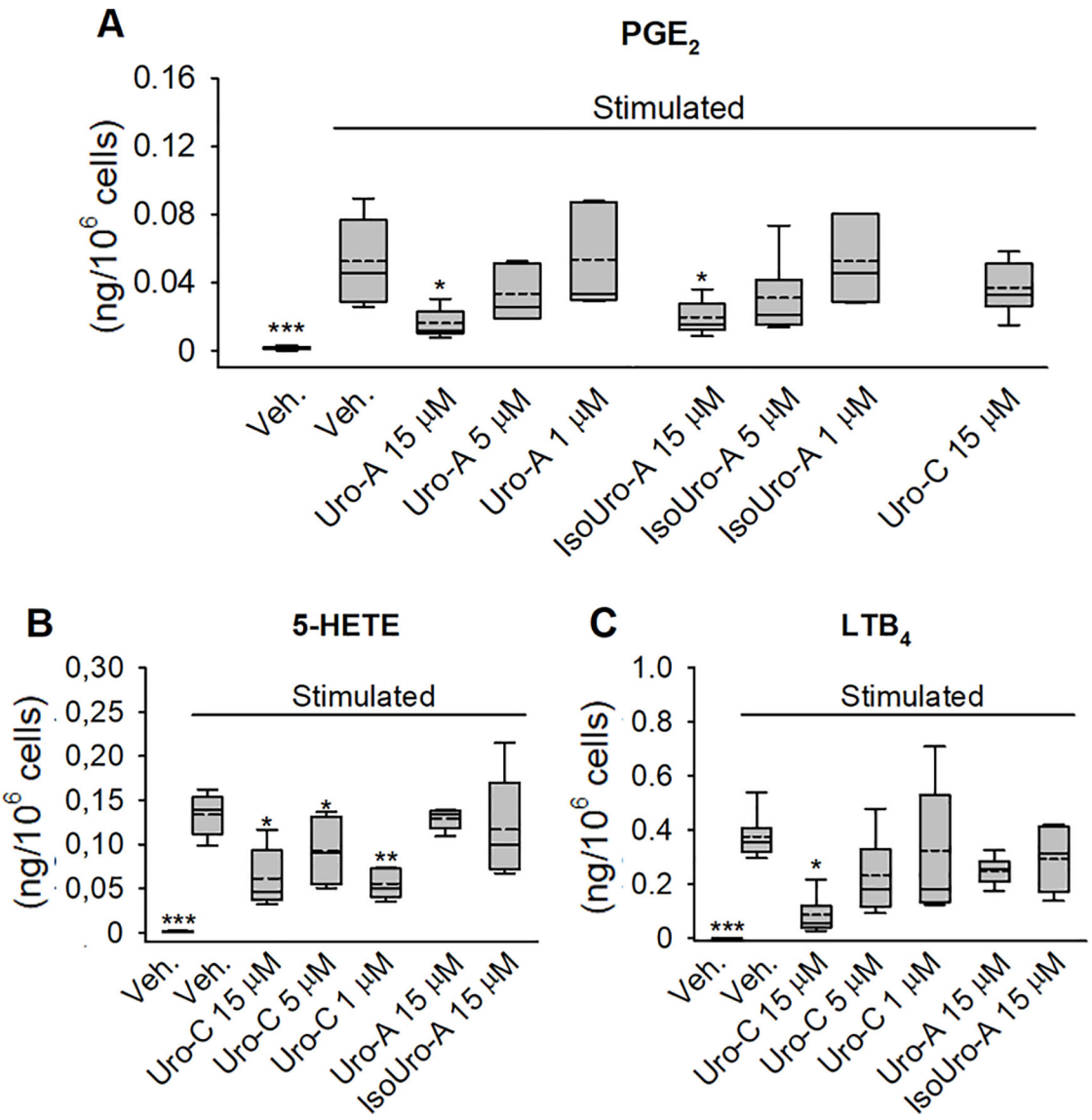

Figure 4.

Dose-dependent effect of Uro-A, IsoUro-A, and Uro-C on COX-2 and 5-LOX product formation in stimulated human leukocytes. PGE_2 (A), 5-HETE (B), and LTB_4 (C) were quantified using LC-MS/MS analysis after AMPP derivatization. The effect of Uro-C on PGE_2 as well as Uro-A and IsoUro-A on 5-HETE and LTB_4 were included as controls. The box plots show the range of data between the 25th and 75th percentiles. Veh., vehicle. Solid horizontal line within bars: median. Dash horizontal line: mean. from 6 volunteers (n = 6). Each treatment was analyzed in duplicate. *p<0.05, **p<0.01, ***p<0.001 compared to the stimulated/veh.-treated group.

