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Running title: Urolithins attenuate lipid accumulation

Abbreviations: AMPK, AMP-activated protein kinase; **ATGL**, Adipose triglyceride lipase; **C/EBPα**, CCAAT/enhancer binding protein α; **EA**, ellagic acid; **ET**, ellagitannin; **FA**, fatty acid; **Fas**, fatty acid synthase; ***h*ASCs**, human adipose-derived stem cells; **OA**, oleic acid; **PPARγ**, peroxisome proliferator-activated receptor gamma; **SCD1**, Stearoyl-CoA desaturase-1; **TG**, triglyceride; **Uro**, urolithins

Key words: ellagic acid, urolithins, adipogenesis, lipogenesis, AMP-activated protein kinase, obesity

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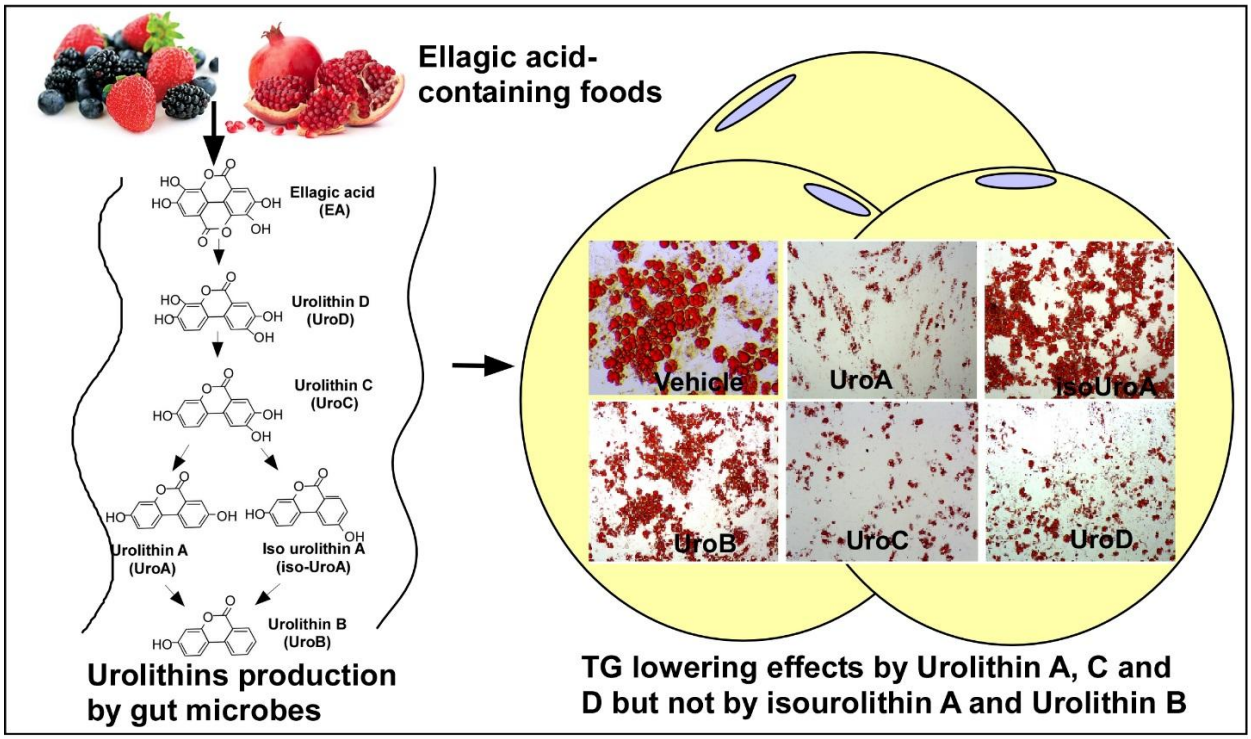
ABSTRACT

Scope: Urolithins (Uro) are ellagic acid (EA)-derived metabolites produced by gut microbes. There is a growing interest in the biological activities of Uro. Our aim was to evaluate the impacts of Uro on regulating triglyceride (TG) accumulation using cultures of primary human adipocytes and hepatoma Huh7 cells.

Methods and Results: UroA, B, C, D, and iso-UroA, were used to determine the effect of Uro on adipogenesis and lipogenesis. Individual Uro (30 μM) were added to human adipogenic stem cells (*h*ASCs) during differentiation. UroA, C and D, but not iso-UroA and UroB, significantly inhibited new fat cell formation by decreasing TG accumulation and adipogenic protein and gene expressions. The regulation of TG synthesis by Uro was investigated via metabolic chasing with radiolabeled precursors. UroA, C, and D attenuated TG accumulation, while increasing the FA oxidation in adipocytes and hepatoma Huh7 cells. Furthermore, UroC, D and A promoted the phosphorylation of AMP-activated protein kinase (AMPK), suggesting that Uro may alter energy-sensing metabolic pathways in primary human adipocytes.

Conclusions: Taken together, our results demonstrated that UroA, C, and D, but not isoUroA and UroB, reduce TG accumulation and increase FA oxidation in adipocytes as well as hepatocytes.

GRAPHICAL ABSTRACT



Ellagic acid is a polyphenolic compound ubiquitously founds in berries and pomegranate. Ellagic is metabolized into urolithins (Uro) by gut microbes. In this study, we investigated the impact of different species of Uro on lipid metabolism by incubating the individual urolithins in human cultures of adipocytes and hepatocytes. We identified that UroA, B and D, but not isoUroA and UroB, exert distinctive lipid-lowering effects by inhibiting adipocyte differentiation, lipogenesis and by promoting beta-oxidation.

1 Introduction

Ellagitannins (ETs) and ellagic acid (EA) derivatives are naturally occurring polyphenols found in pomegranate, berries, and nuts. It has been reported that EA exerts various health benefits including free radical scavenger activity and anti-proliferative effects in various types of cancer *in vivo* and *in vitro* [1-3]. There is a substantial body of evidence that supplementation with pure EA alone or consumption of EA-enriched fruits and nuts attenuates body fat mass and liver lipids [4-6], suggesting that EA possesses lipid-lowering characteristics. Supporting this, our group has recently reported that EA reduces

adipogenesis through the inhibition of co-activator arginine methyltransferase 1 (CARM1) in primary human adipogenic stem cells (*h*ASCs) [7], alters lipid mechanisms both in human adipocytes and hepatocytes [8], and normalizes HF-diet mediated obesity and metabolic dysfunction [6, 8].

