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Formation of a Vitamin C Conjugate of Acrolein and its Paraoxonase-mediated Conversion into 5,6,7,8-Tetrahydroxy-4-oxooctanal

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Abstract

Vitamin C (ascorbic acid) has been reported to participate in Michael addition reactions in vitro to form vitamin C conjugates with α,β -unsaturated aldehydes, such as acrolein. This study shows evidence for the formation and metabolism of the vitamin C conjugate of acrolein (AscACR) in cultured human monocytic THP-1 cells exposed to acrolein diacetate. By using ^{18}O and ^{13}C labeling in combination with liquid chromatography–tandem mass spectrometry, AscACR was shown to undergo hydrolytic conversion of the ascorbyl lactone into an intermediate carboxylic acid. Subsequent decarboxylation of the carboxylic acid yielded 5,6,7,8-tetrahydroxy-4-oxooctanal (THO). When THP-1 cells were pretreated with ascorbic acid (1 mM, 18 hours) and then exposed to acrolein diacetate, THO was detected as its pentafluorobenzyl oxime derivative in the cell lysates and medium. Treatment of THP-1 cells with both ascorbic acid and acrolein diacetate was required for THO formation. The formation of THO from AscACR was facilitated by the lactonase enzymes, human recombinant paraoxonases 1 and 2. THP-1 cells exhibited PON activity which explains the catalytic conversion of AscACR into THO in these cells. THO was formed in addition to metabolites of the glutathione conjugate of acrolein, indicating that THO formation contributes to the elimination of acrolein in a cellular environment.

Introduction

Vitamin C (ascorbic acid, AscH)¹ acts as a cofactor for a number of 2-ketoglutarate dependent dioxygenases, including proline hydroxylase, and also as a biological antioxidant (1–3) and prooxidant (4,5). Less known is the ability of AscH to participate in nucleophilic substitution and in Michael addition reactions. In aqueous solutions at neutral pH, AscH ($\text{pK}_a = 4.2$) is essentially an enolate and therefore capable of forming C–C bonds with electrophiles, for which we here use the term ‘ascorbylation’. The best known and most studied ascorbylated natural product is ascorbigen (6). The natural occurrence, biosynthesis

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Supporting Information Available: ^1H , ^{13}C , COSY, HMBC, and HSQC NMR spectra; and X-ray crystallographic data for AscACR. This material is available free of charge via the Internet at <http://pubs.acs.org>.

¹Abbreviations: ACR, acrolein; ACR(Ac)₂, acrolein diacetate; AscH, ascorbic acid; AscACR, ascorbyl-acrolein conjugate; AUC, area under the curve; CID, collision-induced dissociation; DHC, dihydrocoumarin; FBS, fetal bovine serum; GST, glutathione-S-transferase; GSH-ACR, glutathione-acrolein conjugate; GSH-AA, glutathione-acrylic acid conjugate; GSH-HP, glutathione-hydroxypropyl conjugate; PON, paraoxonase; PFBHA HCl, pentafluorobenzylhydroxylamine hydrochloride; PFB, pentafluorobenzyl; Q, quadrupole; SRM, selected reaction monitoring; THO, 5,6,7,8-tetrahydroxy-4-oxooctanal.

and biological activities of 33 ascorbylated natural products was recently reviewed (7). Ascorbylated products of synthetic origin have also been reported (8–12). One of these compounds is ascorbylated acrolein (AscACR), the Michael addition product of AscH and acrolein (ACR) (Figure 1). This compound was first synthesized by Fodor and co-workers in 1983 (12). The aldehyde group of AscACR may form a hemiacetal with either of the two oxygen atoms at positions 2 or 3, depending on the solvent used. For instance, a 5,5,5-tricyclic spiro compound is formed when AscACR is crystallized from water (13,14) (Figure 1).

Humans are primarily exposed to ACR through cigarette smoking and cooking with vegetable oils in poorly ventilated kitchens (15). ACR is also produced in vivo as a byproduct of protein, polyamine, and glucose metabolism, and lipid peroxidation (16). ACR itself is highly electrophilic and known to adduct to proteins (17) and DNA (18). Exposure to ACR has been associated with the development of lung cancer (15,19). Several research groups have reported protective effects of AscH against ACR-induced toxicity. For example, supplementation of cultured human bronchial epithelial cells with AscH has been shown to strongly inhibit ACR-induced apoptosis, an observation the authors attributed to a general antioxidant effect of AscH and to a 'more direct and specific effect' of AscH (20). Arai and co-workers (21) demonstrated that AscH suppresses ACR modification of apolipoprotein E in human very low density lipoprotein (VLDL) in vitro. The protective effect of AscH against ACR-induced neuronal damage in spinal cord white matter isolated from guinea pigs was also attributed to the antioxidant effects of AscH (22). In these studies, the ability of AscH to react directly with ACR was not addressed as a possible detoxification mechanism.

The metabolism of ACR by enzyme-mediated conjugation with glutathione (GSH) is well documented (23,24). Major metabolites of ACR found in human urine are hydroxypropyl mercapturic acid and carboxyethyl mercapturic acid (25,26). Given the high intracellular concentrations of AscH in humans (≤ 6 mM) (27) and the ubiquitous presence of ACR in vivo (16), we hypothesized that AscACR formation may be biologically significant. However, our previous attempts to detect AscACR in THP-1 cells exposed to AscH and ACR were unsuccessful, which led us to suspect that AscACR is subject to chemical and/or metabolic transformation. Here we show evidence for the formation of AscACR and its biotransformation into 5,6,7,8-tetrahydroxy-4-oxooctanal (THO) in AscH-adequate human monocytic THP-1 cells exposed to ACR diacetate [ACR(Ac)₂], an ACR precursor that provides an intracellular source of ACR. This unusual biotransformation represents a complementary pathway for ACR detoxification that may contribute to the protective effects of AscH against ACR-induced toxicity.

