Supplementary Information

Determination of cellular vitamin C dynamics by HPLC-DAD

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Materials and Methods

Reagents
L(+) -Ascorbic acid (ASC), ascorbate oxidase, cytochalasin B (Cyt B), dehydroascorbic acid (DHA), dithiothreitol (DTT), metaphosphoric acid (MPA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Rabbit erythrocytes were purchased from Nippon Bio-Test Laboratory (Tokyo, Japan). 3',6'-Di(O-acetyl)-4',5'-bis[N,N-bis(carboxymethyl)aminomethyl]fluorescein (Calcein-AM) was purchased from Dojindo (Tokyo, Japan). Dulbecco’s Modified Eagle Medium (DMEM) with or without glucose was purchased from Thermo Fisher Scientific (MA, USA). MIA PaCa-2 were obtained from European Collection of Authenticated Cell Cultures (ECACC). Fetal bovine serum (FBS) was purchased from Biowest (Nuaillé, France). All other chemicals and reagents used were of analytical grade or higher.

Chromatographic (HPLC-DAD) analysis
The HPLC system consisted of a solvent pump (PU 2080 Plus, Jasco International Co. Ltd., Tokyo, Japan), a column oven (CO-2060 Plus, Jasco International Co. Ltd., Hachioji, Tokyo, Japan) and a DAD detector (MD-2018 plus, Jasco International Co. Ltd., Tokyo, Japan). The column (Primesep SB, 4.6 × 250 mm, 100 Å pore size, 5 μm particle size; SiELC Technologies Ltd, Prospect Heights, IL, USA) was eluted using isocratic gradient consisting of the following HPLC solvents: HPLC grade water containing 2 mmol/L ammonium acetate and 0.1 % formic acid. The flow rate was 0.5 ml/min and the column temperature was 50 °C. The column eluent was measured (at 240 nm) by the DAD detector installed at immediate downstream of the column. To verify the assignment on HPLC-DAD chromatograph, UV absorption spectra of DHA and ASC were separately measured by a UV-visible spectrophotometer (V-650 spectorometer, Jasco International Co. Ltd., Tokyo, Japan) for the range of 200-290 nm under the condition identical to the HPLC mobile phase (0.1% formic acid and 2 mM ammonium acetate in water). When standards of ASC and DHA were subjected to HPLC-DAD, each peak was well separated by using the above-described mixed mode column and the mobile phase condition (Fig. 1a, b). In addition, they were not overlaid with the peak of MPA (stabilizer of ASC and DHA during extraction and storage). Standard curves were linear within the concentration range of 0.001-10 nmol (R = 0.9993) for ASC and 0.1-200 nmol (R = 0.9996) for DHA, respectively (Fig. 1e, f). The observed DAD absorption spectra for standards were consistent with those analyzed by UV-visible spectrophotometer with maximal absorptions at 220 nm for DHA and 245 nm for ASC. (Fig. S2). For determination of the detection limits, signal to noise ratio (S/N) of 3:1 was employed.1

Reduction method: measurement of total vitamin C
DHA was reduced to ASC using DTT as a reductant, according to a method previously reported with some modifications 11. In order to assess the effect of pH, reaction solutions were prepared in two ways; each 100 μL aqueous solution containing 0, 0.5 (with 0.5 mM ASC), 5 (with 5 mM ASC) and 10 mM of DHA were mixed with either 50 μL pure water or 50 μL of 1 M Trizma buffer containing DTT (10
mM, 100 mM and 1 M), yielding the solution pHs of below 2 or exactly 6.2 (reportedly the best suited pH for the DHA-ASC reduction by DTT), respectively. To each solution was added 100 mM (at final concentration) DTT to initiate the reactions at room temperature for different periods of time (5, 30, 60, 180 and 360 min) and finally quenched by addition of 50 μL of 0.2 M sulfuric acid. The resulting mixtures were analyzed immediately by HPLC-DAD and the observed ASC peaks in the chromatogram were calculated as the total vitamin C. To study the effect of MPA on the reaction efficiency, the same procedure was carried out in the presence of MPA (0, 1, 10 and 50 %) and systemically compared (Fig. 1i). The reaction time for this assay was 60 min.