Likewise to many other health-promoting polyphenolic compounds, low bioavailability of EA remains paradoxical. Oral administration of pure EA or EA-containing products in both rodents and humans showed that ~ 1 μM of EA can be found in plasma or tissues [9, 10]. This is attributed to the fact that EA undergoes extensive metabolic transformation prior to absorption [11]. In the intestinal lumen, EA is extensively metabolized by gut microbes producing a series of metabolites called urolithins (Uro). Uro are characterized by a common 6H-dibenzo[*b,d*]-pyran-6-one nucleus and a decreasing number of phenolic hydroxyl groups (UroD →UroC →UroA or iso-UroA→ UroB) (Fig. 1A) [12]. Among Uro species, UroA is the major metabolite observed in humans while Iso-UroA and UroB conjugates are also observed in some, but not all, humans [12, 13]. As the result of microbial actions, EA is converted into bioavailable Uro which can reach significant concentrations in plasma and tissues [12, 14-16].

Recently, several studies have demonstrated that Uro have metabolic characteristics of EA showing anti-inflammatory [17], anti-cancer [18, 19], anti-glycative [20] and anti-oxidant [21] properties. However, it is largely unknown if Uro have TG-lowering effects by altering lipid metabolism. To address this issue, we investigated the effects of individual Uro on lipid metabolism in human adipocytes and confirmed augmented fatty acid (FA) oxidation in human hepatoma Huh7 cells. Here, we are the first to report that Uro (30 μM)

possess biological activities to downregulate adipogenesis and lipogenesis similar to EA in human adipocytes. We also demonstrated that Uro alter lipid metabolism via AMPK activation.

2 Materials and methods

2.1 Chemical reagents

All cell culture supplies were purchased from Fisher Scientific. Fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Cellgro Mediatech, Inc. (Herndon, VA). Rosiglitazone (BRL49653) was purchased from Cayman Chemical (Ann Arbor, MI). EA was purchased from Sigma-Aldrich (St. Louis, MO). UroA, B, C, D and iso-UroA were obtained as described elsewhere [22]. All other chemicals and reagents were purchased from Sigma Chemical Co (St. Louis, MO), unless otherwise stated.

2.2 Cell culture

All protocols and procedures were approved by the Institutional Review Board at the University of Nebraska-Lincoln. For isolation of human adipogenic stem cells (*h*ASCs), abdominal adipose tissue was obtained from females with a body mass index (BMI) of ~30 during liposuction or abdominal plastic surgeries. Isolation of *h*ASCs and differentiation of adipocytes was conducted as we described previously [7, 8]. Each independent experiment was repeated at least three times using a pool of *h*ASCs from three or four human subjects to avoid individual variation. Huh7 cells were maintained in Dulbecco's modification of

Eagle's medium (DMEM) containing 1% L-glutamine, 10% fetal bovine serum, 100 units/ml penicillin, 100 g/ml streptomycin in 5% CO2 at 37°C. The medium was changed every 3 days.

2.3 Cell viability assay

The cytotoxic effects of Uro and EA were determined using the XTT Cell Viability Kit (Cell Signaling Technology, Danvers, MA) according to the manufacturer's protocol. Briefly, undifferentiated *h*ASCs, fully differentiated *h*ASCs, and Huh7 cells were cultured in 96-well plates using a seeding density of ~20,000 cells per well. Cells were incubated with either dimethyl sulfoxide (DMSO) or increasing concentrations of Uro and EA for 24 hours (Fig. 2, 5). Culture medium was then replaced with fresh medium containing XTT solution for 3 hours at 37℃ before measurement of OD 450 nm using a Synergy™ H1 hybrid plate reader (BioTek, Winooski, VT).

2.4 Lipid accumulation

To measure the lipid accumulation in human adipocytes, cells were fixed with 10% formalin and stained with Oil Red O (ORO). Bright field images were taken by EVOS® XL microscope (AMG), or ORO dye was extracted by isopropanol to quantify relative TG accumulation (at OD 500 nm).

2.5 [^{14}C]-oleic acid (OA) and [^{3}H]-acetate incorporation into FA and TG

The measurement of TG esterification rate and *de novo* lipid synthesis in cultures of mature adipocytes or human hepatoma Huh7 cells was conducted as described previously

[8]. Briefly, cells were incubated with serum-free low glucose (1,000 mg/L d-(+)-glucose) overnight before the experiment. [^{14}C]-OA (final concentration of 0.5 µCi/mL, Perkin Elmer) and [^{3}H]-acetate (final concentration of 0.5 µCi/mL, Perkin Elmer) were mixed with conditioned media or sodium oleate (Sigma)-Bovine Serum Albumin (BSA, fatty acid free, Sigma) complex, then added to cells for 3 hours. After 3 hours incubation with [^{14}C]-OA and [^{3}H]-acetate, medium (containing unincorporated isotope) was removed by washing with phosphate-buffered saline (PBS). Cellular lipids were extracted using the Bligh and Dyer method [23]. Next, thin layer chromatography was performed to fractionate FA and TG, and the [^{14}C] and [^{3}H] radioactivity was measured by liquid scintillation counting (Tri-Carb 2000TR, Packard). Radioactivity was normalized by protein concentration quantified by bicinchoninic acid (BCA) colorimetric assay (Pierce, Rockford, IL).

2.6 Fatty acid oxidation rate using [^{3}H]-OA

To measure the FA oxidation rate in cultures of mature adipocytes or human hepatoma Huh7 cells, we followed previously published methods [24]. Briefly, cells were incubated with serum-free low glucose (1,000 mg/L d-(+)-glucose) before the experiment. [^{3}H]-OA (final concentration of 0.5 µCi/mL, Perkin Elmer) was mixed with sodium oleate-BSA complex, and then added to cells for 2 hours. [^{3}H] radioactive containing medium was harvested, and precipitated using trichloroacetic acid (TCA) solution. After precipitation, 6N NaOH was added to reach a final concentration of 0.8-1.0N, resulting in an alkaline supernatant. Next, supernatant was put through columns filled with Dowex™ ion-exchange resin (ACROS Organics™) to capture [^{3}H]-H_2O. Finally, radioactivity was measured by liquid scintillation counting.