Experimental Procedures

General Methods

All solvents and reagents used were commercially available, analytical grade quality unless otherwise stated. Ascorbic acid (AscH), reduced L-glutathione (GSH), and acrolein (ACR) were purchased from Sigma-Aldrich (St. Louis, MO). Pentafluorobenzyl hydroxylamine hydrochloride and acrylic acid were purchased from TCI America (Portland, OR). L-[1-¹³C]-AscH and L-[¹³C₆]-AscH were purchased from Omicron Biochemicals (South Bend, IN). H₂¹⁸O was obtained from Cambridge Isotope Laboratories (Andover, MA). HPLC-grade acetonitrile and water were purchased from Honeywell, Burdick and Jackson (Muskegon, MI). Formic acid was obtained from Fluka (Buchs, Switzerland) and K₂CO₃ was purchased from Mallinckrodt Baker (Phillipsburg, NJ). Acrolein diacetate (allylidene diacetate) was purchased from Pfaltz & Bauer (Waterbury, CT). ¹H NMR spectra (400 MHz) and ¹³C NMR spectra (100 MHz) were recorded on a Bruker DPX 400 MHz instrument. The solvent peak was used as an internal standard for reporting chemical shifts.

Two-dimensional ^1H - ^1H COSY, ^1H - ^{13}C HMBC, and ^1H - ^{13}C HSQC experiments were also performed on the Bruker DPX 400 MHz instrument (see Supporting Information for spectra).

Liquid Chromatography-Tandem Mass Spectrometry

The HPLC system consisted of two Shimadzu Prominence LC-20AD pumps, a DQU-20A₅ degasser, and a Shimadzu SIL-HTc autosampler equipped with two switching valves (Shimadzu, Kyoto, Japan). Three chromatographic systems were employed. System 1 used a Thermo Betasil diol column (150 × 2.1 mm i.d.; particle size, 5 μm; pore size, 100Å; Thermo Fisher Scientific, Waltham, MA) and a linear solvent gradient from 100% solvent B (MeCN containing 0.1% HCOOH) to 5% B in solvent A (0.1% aqueous HCOOH) over 10 min at 0.2 mL/min. The first 2 min of each LC run was diverted to waste. In System 2, the HPLC column was a Synergi HydroRP C18 column (250 mm × 1 mm i.d.; particle size, 4 μm; pore size, 80Å; Phenomenex, Torrance, CA). The HPLC solvents were the same as in System 1. The column was eluted with 5% solvent B in A during the first minute, followed by a linear solvent gradient from 5% B to 95% B over 9 min and then with 95% B for 5 min. After returning to 5% B in 1 min, the column was equilibrated for 10 min before the next injection. The flow rate was 0.1 mL/min. The column effluent was directed to the mass spectrometer between 5 and 20 min of the LC run and to a waste container during the remainder of the LC run. In System 3, chromatographic separations were achieved on a Synergi MaxRP C12 column (250 mm × 2 mm i.d.; particle size, 4 μm) (Phenomenex) at a flow rate of 0.2 mL/min. The HPLC solvents were the same as in HPLC System 1. A linear solvent gradient was used, running from 10% B to 40% B in 10 min, 40 to 90 % B over the next 2 min, held constant at 90% B for 7 min, returned to 10 % B over 1 min, and equilibrated at 10% B for 5 min before the next injection.

The LC-MS/MS instrument consisted of a hybrid triple quadrupole/linear ion trap (4000 QTrap) mass spectrometer equipped with a pneumatically assisted electrospray (Turbo V) source operated at 450 °C (Applied biosystems /MDS Sciex, Concord, Ontario, Canada). Liquid nitrogen was used as the source of heating/nebulizing, curtain, and collision gas. The spray needle was kept at -4.5 kV in the negative ion mode. Q1 mass spectra were recorded by scanning in the range m/z 100–300 at a cycle time of 1 s with a step size of 0.2 u. MS/MS experiments (product ion scan and selective reaction monitoring, SRM) were conducted at unit resolution for both Q1 and Q3 with collision gas set at “medium”, a collision energy of 17 eV, and a declustering potential of 70 V. Peak areas were measured using Analyst 1.4.2 software (Applied Biosystems). The following LC-MS/MS characteristics were used for analysis of cell media and lysates (the first SRM transition was used for quantitative purposes, and subsequent SRMs were used for additional identity confirmation): THO-PFB oxime, t_R 10.4 min (System 2), m/z 400→310, m/z 400→167, m/z 400→112; $^{13}\text{C}_5$ -labeled THO-PFB oxime, t_R 10.4 min (System 2), m/z 405→312, m/z 405→167, m/z 405→114; AscACR-PFB oxime, t_R 11.8 min (System 2), m/z 426→115, m/z 426→366, m/z 426→157; GSH-ACR, t_R 5.0 min (System 3), m/z 362→143, m/z 362→306, m/z 400→272, m/z 362→128, m/z 362→179, m/z 400→254; GSH-HP (System 3), t_R 5.0 min, m/z 364→143, m/z 364→128, m/z 364→143; GSH-AA, t_R 5.1 min (System 3), m/z 378→306, m/z 378→143, m/z 378→128.