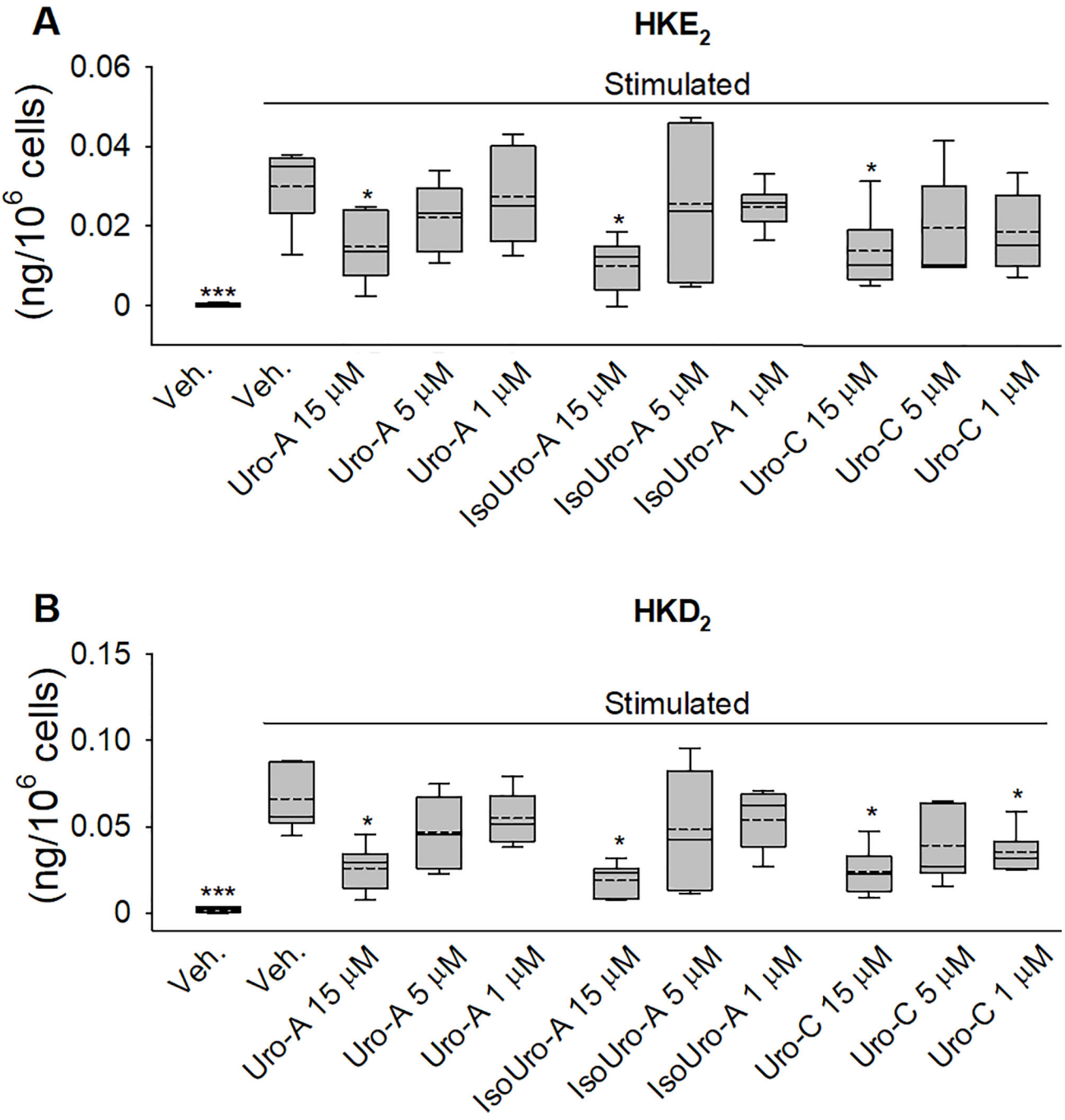

Figure 5.

Dose-dependent effect of Uro-A, IsoUro-A, and Uro-C on hemiketal (HK) formation by the 5-LOX/COX-2 cross-over pathway in stimulated human leukocytes. HKE_2 (A) and HKD_2 (B) were quantified using LC-MS/MS analysis after AMPP-derivatization. The box plots show the range of data between the 25th and 75th percentiles. Veh., vehicle. Solid horizontal line within bars: median. Dash horizontal line: mean. (SD) from 6 volunteers. Each treatment was analyzed in duplicate. *p<0.05, ***p<0.001 compared to the stimulated/veh.-treated group.