2.7 qPCR

Gene-specific primers for qPCR were obtained from Integrated DNA Technologies (Chicago, IL). Total RNA was isolated using TRIzol® reagent (Invitrogen). To remove any potential genomic DNA contamination, mRNA was treated with DNase (Mediatech), and 2 μg of mRNA was converted into cDNA in a total volume of 20 μl (iScript™ cDNA Synthesis Kit, Bio-Rad). Gene expression was determined by real-time qPCR (ABI7300, Applied Biosystems), and relative gene expression was normalized by 36B4 (primer sequences available in Supplemental Table 1).

2.8 Western blot analysis

To prepare total cell lysates, monolayers of *h*ASC cultures and fully differentiated adipocytes were scraped with ice cold radioimmune precipitation assay (RIPA) buffer (Thermo Scientific) containing protease inhibitors (Sigma) and phosphatase inhibitors (2 mM Na_3VO_4, 20 mM β-glycerophosphate and 10 mM NaF). Proteins were fractionated using 8 or 10% SDS-PAGE, transferred to PVDF membranes, and incubated with the relevant antibodies. Chemiluminescence from ECL (Western Lightning) solution was detected using a FluorChem E Imaging System (Cell Biosciences). Polyclonal or monoclonal antibodies targeting phospho- AMP-activated protein kinase (AMPK) (Ser473, #4060), total AMPK (#9279), CCAAT/enhancer binding protein α (C/EBPα) (#2295), fatty acid synthase (Fas) (#3180), and β-actin (#4967) were purchased from Cell Signaling Technology. The mouse monoclonal antibodies for fatty acid binding protein 4 (aP2) (sc-271529) and PPARγ (sc-7273) were purchased from Santa Cruz Biotechnology (Dallas, TX).

2.9 Statistical analysis

Results are presented as means ± SEM. The data were statistically analyzed using one-way ANOVA with Tukey's multiple comparison tests. All analyses were performed with GraphPad Prism 5 (Version 5.04).

3 Results

3.1 Urolithins do not affect the viability of primary human adipogenic stem cells (*h*ASCs) at concentrations of 0-30 μM

Currently, it is unknown whether Uro affect the cell viability of human adipocytes. To address this question, cytotoxic effects of individual Uro were determined both in undifferentiated *h*ASCs and fully differentiated adipocytes. UroA, B, C, D, iso-UroA and EA were incubated with either *h*ASCs or mature adipocytes for 24 hours before XTT assay. Similar to EA, Uro treatment slightly affected cell bioavailability (~80%) but no specific cytotoxic effects were observed in up to 30 μM concentrations (Fig. 1B-G). Based on these results, for the remaining experiments we used the 30 μM of Uro in order to augment the potential action but without causing cellular damage.

3.2 Urolithins inhibit adipogenesis in *h*ASCs

We previously demonstrated that EA suppressed adipogenic differentiation in *h*ASCs [7, 8]. To determine whether Uro were able to inhibit adipogenesis, 30 μM individual Uro was added to *h*ASCs during differentiation (Fig. 2A). The presence of Uro, but not iso-UroA,

caused a significant reduction of TG accumulation measured by ORO staining (Fig. 2A, B). The rank order of anti-adipogenic potential was UroA, C, D >> UroB > iso-UroA as assessed by TG accumulation using ORO staining. To further confirm the anti-adipogenic effects of Uro, adipogenic gene and protein expression levels were determined by qPCR and Western blot. Consistent with reduced TG accumulation, Uro treatment, except for iso-UroA, significantly suppressed adipogenic gene expression including PPARγ and Fas (Fig. 2C). Adipogenic protein expression including aP2, Fas, PPARγ, and C/EBPα were also dramatically reduced in cultures treated with UroC and D and to a lesser magnitude with UroA (Fig. 2D). To gain insight into whether Uro alter epigenetic marks similar to their parent compound EA [7], histone 3 arginine 17 methylation (H3R17me2), and histone acetylation (AcH3) levels were examined. Interestingly, only UroC incubation, but no other Uro treatment, markedly suppressed the epigenetic markers (Supplemental Fig. 1).

Several phytochemicals are known to inhibit adipogenesis through the mechanism related to AMPK activation [25-28]. AMPK is a major energy-sensor triggering a variety of catabolic processes and suppressing anabolic pathways simultaneously [29]. Treatment with EA increased AMPK phosphorylation dose-dependently (Fig. 2E). To further identify whether Uro are also able to regulate AMPK activation, we examined phosphorylated and total AMPK levels. Interestingly, UroA, C, and D increased phosphorylated AMPK levels (Fig. 2F), which is consistent with the trend of the anti-adipogenic role of Uro.

3.3 Urolithins attenuate lipid accumulation in cultures of human adipocytes

Next, we asked whether Uro could antagonize adipocyte hypertrophy. To examine this, fully differentiated cultures of human adipocytes were exposed to Uro for seven days

based on the experimental design (Fig. 3A, upper). Exposure to 30 μM Uro for seven days caused a significant reduction of TG accumulation, but not UroB and iso-UroA, as measured by ORO staining (Fig. 3A, below). To test whether the reduction of TG accumulation was aligned with lipogenic transcriptional inhibition, we measured mRNA expressions. Uro treatment (A, C, and D) decreased lipogenic-specific gene expression including PPARγ, Fas, adipose triglyceride lipase (ATGL) and steroyl-CoA desaturase-1 (SCD-1) compared to vehicle control (Fig. 3B). UroC and D are potent EA-derived metabolites in upregulating AMPK activation in fully differentiated adipocytes (Fig. 3C).