Preparation of AscACR

AscACR was synthesized following the method published by Fodor (12). ACR (1 mmol) was added to a solution of AscH (1 mmol) in water (1 mL). After 2 h stirring at room temperature, the solution was placed at 4 °C for 16 h. The resulting precipitate was filtered, washed with cold water, and dried. NMR spectra of the precipitate were in agreement with published data for AscACR (12): ^1H NMR (400 MHz, DMSO- d_6) δ_{H} 6.6 (1H, s), 6.53 (1H,

d, $J=5.6$ Hz), 5.61 (1H, d, $J=4.4$ Hz), 5.56-5.53 (1H, m), 4.45 (1H, s), 4.28-4.26 (1H, m), 4.21-4.19 (1H, m), 3.86 (1H, dd, $J=4.8, 4.4$ Hz), 2.45-2.40 (1H, m), 2.02-2.00 (1H, m), 1.87-1.83 (1H, m), 1.81-1.78 (1H, m); ^{13}C NMR (100 MHz, DMSO- d_6) δ_{C} 175.08, 106.13, 99.68, 87.67, 85.34, 74.87, 73.72, 32.06, 29.39. [$^{13}\text{C}_6$]- and [$1\text{-}^{13}\text{C}$]-AscACR were produced by treating 1 mg [$^{13}\text{C}_6$]-AscH and [$1\text{-}^{13}\text{C}$]-AscH, respectively, with an equimolar amount of ACR in aqueous solution.

Structure Determination of AscACR by X-ray Diffraction Analysis

ACR (5 mmol) was slowly added to a stirred solution of AscH (5 mmol) in water (5 mL). The reaction mixture was stirred for 2 h at room temperature and then placed at 4 °C for 5 days. After this period, a crystalline precipitate was collected and recrystallized from water. The structure of the material was determined to be the 5,5,5-tricyclic form of AscACR (Figure 1) by single crystal X-ray diffraction (see Supporting Information), in agreement with the structure of AscACR published by Eger and colleagues (28).

Hydrolysis of AscACR

An aliquot of an aqueous solution of AscACR (10 μL , 100 μM) was added to 200 μL H_2^{18}O . The solution was immediately analyzed by LC-MS using HPLC System 1. The sample was analyzed at 30 minute intervals over 4 h using Q1 scanning from m/z 100 to 300.

Decarboxylation of AscACR Acid to obtain THO

AscACR (0.11 mg) was dissolved in 1 mL of an aqueous solution of K_2CO_3 (0.18 M). After 2 h of incubation at room temperature, the sample containing THO was analyzed by LC-MS using HPLC System 1.

Derivatization of THO and AscACR

A 10 μL aliquot of the above solution containing THO was treated with 1 mL of a 500 mM solution of pentafluorobenzyl hydroxylamine hydrochloride (PFBHA HCl) in NaOAc buffer (pH 5.5, 1 M) for 1 h at room temperature. Similarly, [$^{13}\text{C}_5$]-labeled THO was prepared by treating 10 μL of [$^{13}\text{C}_6$]-AscACR with aqueous K_2CO_3 (0.18 M; 200 μL) for 2 h. The reaction mixture containing [$^{13}\text{C}_5$]-labeled THO was subsequently treated with 1 mL of a 500 mM solution of PFBHA HCl in NaOAc buffer (pH 5.5, 1 M) for 1 h at room temperature. Treatment of sample solutions containing AscACR with PFBHA resulted in the conversion of AscACR into its PFB oxime. PFB oxime derivatives were analyzed by LC-MS using HPLC System 2. Exact mass calculated for $\text{C}_{15}\text{H}_{15}\text{NO}_6\text{F}_5$ (THO-PFB oxime, $[\text{M}-\text{H}]^-$): 400.0820 (Found 400.0819). Exact mass calculated for $\text{C}_{12}\text{H}_9\text{NO}_3\text{F}_5$ (prominent fragment ion, Figure 5): 310.0503 (Found 310.0484).

Metabolic Transformation of ACR(Ac) $_2$ by THP-1 Cells

THP-1 cells, obtained from the American Type Culture Collection (Manassas, VA), were grown as suspension cultures in RPMI 1640 medium supplemented with penicillin (100 U/mL), streptomycin (100 $\mu\text{g/mL}$), 0.05 mM 2-mercaptoethanol and 10% fetal bovine serum (FBS) at 37 °C in an atmosphere of 95% air and 5% CO_2 . The cells (3×10^6 cells/mL; 2 mL/well) were pretreated with 1 mM AscH for 18 h in phenol red-free RPMI medium with supplements, centrifuged at $500 \times g$ for 5 min, and then co-treated with freshly prepared 1 mM AscH and 0.1 mM ACR(Ac) $_2$ in fresh phenol red-free RPMI medium with supplements. ACR(Ac) $_2$ was prepared as a 100 mM stock solution in 100% ethanol before addition to the culture medium (2 μL ACR(Ac) $_2$ / 2 mL medium, 0.1 mM final concentration). A stock solution of 50 mM AscH was freshly prepared in Dulbecco's Phosphate-Buffered Saline (D-PBS, Invitrogen Cat. no. 14190250) and neutralized with sodium hydroxide prior to use. Control cells were incubated with D-PBS and ethanol (0.1%)

in phenol red-free RPMI medium with supplements. No-cell controls consisted of complete RPMI medium containing 1 mM AscH and 0.1 mM ACR(Ac)₂. After 3, 6, 12, and 24 h of incubation, the cells were harvested by centrifugation at $500 \times g$ for 5 min and the media were collected. No-cell controls were terminated by placing the treated media on ice and immediately frozen at -80°C prior to analysis of ACR metabolites. The cell pellet was washed by resuspension in D-PBS and re-centrifugation at $500 \times g$ for 5 min. The pellet was then resuspended in D-PBS and lysed by sonication. The experiments were conducted in replicates of five. The samples were derivatized as described below and then analyzed for THO formation and residual AscACR by LC-MS/MS using HPLC System 2.