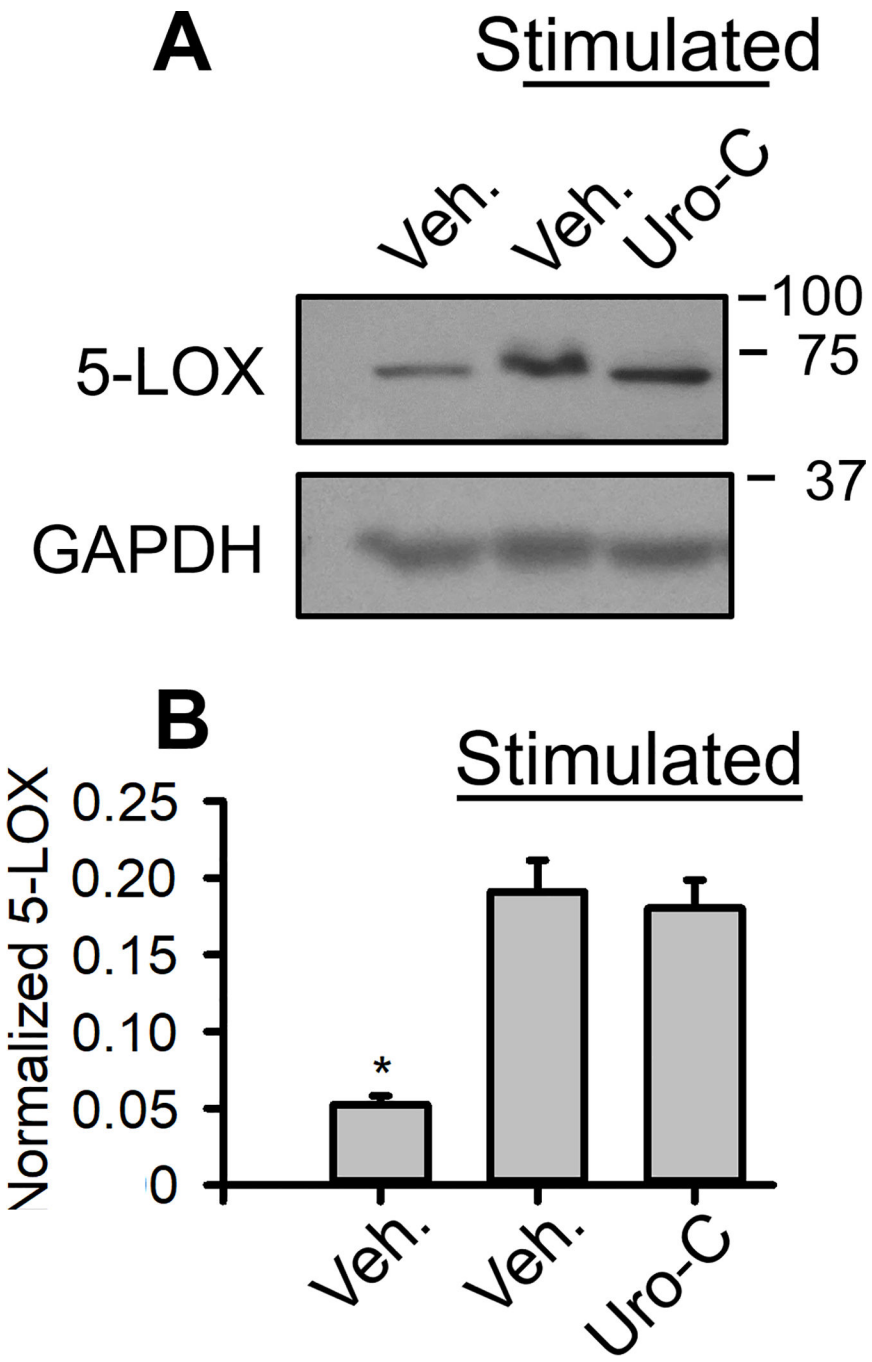

Figure 6.

Western blot analysis of 5-LOX expression in human leukocytes. 5-LOX expression levels were quantified by densitometry analysis using ImageJ software. Veh.: vehicle. The experiment was repeated in leukocytes from 6 volunteers. *p<0.05, compared to the stimulated/veh.-treated group.