3.4 Urolithins regulate lipid metabolisms in cultures of human adipocytes

We then investigated whether lipid-lowering effects of Uro are due to an alteration of lipogenic pathways. Fully differentiated adipocytes were incubated with radioactive precursors of [^{3}H]-acetate and [^{14}C]-OA to measure their conversion into [^{3}H]-TG (*de novo* synthesis of TG) and [^{14}C]-TG (esterification of TG). The conversion of [^{3}H]-acetate into [^{3}H]-TG was significantly dampened in UroA and UroD treated adipocytes (Fig. 4A). Consistently, UroA and D incubation was associated with a significant decrease of conversion of [^{14}C]-OA into [^{14}C]-TG, indicating a decrease of TG esterification by UroA and D (Fig. 4B). To determine whether FA oxidation is involved in lipid-lowering effects of Uro, we measured FA oxidation rate in Uro-treated adipocytes. FA oxidation by determining [^{3}H]-H_2O release from [^{3}H]-OA clearly showed that UroA, C, and D markedly increased FA oxidation, but not in iso-UroA and UroB treated adipocytes (Fig. 4C). Taken together, these data implicate that the inhibition of *de novo* synthesis of TG and FA esterification are accompanied by transcriptional regulation of lipogenic gene expression in Uro-treated human adipocytes.

3.5 Urolithins regulate lipid metabolism in Huh7 cells

We further examined the effects of Uro on TG esterification, *de novo* synthesis and FA oxidation in human hepatoma Huh7 cells. Prior to analysis of lipid metabolism, we first measured the cytotoxic effects of individual Uro in Huh7 cells. The concentrations (0-30 μM) of UroA, B, C, D, iso-UroA and EA treatment slightly affected cell bioavailability (~80%) but no specific cytotoxic effects were observed in up to 30 μM concentrations (Fig. 5A). Next, to test whether Uro alter the lipogenic pathways, radiolabeled precursors were incubated with Huh7 cells, as with human adipocytes. Incorporation of [^{3}H]-acetate into [^{3}H]-FA and [^{3}H]-TG were markedly decreased with UroC and D (Fig. 5B and C). Conversion of [^{14}C]-OA into [^{14}C]-TG was clearly inhibited by UroC treatment (Fig. 5D). FA oxidation rate was increased by UroA and C treated Huh7 cells, which was confirmed by [^{3}H]-H_2O release from [^{3}H]-OA (Fig. 5E).

4 Discussion

Uro are gut microbiota-derived metabolites of EA and ETs. Different types of Uro (Fig. 1A) are produced by intestinal microbes, which may reflect the metabolic health status of hosts [13]. Although it has long been known that Uro may be critical determinants of the effectiveness of EA supplementation, it is largely unknown whether different species of Uro possess different biological activities in humans. Recently, a number of studies have reported the unique role of Uro species by evaluating their efficacy in various cancer [18, 30], and inflammatory diseases [17, 31]. One recent study [13] and other unpublished

observation correlate a higher prevalence of the metabotype producing iso-UroA and UroB in overweight and obese individuals, while UroA is associated with healthy individuals. However, no studies have been conducted to determine the function of individual Uro in human obesity. The present study was specifically designed to assess the role of individual Uro species in manipulating lipid metabolism using primary human adipocytes. Here we demonstrated that UroA, C and D are effective in attenuating TG accumulation and increasing FA oxidation through AMPK-dependent mechanisms in primary human adipocytes as well as in Huh7 cells. These results may provide a novel insight into a 'structure and function' relationship among Uro, which may lead to a unique therapeutic design to control adiposity.

An accumulating body of evidence suggests that the gut microbial community affects the host's energy homeostasis by altering energy metabolism [32-34]. To corroborate this concept, production of different Uro may comprise a part of the mechanism in which gut microbes regulate the host's energy metabolism. In other words, metabolically healthy subjects may possess microbiota that are able to generate mainly active Uro such as UroA (i.e. subjects belonging to the so-called 'metabotype A' [13]). In contrast, metabolically unhealthy humans may have bacterial communities producing UroA but also other less active urolithins such as iso-UroA and UroB (i.e., subjects with 'metabotype B' [13]). García-Villalba et al. reported that there were compositional differences in the gut microbiome between human subjects who produce UroA (effective Uro) and who produce iso-UroA and B (less active Uro) [11, 35]. Also, subjects who have a higher risk of chronic illness produce iso-UroA and UroB [13], the two inactive EA metabolites in our experimental setting (Fig. 2-4). Recently, *Gordonibacter urolithinfaciens* sp. nov. has been identified as a novel bacterial

species responsible for converting EA into UroM5 and UroC [36]. This bacterium belongs to the family *Coriobacteriaceae* a family that is associated with benefits in obesity [37]. Since the bacterial phylum that specifically transform EA or its intermediate metabolites into UroA vs. iso-UroA/UroB have not yet been identified, it could be of interest for future studies.

Numerous studies in animals and humans have demonstrated that free EA can be found in no more than 100 nM concentrations after oral administration of EA or ET-containing foods [9, 10]. The low bioavailability of EA is largely due to limited solubility and its rapid metabolism into Uro by the gut microbiome [10]. As a metabolic consequence, Uro can reach relevant concentrations in the blood stream and human colon microenvironment [12, 14]. Although Uro might not be accumulated in metabolic tissues, i.e., adipose tissue, liver or muscle, a few reports have shown that Uro are able to circulate enterohepatically [38]. In terms of cytotoxicity, 50-150 μM of Uro have exhibited anti-cancer properties by inhibiting cell proliferation and cell cycle progression in various carcinoma cell lines [18, 39]. In this study, we first assessed the cytotoxicity of 30 μM concentration in *h*ASCs as well as differentiated cultures of adipocytes. However, Uro treatment does not seem to affect cell viability in non-carcinogenic cells [31], implying that Uro might induce apoptosis in malignant tumor cells but not in mitogenically quiescent cells. Consistently, our results showed that 30 μM Uro treatments showed no sign of cytotoxicity in time frames of both early- and terminal-stages of adipogenesis. The anti-adipogenic and anti-lipogenic effects of Uro in our study were not due to the apoptotic property of Uro (Fig. 1B-G). Thus, we regarded 30 μM of Uro as a non-toxic concentration to assess potency of individual Uro in regulating lipid metabolism in human adipocytes. This concentration is selected to be comparable to our previous studies showing that 10 μM of EA treatment lowers TG