Derivatization of THP-1 Samples

An aliquot (200 μL) of each cell medium sample was transferred to an HPLC autosampler vial containing 50 μL PFBHA HCl (500 mM) in NaOAc buffer (pH 5.5, 1 M). Cell lysate samples were vortexed and centrifuged. An aliquot of the supernatant (150 μL) was transferred to an HPLC autosampler vial with a glass insert containing 50 μL of a 500 mM solution of PBFHA HCl in NaOAc buffer (pH 5.5, 1 M). After 1 h of incubation at room temperature, the samples were analyzed by LC-MS/MS using HPLC System 2.

Lactonase Activity of THP-1 cell lysate, FBS, Human Serum and Recombinant Paraoxonases

A venous blood sample (10 mL) was taken from a 43 year old volunteer and centrifuged at $3000 \times g$ for 5 min at room temperature to obtain serum (Study #2599, approved by the Institutional Review Board of Oregon State University). Lactonase activities of THP-1 cell lysate, FBS, human serum, recombinant human paraoxonase-1 (PON1, ProSec, Rehovot, Israel) and recombinant human paraoxonase-2 (PON2, Prospec, Rehovot, Israel) were measured using dihydrocoumarin (DHC) as the substrate (29) with some modifications. The incubation was carried out on a 96-well UV plate and the reaction mixture consisted of 20 μL of serum [diluted 1:10 in 10 mM Tris-HCl, pH 8, with 1 mM CaCl_2 or in RPMI 1640 (for FBS)], THP-1 cells (50 μg protein/well), PON1 or PON2 (0.05 μg protein/well), 1 mM DHC, 1 mM CaCl_2 , and 50 mM Tris-HCl (pH 8.0) in a total volume of 0.2 mL/well. The reaction was monitored at 30°C by the increase in absorbance at 270 nm over a 10 min period after substrate addition. Non-enzymatic hydrolysis of DHC was run on microplate wells without THP-1 cells, serum or recombinant enzyme. Vehicle control wells contained methanol instead of DHC. Lactonase activity was calculated as $\mu\text{moles DHC hydrolyzed/min per mL serum or per mg protein}$ (for THP-1 cells, PON1 and PON2) using an extinction coefficient of $1295 \text{ M}^{-1} \text{ cm}^{-1}$. Only the initial linear portion of the curve was used for calculations and all assays were run in quadruplicate.

Metabolic Conversion of AscACR to THO by Human Serum, PON1 and PON2

The incubation mixture contained 0.1 mM AscACR (from a 50 mM stock, dissolved in ethanol:water, 2:1 v/v), 50 mM Tris-HCl (pH 8.0), 1 mM CaCl_2 , and 25 μL of serum (diluted 1:10), in a total volume of 0.25 mL. Other incubations contained 0.2 μg PON1 or 0.2 μg PON2, instead of serum. Control incubations that contained appropriate combinations of reagents and either no enzyme or heat denatured enzyme (boiled at 100°C for 10 min) were also conducted. The reaction was carried out for 3 min, 30 min and 3 h at 37°C . At each time point, an aliquot (200 μL) of the reaction mixture was transferred to an HPLC autosampler vial containing 50 μL PFBHA HCl (50 mM) in NaOAc buffer (pH 5.5, 1 M). After 1 h of incubation at room temperature, the samples were analyzed for THO formation by LC-MS/MS using HPLC System 2.

Preparation of GSH Adducts

GSH adducts of ACR (GSH-ACR) and acrylic acid (GSH-AA) were prepared and characterized by LC-MS/MS following the method of Miranda et al. (30). Briefly, a solution of GSH (10 mM) was prepared in 0.1 M phosphate buffer (pH 8). To 100 μ L aliquots were added 400 μ L of the same phosphate buffer and 400 μ L of water. These solutions (900 μ L) were mixed with 100 μ L of a 1 mM solution of ACR or acrylic acid in EtOH, and the reaction mixtures stirred for 2 h at 37 °C and then acidified to pH 3 with 1 M HCl. Work-up of the reaction mixtures utilized Strata-X solid-phase extraction (SPE) columns (60 mg; Phenomenex, Torrance, CA) that were preconditioned with 1.2 mL of MeCN containing 0.1% HCOOH and equilibrated with 1.2 mL of 0.1% aqueous HCOOH. After sample loading and washing with 0.1% aqueous HCOOH (1.2 mL), the SPE column was eluted with MeCN-0.1% aqueous HCOOH (1:1, v/v) to obtain the GSH adducts. Hydroxypropyl-S-GSH (GSH-HP) was prepared by reduction of GSH-ACR adduct with 10 μ L of a 5 M sodium borohydride solution in 1 M NaOH. The reaction mixture was stirred for 30 min at room temperature and then acidified to pH 3 with 1 M HCl. The reduction product was isolated by SPE as described above.

LC-MS/MS Analysis of GSH Adducts in Cell Lysates and Media

Cell lysate (50 μ L) and medium (400 μ L) were mixed with a 2-fold volume of MeCN containing 0.1% HCOOH and centrifuged. The supernatant was analyzed by LC-MS/MS using HPLC System 3.