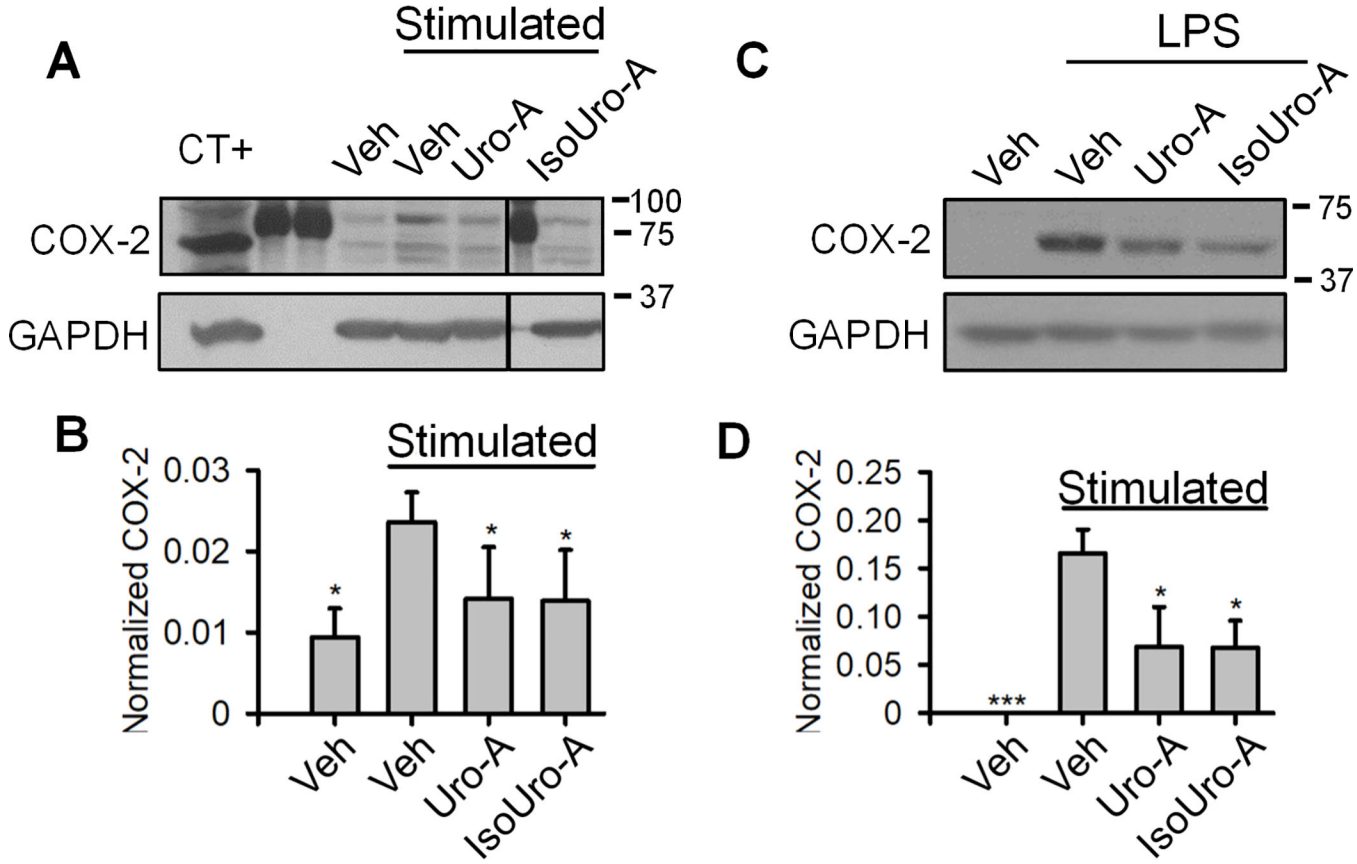

Figure 7.

Western blot analysis of COX-2 expression in stimulated human leukocytes (A) and murine RAW264.7 macrophages (C). The COX-2 expression levels in leukocytes (B) and macrophages (D) were quantified by densitometry analysis using ImageJ software. The lanes 2, 3, and 7 in (A) were loaded with protein molecular weight markers. The size of each marker (kDa) is given at the right of the figure. Veh.: vehicle. CT+: cell lysate obtained from LPS-treated RAW264.7 cells used as positive control. The vertical line in (A) indicates non-consecutive lanes in the same membrane. The experiment was performed using leukocytes from 6 volunteers. In RAW264.7 cells, the assay was repeated 3 times. *p<0.05 compared to the stimulated/veh.-treated group.