deposition in the same cells, i.e., primary human adipocytes and human hepatoma cell lines [8]. Despite that detectable plasma level of Uro in humans is obviously lesser than 30 μM, our assay conditions using 30 μM of Uro is rationed based on the facts that 1) treatment of high dose of polyphenolics are valid when we characterize the biological significance of polyphenolic molecules (such as resveratrol) using in vitro systems, and 2) in the context of urolithins, the reported systemic concentrations of urolithins can reach up to micro molar levels [14]. These concentrations can be much higher in the gut [16] and also in the bile [38]. In our study, 30 μM of Uro treatment in adipocytes was associated with at least three metabolic outcomes: 1) attenuating adipogenesis, 2) inhibiting TG accumulation, and 3) increasing AMPK activation.

The major distinctive metabolic consequences that we immediately noticed was that Uro differentially impacted adipogenesis in *h*ASCs. UroA, C and D treatment, but not iso-UroA or UroB, interfered with adipogenic differentiation (Fig. 2). Recently, estrogen receptor α (ER α) has been proposed as a negative regulator of adipocyte development in preadipocytes [40]. Interestingly, Larrosa et al. showed that UroA possesses higher ER α binding affinity than UroB in breast cancer cells suggesting that UroA plays a role as a phytoestrogen (ER binding affinity of UroA: α > β) [41]. In terms of structural differences, EA, UroA, C and D have common hydroxyl position at number 8 carbon (-OH at C8) compared to iso-UroA or UroB. González-Sarrías et al. also proposed that phase II phenolic enzymatic activities are crucially affected by reactive moieties such as hydroxyl groups [18]. This interpretation aligns with our results showing that the absence of -OH moiety at C8 position of Uro, due to isomerism (iso-UroA) or metabolic loss (UroB) is inversely correlated with the ability to downregulate adipogenic differentiation. Thus, it may also be reasonable

to assume that -OH moiety at C8 position might affect binding affinity to ER. Further studies are necessary to validate the aforementioned structure-function relationship in adipogenic differentiation. The other possible mechanism of Uro's anti-adipogenic effect could be explained by its ability to alter epigenetic marks. We have previously shown that EA modified histone methyltransferase (CARM1) enzyme activities during adipogenesis [7]. Consistently, we were able to observe that UroC downregulated histone arginine 17 methylation during adipogenesis (Supplemental Fig. 1). UroC was also reported to reduce TNFα-induced inflammation through inhibition of histone acetyltransferase (HAT) activity in the monocyte cell [42]. At this point, we speculate UroC may have stronger DNA binding affinity comparable to EA [43, 44] than the other Uro intermediates, conferring ability to edit epigenetic codes such as histone methylation and/or acetylation. Collectively, differential regulation of adipogenesis by Uro likely originates from their structural difference of Uro. Future studies are required to scrutinize the metabolic contribution of C8 hydroxyl moiety of Uro to ER binding affinity, CARM1 activity, and AMPK activation.

Subsequently, we examined the potential role of Uro in regulating lipid metabolism in fully differentiated adipocytes and hepatoma Huh7 cells. Previously, we showed that EA modulates global lipid metabolism by attenuating TG accumulation in both adipocytes and hepatocytes [8]. This TG-lowering effect seems to be conserved in mammalian cells [8] and even in yeasts (data not shown). In this current research setting, we found that Uro effectively reduced *de novo* synthesis and TG esterification and enhanced FA oxidation in adipocytes (Fig. 3, 4). These data revealed that EA apparently gradually lost its ability to downregulate lipid accumulation while EA is successively metabolized by gut microbes until its conversion to the least effective UroB. Therefore, the potency of Uro in regulating TG

metabolism could be ranked as earlier metabolites (UroC and D) > intermediate metabolite (UroA) > later metabolite (UroB). Agreeing to the fact that EA shows a global impact in lowering lipids in various cells, Uro were also effective in attenuating TG accumulation in Huh7 cells (Fig. 5). The simultaneous lipid-lowering action in both adipocytes and hepatocytes is one of the ultimate goals for weight loss as dietary intervention agents. To accomplish this goal, upregulation of FA oxidation is critical; otherwise the failure of FA accumulation in adipose tissue triggers lipotoxicity and hepatic steatosis. It is interesting that UroC was more potent in modulating TG accumulation than UroD in hepatocytes. More noticeably, UroA, C and D significantly elevated FA oxidation ($[^3H]$-OA$\rightarrow[^3H]$-H_2O) in hepatocytes, satisfying the criteria for effective dietary supplementation to mitigate lipid accumulation both in adipocytes and hepatocytes. Further studies should be conducted to determine the efficacy of UroC, D and A *in vivo*.

Lastly, we have found AMPK activation could be a link mediating anti-adipogenic and anti-lipogenic effects of Uro. AMPK is an energy sensor that shuts down anabolic pathways [45]. Phosphorylation of AMPK inhibits transcriptional activation for adipogenesis, TG synthesis, and FA oxidation [46]. Here, we showed EA promotes AMPK activation during adipogenesis of *h*ASC (Fig. 2E); Wang et al. also showed the implication of AMPK activation by EA in 3T3-L1 cells [47]. Consistently, our results showed that upregulation of AMPK phosphorylation by UroA, C, and D attenuated: i) adipogenesis (Fig. 2); ii) *de novo* lipogenesis (gene expressions and metabolic conversion of radiolabeled precursors) and TG esterification (Fig. 3, 4, 5); and iii) FA oxidation (Fig. 4, 5). Consistently, the recent animal study showed that punicalagin (ET)-enriched pomegranate consumption prevents obesity associated-cardiac metabolic disorders through AMPK activation [48]. The regulation of AMPK by the structural

specificity of EA and Uro will be of interest for future study to further elucidate the ultimate role of EA in regulating lipid metabolism.