Calculation of Intracellular Metabolite Levels

Intracellular levels of metabolites were presented as peak areas and calculated using a THP-1 cell volume of 473 μ m³ or 4.73×10^{-7} μ L per cell (31). Cells were counted using a hemacytometer.

Statistical analysis

Statistical differences were determined by ANOVA followed by Tukey-Kramer multiple comparison tests or by the Student's *t*-test. Comparisons between the amounts of metabolites in different incubations were based on areas under the curve (AUC) between the start and the end of the incubation periods (Figure 7 and Figure 9). Values of *p* < 0.05 were considered to be statistically significant.

Results

The investigations described below were driven by two observations, 1) AscH readily reacts with ACR in aqueous solution to form AscACR, but AscACR is not detectable in AscH-adequate THP-1 cells exposed to ACR, and 2) synthetic AscACR is detectable in aqueous solution at neutral pH for several hours but it rapidly disappears when added to THP-1 cells or to human serum. These findings led us to examine the fate of AscACR in aqueous solution, in THP-1 cells and in human serum. After identification of THO as a degradation product of AscACR in alkaline solution, we were able to detect THO as a metabolite formed from ACR and AscH in THP-1 cells.

Hydrolysis of AscACR

When dissolved in water at neutral pH, AscACR produced an LC-MS signal with *m/z* 231([M-H]⁻) that decreased over a period of hours. A simultaneous increase of a new signal with *m/z* 249 was observed that corresponds to the addition of a water molecule to AscACR (Figure 2A). The addition of a water molecule reached equilibrium after approximately 24 h. We hypothesized that the addition of a water molecule was due to hydrolysis of the lactone

moiety and not due to hydration of the aldehyde group. To test this hypothesis, AscACR was dissolved in H₂ ¹⁸O and the incorporation of ¹⁸O was monitored. The aldehyde group of AscACR is in equilibrium with a spiro-hemiacetal moiety (Figure 1) and is expected to incorporate one ¹⁸O atom following ¹⁶O/¹⁸O exchange. Likewise, the oxygen atom at carbon-3 of the hemiketal group is exchangeable. After 3 h of incubation with H₂ ¹⁸O, AscACR indeed showed incorporation of one or two ¹⁸O atoms, giving rise to isotopomers with *m/z* 233 and *m/z* 235 (Figure 2B). The molecular species with *m/z* 249 produced three ¹⁸O isotopomers upon ¹⁶O/¹⁸O exchange, consistent with hydrolysis of the lactone moiety in addition to ¹⁸O incorporation at the hemiacetal and hemiketal sites.

When the H₂O addition product of AscACR with *m/z* 249 was subjected to collision-induced dissociation (CID), the MS/MS spectrum showed a fragment ion with *m/z* 205 (=249-44), consistent with loss of CO₂. The product's ¹⁸O₃-isotopomer with *m/z* 255 produced a fragment ion with *m/z* 209 (=255-46), consistent with loss of ¹⁸OC¹⁶O. To identify the origin of the carbon atom that is lost as carbon dioxide, the exchange experiments were repeated with [1-¹³C]-AscACR in unlabeled H₂O. Upon CID, the H₂O addition product of [1-¹³C]-AscACR with *m/z* 250 produced a fragment ion with *m/z* 205 (=250-45), consistent with loss of ¹³CO₂ (Figure 3).

Formation of AscACR-acid (Figure 1) is consistent with the incorporation of three ¹⁸O atoms in the ¹⁶O/¹⁸O exchange experiment. Hydration of the aldehyde moiety is not in agreement with the loss of ¹⁸OC¹⁶O from the [¹⁸O₃]-isotopomer of the H₂O addition product of AscACR, because the hemiacetal and hemiketal oxygens of AscACR were both exchangeable and would also be exchangeable in the H₂O addition product of AscACR, leaving the newly formed COOH group as the only possible third site of ¹⁸O incorporation. Taken together, these findings indicate that AscACR undergoes hydrolysis of the lactone to form AscACR-acid.

Decarboxylation of AscACR

When AscACR was dissolved in a solution of K₂CO₃ (0.18 M), the LC-MS signal with *m/z* 231 [M-H]⁻ rapidly declined and a new chromatographic peak with *m/z* 205 [M-H]⁻ appeared. The newly formed species with *m/z* 205 (= 231+H₂O-CO₂) did not arise from in-source fragmentation of AscACR-acid because its retention time was different from that of AscACR-acid under the conditions of HPLC System 1. When the incubation experiment was repeated with [¹³C₆]-AscACR, the corresponding new product appeared with a peak at *m/z* 210 (= 237+H₂O-¹³CO₂) in the Q1 mass spectrum. These findings indicate that AscACR undergoes hydrolysis and decarboxylation in alkaline solution to form THO (Figure 1) and that [¹³C₆]-AscACR forms [¹³C₅]-THO due to hydrolysis and loss of ¹³CO₂. The MS/MS spectrum of THO (*m/z* 205 [M-H]⁻) shows a series of fragment ions that can be rationalized by multiple loss of H₂O neutrals and α-cleavage of enolate products (Figure 4).

In order to be able to analyze the hydrophilic THO by LC-MS using reversed-phase LC columns, samples containing THO were treated with pentafluorobenzyl hydroxyl amine (PFBHA) to convert THO into its PFB oxime. THO-PFB oxime was retained on a Synergy HydroRP C18 column (HPLC System 2) and showed a molecular ion with *m/z* 400 in its Q1 mass spectrum. Mass fragmentation of THO-PFB oxime gave rise to fragment ions resulting from cleavage of the oxime to form nitrile species and α-cleavage of enolate products. The odd-mass fragment with *m/z* 167 is readily identified as the pentafluorobenzyl anion (Figure 5). In subsequent LC-MS/MS experiments, the prominent fragment ions with *m/z* 310 and *m/z* 112 were chosen for selected reaction monitoring (SRM) to detect and semi-quantify THO-PFB oxime in biological samples.