In our study, we revealed the previously unknown function of Uro, gut metabolites of EA, as potential regulators of lipid metabolism in adipocytes. We also compared whether individual Uro could exert a differential impact on lipogenesis and FA oxidation in adipocytes and hepatocytes. We identified that UroA, C, and D are more plausible to reduce TG accumulation, but not iso-UroA and UroB. Whether the differential impact of Uro in lipid metabolism is dictated by structural differences of Uro, and these initial findings *in vitro* will be confirmed *in vivo*, must await future studies. Using high dose of Uro (30 μM), that may not easily attainable in human physiological conditions, is an obvious limitation of this study. As a seminal investigation, it was inevitable to simulate the chronic dietary responses using the cell-based platform in short term. Studies whether lower-dose Uro with prolonged treatment can recapitulate the current results should be confirmed in future studies. In addition, following studies should define whether the mode of action from the tentative mixtures of Uro (that are pertinent to human physiology) would be additive, synergistic and/or counteractive. Nevertheless, we believe that our study sheds new insight into Uro, gut metabolites of EA, as new dietary strategies to attenuate adiposity.

SUPPORTING INFORMATION

mnfr201500796-sup-0001-Supplemental Fig1.docx

Supplemental Fig. 1. Cultures of *h*ASCs were induced to differentiation (d1) in the presence of either DMSO (vehicle control) or 30 μM Uro for 10 days. Nuclear extracts from Uro

treated *h*ASCs were immunoblotted for histone modification enzyme (H3R17me2, AcH3, and PPARγ).

mnfr201500796-sup-0002-Supplemental Table1.docx

Supplemental Table 1. Primer sequences for qPCR

Gene	Forward/Reverse	Sequence (5'-3')
h36B4	Forward	GAAGGCTGTGGTGCTGATG
	Reverse	GTGAGGTCCTCCTTGGTGAA
hATGL	Forward	CTGACCACCCTCTCCAACAT
	Reverse	ACCAGGTACTGGCAGATGCT
hFas	Forward	GGCAAGCTGAAGGACCTGTCTA
	Reverse	AATCTGGGTTGATGCCTCCGT
hPPARγ	Forward	TGCTGTTATGGGTGAAACTCTG
	Reverse	TCAAAGGAGTGGGAGTGGTC
hSCD1	Forward	GGGTGAGGGCTTCCACAACTA
	Reverse	CGGCCATGCAATCAATGAA

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Author contributions

I.K., and S.C designed and performed experiments, analyzed data and wrote the manuscript. I.K. and Y.K. performed experiments and acquired data. F.T-B., and J.C.E. provided Uro and critically reviewed the manuscript. S.C. is the guarantor of this work and had full access to all the data in the study and takes responsibility of the integrity of the data and accuracy of the analysis. All authors read and approved the final manuscript.

Conflict of interest statement

The authors have declared no conflict of interest.

Figure Legends

Figure 1. Metabolism of EA to produce Uro and the effects of Uro on cell viability in primary human adipocytes.

(A) Microbial enzymatic transformation to produce a series of Uro metabolites from EA. Chemical structures of test compounds are shown. Human culture of undifferentiated *h*ASCs (open circle) and fully-differentiated adipocytes (closed circle) were treated with 0, 5, 10, 30 μM of EA (B) or UroA (C), iso-Uro A (D), UroB (E), UroC (F), and UroD (G) for 24 hours. XTT reagent was added 3 hours before measurement of OD 450nm. Data are expressed as a percentage of the vehicle control (DMSO). Each data point represents the mean ± S.E.M (n=3).

Figure 1

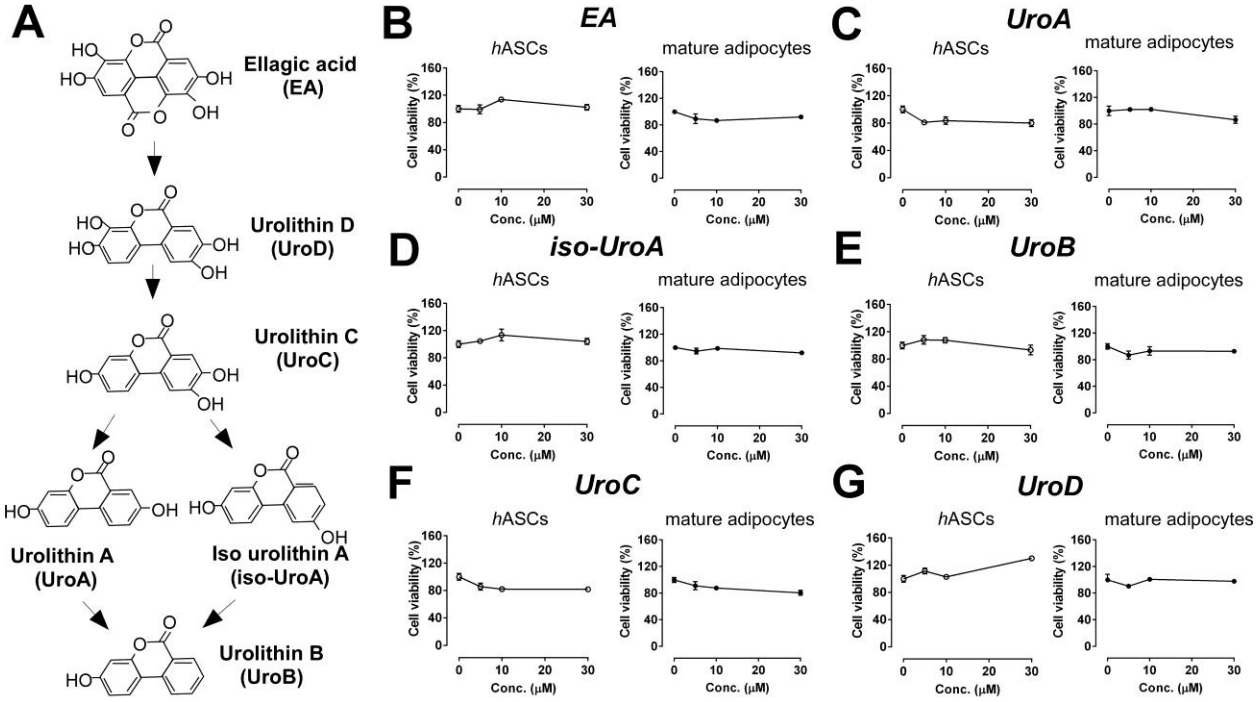

Figure 2. Uro, but not iso-UroA, suppressed adipogenesis in *h*ASCs.

(A, upper) Experimental Scheme: *h*ASCs were seeded the day before differentiation (d0). Cultures of *h*ASCs were induced to differentiation (d1*) in the presence of either DMSO (vehicle control) or 30 μM Uro for 10 days. (A, below) Triglyceride accumulation was visualized by Oil Red O staining and representative images from three separate experiments are shown. (B) Extracted staining was quantified (OD 500 nm) and relative TG accumulations to the DMSO control are shown. (C) Adipogenic gene expression of PPARγ, and Fas by qPCR (n=5). (D) Adipogenic protein expressions of aP2, Fas, PPARγ, and C/EBPα by Western blot analysis. (E) Culture of *h*ASCs was differentiated for 2 days in the presence or absence of 0, 4, and 8 μM EA. Total cell extracts were immunoblotted with phosphor or total antibodies targeting AMPK. (F) Immunoblot of phosphor or total antibodies targeting AMPK in Uro

treated *h*ASCs. All values are presented as the mean ±S.E.M. Bars with different letters are significantly different by one-way ANOVA.

Figure 2

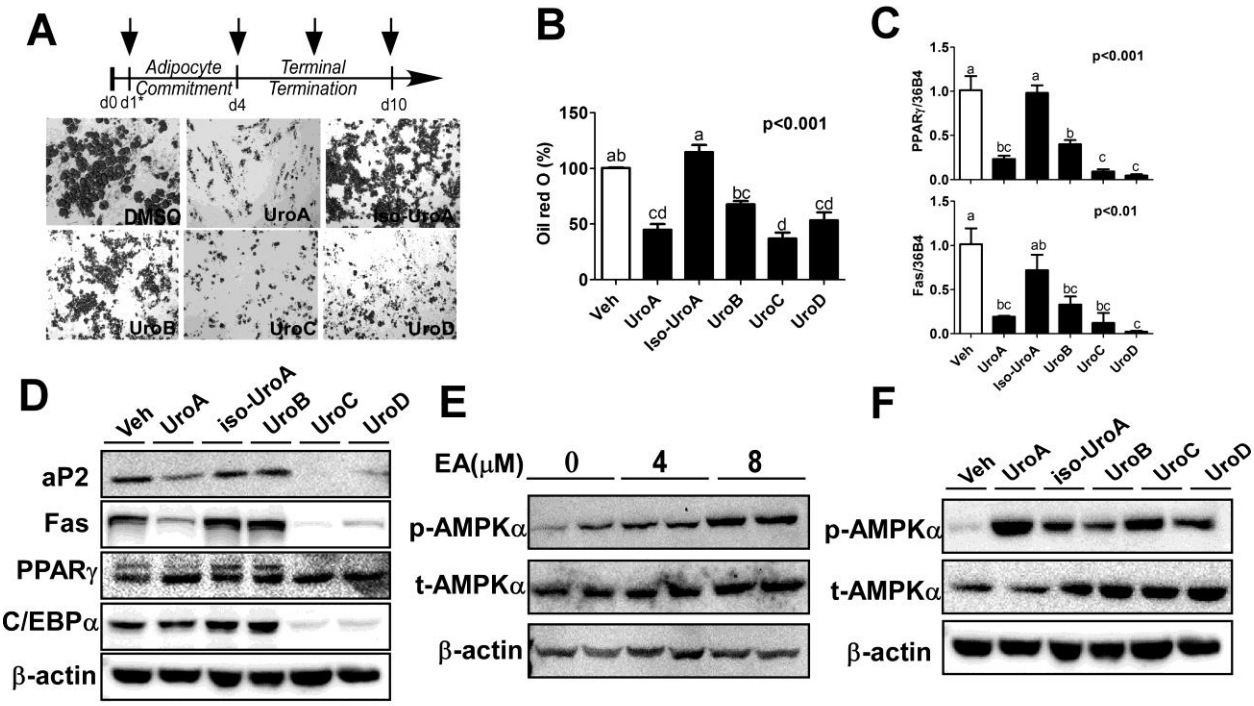

Figure 3. Uro, but not iso-UroA, suppressed lipogenesis in mature human adipocytes.

(A, upper) Experimental Scheme: *h*ASCs were seeded the day before differentiation (d0) and induced to differentiation (d1*). *h*ASCs were kept differentiated into fully differentiated adipocytes until d10. 30 μM Uro was added to the fully differentiated human adipocytes (d10) and incubated for 7 days. (A, below) Relative TG accumulations quantified by Oil Red O. (B) Relative gene expressions levels of PPARγ, Fas, ATGL and SCD-1 by qPCR (n=5). (C) Cultures of fully differentiated adipocytes were treated with 30 μM Uro for 3 days. Total cell extracts were immunoblotted with phosphor or total antibodies targeting AMPK. All values

are presented as the mean ±S.E.M. Bars with different letters are significantly different by one-way ANOVA.