Transformation of ACR(Ac)₂ into THO

Human monocytic THP-1 cells were pretreated with 1 mM AscH in the culture medium for 18 h and subsequently exposed to 100 μ M ACR(Ac)₂ and freshly added AscH (1 mM). At various times following ACR(Ac)₂ exposure, culture medium and cell lysates were analyzed by LC-MS/MS using SRM. In the experiment of Figure 6, the samples were also spiked with [¹³C₅]-THO, prepared by alkali treatment of [¹³C₆]-AscACR, and then immediately treated with PBFHA. Figure 6 shows the SRM ion currents for two of the most prominent ion transitions of THO-PFB oxime (panel A) and the corresponding SRM ion currents of [¹³C₅]-THO-PFB oxime (panel B) in a medium sample. Panels A and B show the same chromatographic peak with a retention time of 10.4 min. We attribute the observed peak splitting to the presence of diastereoisomers (Figure 1). The SRM ion currents shown in panel A were not observed for a solution of [¹³C₅]-THO-PFB oxime alone, and neither for culture medium or cell lysate samples prepared from cells that were not pretreated with AscH or not exposed to ACR(Ac)₂. These findings demonstrate that THO is formed from AscH and ACR(Ac)₂.

The relative levels of THO were measured by LC-SRM in culture medium and cell lysate samples prepared from AscH-pretreated THP-1 cells following ACR(Ac)₂ exposure. In addition, THO formation was measured in FBS-containing and FBS-lacking culture media that were co-incubated with AscH and ACR(Ac)₂ in the absence of cells. Comparison of areas under the curve (AUCs_{0–24h}, Figure 7A) revealed that the relative amounts of intracellular THO were significantly higher than the amounts of THO in medium surrounding the cells ($p < 0.001$) and higher than in the no-cell controls (medium with FBS, $p < 0.01$; medium w/o FBS, $p < 0.001$).

Our LC-SRM method allows detection of AscACR as its PFB oxime, but it was not detected in culture medium and cell lysate samples prepared from AscH-adequate THP-1 cells exposed to ACR(Ac)₂. We did detect AscACR in FBS-containing and FBS-lacking culture media that were co-incubated with AscH and ACR(Ac)₂ in the absence of cells. The AscACR concentration was maximal at 3 h and significantly higher in the presence of FBS (Figure 7B).

Incubation of AscACR with Human Serum and Paraoxonase (PON) 1 and 2

AscACR and THO were both measured by LC-MS/MS in samples of AscACR incubated with human serum, buffer (control experiment), and buffer containing PON1 or PON2. At 3 minutes of incubation, the serum and PON incubations contained 10-fold higher concentrations of THO compared to the buffer control (Figure 8A), indicating that PON1 and PON2 both accept AscACR as a substrate. At 30 minutes of incubation, AscACR was completely consumed in all incubations, including the buffer control (Figure 8B). The initial 10-fold difference in THO concentration between the buffer and PON incubations disappeared during the 3–30 minute incubation period (Figure 8A). After complete consumption of AscACR at 30 min, the concentration of THO continued to increase between 30 min and 3 h of incubation in all samples, which is explained by the transient formation of AscACR-acid (Figure 1, not measured) and its decarboxylation to form THO. The corresponding control reaction comparing the amount of THO produced when AscACR is incubated with buffer and buffer containing heat-denatured PON1 or PON2 showed no statistical difference at any of the three time points ($p > 0.05$ for each comparison at a specific time point).

Lactonase Activity of THP-1 Cell Lysate, FBS, Human Serum and Recombinant Paraoxonases

Human serum, FBS, THP-1 cell lysate, PON1 and PON2 exhibited lactonase activity as shown by their ability to hydrolyze DHC (Table 1). DHC was chosen as a substrate to demonstrate lactonase activity of the various samples because this compound has been used to detect lactonase activity of human PON1, PON2 and PON3 (38).

GSH Conjugates of ACR in THP-1 Cell Lysates and Media

GSH conjugates were measured in culture medium and cell lysate samples prepared from THP-1 cells exposed to ACR(Ac)₂, either with or without AscH pre-incubation. GSH-ACR, GS-AA, and GSH-HP were all found to be produced following exposure of cells to ACR(Ac)₂. Intracellular levels of these GSH metabolites were maximal at 3 h and subsequently declined over a period 24 h. GSH-HP (Figure 9A) was found to be the major metabolite in both the cell lysates (Figure 9B) and the media (Figure 9C), with a much higher concentration found in the cells relative to the media. GSH conjugates were not detected in culture medium exposed to ACR(Ac)₂ in the absence of cells.

Comparison of the areas under the curve (AUC_{0-24h}) revealed a slightly lower amount of GSH-HP in cells pretreated with AscH compared to AscH-deficient cells (Figure 9B, Student's *t*-test, *p* = 0.037 with *n* = 5). In Figure 9C, the average amount of GSH-HP found in cell medium (AUC_{0-24h}) was higher for the AscH-adequate cells but the difference from AscH-deficient cells did not reach significance (Student's *t*-test, *p* = 0.28 with *n* = 5).

Discussion

THP-1 cells provide an ideal biological environment in which to investigate the covalent interaction between AscH and ACR because these cells accumulate AscH at concentrations of up to 9 mM and are capable of forming GSH conjugates of 2-alkenals and their metabolites (30) via enzyme-mediated pathways that will compete with AscACR formation. ACR(Ac)₂ was used as a 'pro-drug' form of ACR to minimize adduct formation of ACR with components of the cell culture medium and to maximize intracellular concentrations of ACR by enzyme-mediated release of ACR from ACR(Ac)₂. Another reason for not using free ACR is that it could react with AscH in the medium despite presence of cells. Exposure of AscH-adequate THP-1 cells to ACR(Ac)₂, however, did not yield detectable concentrations of AscACR in the medium or cell lysates. These findings suggest that either AscACR is not formed due to competing pathways such as glutathione-S-transferase (GST)-mediated GSH conjugation and subsequent metabolism of the GSH conjugates, or that AscACR is formed but not detectable due to degradation or metabolism. The second hypothesis was tested by investigating the fate of AscACR in neutral and in alkaline solution. By using ¹⁸O- and ¹³C-labeling of AscACR, we found that AscACR undergoes hydrolysis of the lactone moiety at neutral pH to form AscACR-acid (Figure 2). The carboxyl group of AscACR-acid spontaneously dissociates from the ascorbyl moiety in the collision cell of the mass spectrometer (Figure 3) and in alkaline solution to form THO (Figure 4). THO was detected in the medium and lysate prepared from AscH-adequate THP-1 cells exposed to ACR(Ac)₂ (Figure 6). Taken together these results suggest that AscACR is indeed formed but converted into THO.

A similar transformation has been observed for ascorbigen, the most well-known and studied ascorbylated product. Ascorbigen is formed during the degradation of the indole glucosinolate, glucobrassicin, in the presence of AscH and is found in many species of Brassicaceae (6,32). Studies by Preobrazhenskaya and coworkers (33) have demonstrated that ascorbigen undergoes hydrolysis of the lactone moiety and subsequent decarboxylation

to form a mixture of 1-indolyl-1-deoxytagatose and 1-indolyl-1-deoxysorbose. Both 1-indolyl-1-deoxyketohexoses were found in bovine serum exposed *ex vivo* to ascorbigen and in urine of mice after *i.p.* administration of ascorbigen (34). The chemical transformation reported for ascorbigen in bovine serum is essentially identical to the transformation of AscACR into THO in cell culture medium and in human serum. The observed splitting of the chromatographic peak that represents THO (Figure 6) may be due to the formation of cyclic hemiketal isomers, analogous to the degradation of ascorbigen in bovine serum (34).

AscACR was detected in culture medium that was co-incubated with AscH and ACR(Ac)₂ (Figure 7B), but not in culture medium when THP-1 cells were present. Furthermore, the maximum concentration of AscACR was higher when the culture medium contained FBS. These findings indicate that ACR(Ac)₂ hydrolyzes spontaneously in culture medium and that the hydrolysis is facilitated by components of FBS, presumably by enzymes with esterase activity. In the presence of cells, levels of THO in the media surrounding the cells were much lower than the intracellular concentrations of THO (Figure 7A), suggesting an intracellular source of THO via hydrolysis of ACR(Ac)₂, formation of AscACR and metabolic conversion of AscACR into THO.

The conversion of AscACR into AscACR-acid involves hydrolytic opening of the lactone ring of the ascorbyl moiety (Figure 1). Therefore we hypothesized that the conversion is mediated by an enzyme or enzymes with lactonase activity. Paraoxonases are a family of enzymes capable of hydrolyzing the organophosphate paraoxon, the bacterial *N*-3-oxododecanoyl homoserine lactone, the δ -lactone dihydrocoumarin, and, with the exception of PON2, a series of γ - and δ -hydroxy alkanolic acid lactones (29,35–38). PON1 is found in serum associated with high-density lipoprotein (HDL) while PON2 is found in human and murine macrophages but not in serum (39). We tested the hypothesis that AscACR is a substrate for PON1 and PON2. Both paroxonase isoenzymes exhibited lactonase activity with DHC as a substrate (Table 1) and facilitated the conversion of AscACR into THO (Figure 8). Human serum exhibited lactonase activity by facilitating the hydrolysis of the PON-specific substrate DHC (Table 1) as well as by the conversion of AscACR into THO (Figure 8), suggesting that catalytic formation of THO from AscACR in human serum is due to PON1. PON activity was also detected in THP-1 cell lysates and in FBS using DHC as the substrate (Table 1). The activity detected in the THP-1 cell experiments was comparable to the activity levels detected previously by other researchers (40,41). These results suggest that PON mediates the conversion of AscACR into THO in THP-1 cells and FBS.

The detection of the major GSH metabolite, GSH-HP, and the minor metabolites, GSH-ACR and GSH-AA, in culture media and cell lysates (Figure 9) shows that THP-1 cells are capable of GSH conjugation of ACR and phase I metabolism of GSH-ACR, while also producing THO via AscACR. The gradual decrease over time of the intracellular concentration of GSH-HP is most likely due to excretion of GSH-HP into the medium. The progressive decrease of GSH-HP in the medium is best explained by extracellular conversion of GSH-HP into Gly-Cys-HP by γ -glutamyltransferase, a membrane protein with its catalytic site faced extracellularly (42). The intracellular levels of GSH-HP were slightly but significantly lower in the AscH-adequate cells compared to the AscH-deficient (Student's *t*-test of AUC_{0–24h}, *p* = 0.037, Figure 9B). This difference can be explained by the effect of AscH on preserving the GSH-HP export activity of the ATP-dependent multi-drug resistance-associated protein (MRP) and on maintaining ATP levels following electrophile stress in THP-1 cells (30). An alternative explanation for the lower cellular levels of GSH-HP in AscH-adequate cells is that AscACR formation competes with GSH conjugation of ACR. Although we are unable to distinguish between the relative contributions of the two pathways to the fate of ACR without absolute quantification of all

possible metabolites of ACR, our results demonstrate that the two pathways occur concurrently.