Figure 3

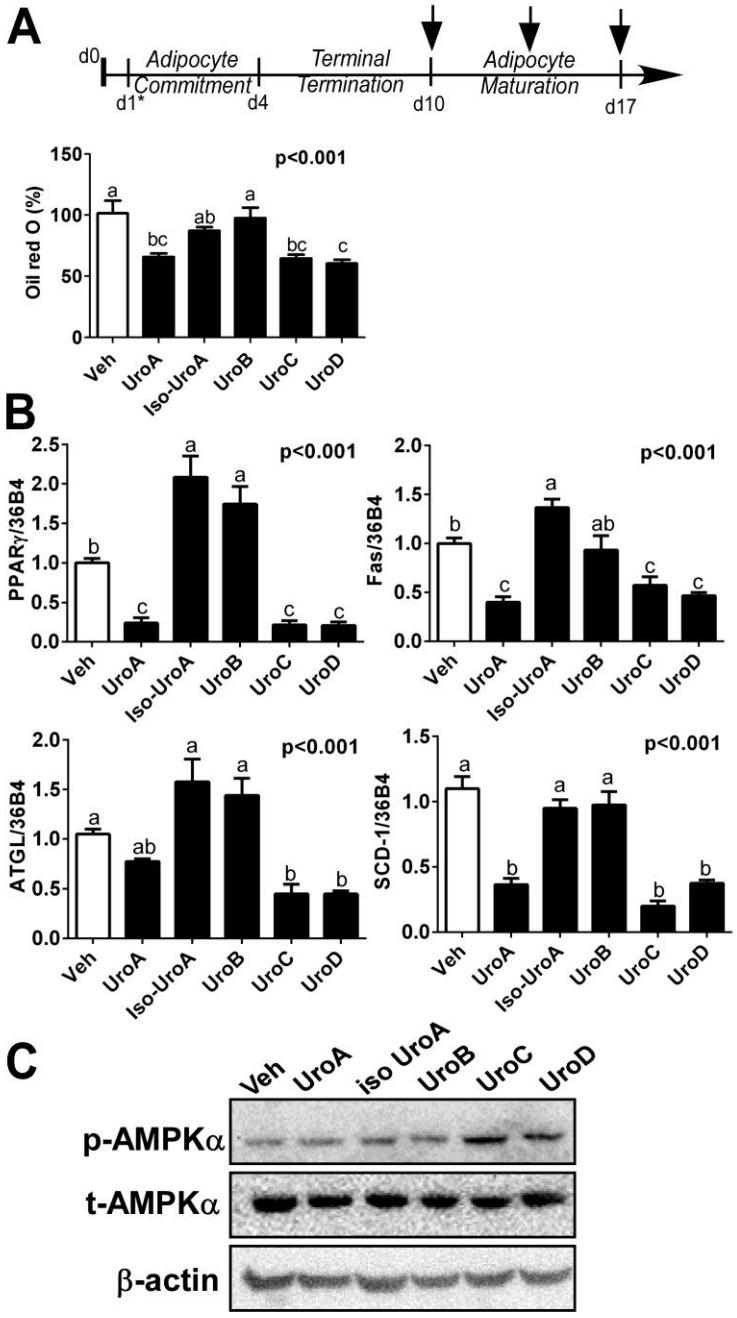

Figure 4. Uro regulated lipid metabolisms in human adipocytes.

Uro (30 μM) was added to the differentiated human adipocytes (d10) and incubated for 3 days.

(A) Conversion of [^{3}H]-acetate into [^{3}H]-TG. (B) Conversion of [^{14}C]-OA into [^{14}C]-TG. (C) [^{3}H]-OA into [^{3}H]-H_2O. All values are presented as the mean ±S.E.M. (n=6/group) Bars with different letters are significantly different by one-way ANOVA.

Figure 4

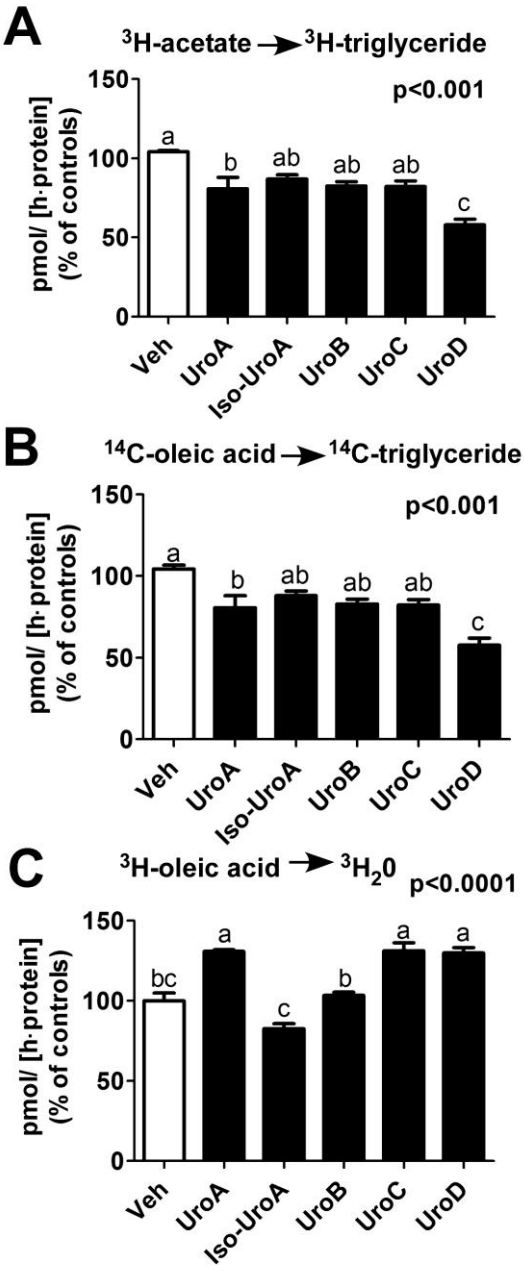

Figure 5. Uro, but not iso-UroA, regulated lipid mechanisms in human hepatoma Huh7 cells.

(A)Cultures of Huh7 cells were treated with either 10 μM EA or 30 μM UroA, iso-Uro A, UroB, UroC, and UroD for 24 hours. XTT reagent was added 3 hours before measurement of OD 450nm. Huh7 cells were pre-incubated with Uro (30 μM) for 48 hours and 0.4 mM BSA-OA complex was loaded for 3 hours with radiolabeled precursors ([^{3}H]-acetate, [^{14}C]-OA, [^{3}H]-OA). (B) Conversion of [^{3}H]-acetate into [^{3}H]-FA. (C) Conversion of [^{3}H]-acetate into [^{3}H]-TG. (D) Conversion of [^{14}C]-OA into [^{14}C]-TG. (E) Conversion of [^{3}H]-OA into [^{3}H]-H_2O. All values are presented as the mean ±S.E.M. (n=6/group for each experiment) Bars with different letters are significantly different by one-way ANOVA.

Figure 5