Conclusions

We have shown that ascorbylation of ACR and subsequent transformation of AscACR into THO is a pathway for elimination of ACR that co-exists with GSH conjugation of ACR in THP-1 cells. The conversion of AscACR into THO is catalyzed by the lactonase activity of recombinant human PON1 and PON2. Human serum, FBS and THP-1 cells were shown to exhibit lactonase activity using the PON-specific substrate DHC and to facilitate the conversion of AscACR into THO, suggesting that PON is involved in the conversion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Formation of THO from AscH and ACR via AscACR and AscACR-acid. The configuration of carbon atoms 6 and 7 of THO is determined by the configuration of the corresponding carbon atoms in L-AscH. THO is likely to exist as a mixture of 5- and 6-membered cyclic hemiketals and hemiacetals in solution, but not shown here for simplicity.

Figure 2.

Hydrolytic conversion of AscACR into AscACR-acid monitored by LC-MS using HPLC System 1. (A) Q1 Mass spectrum of a co-eluting mixture of AscACR and AscACR-acid obtained by LC-MS of a freshly prepared solution of AscACR in water. (B) Q1 Mass spectrum of a co-eluting mixture of AscACR and AscACR-acid obtained by LC-MS analysis of a 3 h incubation of AscACR in H_2^{18}O . The ions with m/z 205 in panel A and with m/z 205 and m/z 207 in panel B are due to in-source fragmentation of AscACR-acid.

Figure 3. Product ion mass spectrum of the m/z 250 $[\text{M}-\text{H}]^-$ ion of $[1-^{13}\text{C}]\text{-AscACR-acid}$ obtained by LC-MS/MS analysis of a solution of $[1-^{13}\text{C}]\text{-AscACR}$ in H_2O .

Figure 4. Product ion mass spectrum of the m/z 205 $[M-H]^-$ ion of THO obtained by LC-MS/MS analysis of a 2 h incubation of AscACR in an aqueous solution of K_2CO_3 (0.18 M).

Figure 5.

Product ion mass spectrum of the m/z 400 $[M-H]^-$ ion of the pentafluorobenzyl (PFB) oxime derivative of THO obtained by LC-MS/MS analysis (HPLC System 2) of an aqueous solution containing THO and treated with PFB hydroxylamine for 2 h at room temperature.

Figure 6.

LC-MS/MS analysis of culture medium obtained from AscH-adequate THP-1 cells exposed to ACR(Ac)₂ for 3 h, spiked with [¹³C₅]-THO, and treated with PFB hydroxylamine. (A) Detection of THO-PFB oxime by selected reaction monitoring (SRM). (B) Simultaneous detection of spiked [¹³C₅]-THO-PFB oxime by using SRM. THO in the medium formed from AscH and ACR is chromatographically indistinguishable from spiked [¹³C₅]-THO.

Figure 7. Relative concentrations of THO and AscACR following exposure to AscH and ACR(Ac)₂. (A) AscH-adequate THP-1 cells and the surrounding media were analyzed at various time points for AscACR and THO by LC-MS/MS using SRM. AscACR was not detected in the presence of THP-1 cells. In the absence of cells, THO (panel A) and AscACR (panel B) were both detected in FBS-containing and FBS-lacking media that were co-incubated with AscH and ACR(Ac)₂. Symbols represent means $\pm$ SEM of five replicates ($n = 5$).

Figure 8. Biotransformation of AscACR into THO. The formation of THO was monitored after incubation of AscACR with human serum, PON-1, PON-2, and buffer only (panel A). Panel B shows the decrease of the AscACR concentrations in the same incubations. The increase of THO following complete consumption of AscACR within 30 min is presumably due to the formation of the intermediate product, AscACR-acid. Symbols represent means $\pm$ SEM of three replicates ($n = 3$).

A GSH-HP is a major metabolite of GSH-ACR **B** GSH-HP in THP-1 cells **C** GSH-HP in cell medium **Figure 9.**

Relative concentrations of GSH-HP in THP-1 cells (panel B) and surrounding media (panel C) following exposure to ACR(Ac)₂, in the presence and absence of AscH. GSH-HP was measured by LC-MS/MS using SRM and HPLC System 3. Symbols represent means $\pm$ SEM of five replicates ($n = 5$).

Table 1

Lactonase activity of human serum, fetal bovine serum (FBS) and recombinant paraoxonases using dihydrocoumarin (DHC) as a substrate

Enzyme source	Lactonase activity ^a
Human serum	6.25 ± 0.16
FBS	0.172 ± 0.005
THP-1	0.08 ± 0.01
PON1	34.3 ± 2.2
PON2	58.9 ± 3.8

^aData are expressed as mean ± SE (*n* = 4). Lactonase activities of serum (human and FBS) and recombinant PON1 or PON2 are expressed as μmol DHC hydrolyzed/min/mL serum and μmol DHC hydrolyzed/min/mg protein, respectively. Lactonase activity of THP-1 cells is expressed as μmol DHC hydrolyzed/min/mg protein.