**Analysis of DHA uptake by erythrocytes**

Rabbit erythrocytes were washed three times with centrifugation at 1300 g for 15 min at 4 °C with phosphate-buffered saline (PBS, pH 7.4) to prepare packed cells. Each 350 μL suspension of packed cells was incubated with 750 μL of PBS containing ASC or DHA (0, 1 and 10 mM) for different periods of time (0, 1, 5, 10 and 30 min). For the CytB treated group, the erythrocytes were incubated with 10μM CytB for 1 hour and then washed with excess PBS before analysis. At the end of each incubation, samples were centrifuged at 1300 g for 15 min at 4 °C and supernatants were collected. For extraction of DHA and ASC, remaining erythrocytes were washed three times with PBS to remove extracellular components. Thereafter, 350 μL of 10% MPA solution was added to each sample to protect DHA and ASC from degradation and oxidation, which was then vortexed for 5 min to induce the erythrocytes’ hemolysis and precipitation of proteins. The solution was centrifuged at 10000g for 10 min and the supernatants containing ASC and DHA were collected and stored at −30 °C or analyzed immediately by the HPLC-DAD. To confirm the recovery of DHA and ASC, these were spiked to erythrocytes, which were then extracted and analyzed in the same manner as described above. The recovery rate for DHA spikes at the concentrations of 1 mM, 5 mM and 10 mM were 92.88±4.55, 94.19±3.38 and 95.47±1.61 % (n=3), respectively. Likewise, 1 mM, 5 mM and 10 mM ASC spikes were recovered at 86.35±0.54, 83.04±5.52 and 85.97±1.28 % (n=3), respectively.

**Cell culture and VC extraction**

Human pancreatic carcinoma (MIA PaCa-2) cells were seeded at 10⁶ cells/10 ml DMEM medium containing 10 % FBS in 10 cm dishes and incubated in CO₂ incubator at 37 °C for 48 h until the cells become semi confluent. Thereafter, the medium was removed, to which was added the DMEM medium (without FBS) containing DHA or ASC with different amounts (0, 1 and 10 mM) with or without glucose (4.5 g/L). At the end of each incubation (0, 5, 15, 30, 60, 360 min), the cells were counted by haemocytometer. Then, the cells were lysed and extracted by 300 μL of 10% MPA solution containing 0.2 % SDS. The solution was centrifuged at 10000g for 10 min and the supernatants containing ASC and DHA were collected and stored at −30 °C or analyzed immediately by HPLC-DAD.

**Cell viability study**
To study the effect of DHA on the viability of MIA PaCa-2, the cells were seeded in 96 well plates at 1000 cells/well in DMEM containing 10 % FBS. After overnight incubation, the medium was replaced by DMEM without FBS containing various amount of DHA (0, 1 and 10 mM), with and without glucose (4.5 g/L). After 24 h of incubation, the cells were washed with PBS twice, to which was added PBS containing 2μmol/l Calcein-AM and incubated in CO₂ incubator at 37 °C. Finally, the cells were washed with PBS and assessed for their florescence intensity (ex: 480 nm, em: 525 nm) on a plate-reader A (Tecan Microplate Reader: Tecan infinite 200, Zürich, Switzerland) to assess the viability.

Statistics
Results are expressed as mean ±S.D.. The data were analyzed using one-way ANOVA, followed by Tukey's Multiple Comparison test. Differences with **P < 0.01 were considered statistically significant.
**Supplementary Tables and Figures**

**Fig. S1** In vitro validation of “direct method” as a platform for DHA-uptake kinetic study. a,b; HPLC-DAD chromatograms of erythrocyte extracts after 10-minute incubation with 5 mM DHA (a: red) and 5 mM ASC (b: blue), respectively. The inlets show DAD spectra taken at each vitamer assigned elution time (DHA, 6.91 min; ASC, 9.11 min). c; DHA-uptake kinetics by erythrocytes for various DHA-exposure conditions. Data are presented as means ± SD, n=3. d,e; HPLC-DAD chromatograms taken for erythrocyte-suspending PBS after 10-minute incubation (studied in parallel to a and b) with 5 mM DHA (d: red) and 5 mM ASC (e: blue), respectively. f,g; Quantification of DHA (f: red) and ASC (g: blue) in the
erythrocyte-suspending PBS for various exposure concentrations. Data are presented as means ± SD, n=3. Means significantly differed at **P < 0.01. Fig. S1 provides in vitro validation of the “direct method” as a means to monitor DHA-uptake kinetics by (metabolically inactivated) rabbit erythrocytes, the best-documented target in the literature.4,5 Both DHA (red) and ASC (blue) were applied to the erythrocytes at various doses and their intra- and intercellular concentrations were measured over time (Fig. S1a-e). It is well established that erythrocytes undergo GLUT-1-dependent uptake specific to DHA. In accordance, we do observe such DHA-specific and the dose-dependent cellular internalization and complete blockade of ASC (Fig. S1c). In this experiment, no event of intracellular DHA-ASC conversion is observed due to the absence of metabolic activity of the erythrocytes in use, resulting in a continuous intracellular accumulation of DHA. The extracellular stability of the vitamers, which is often a matter of debate in interpretation of the VC uptake behavior and cytotoxicity, has also been confirmed in Fig. S1f and S1g; the abundance of these vitamers stably persists throughout the experiment.
**Fig. S2** UV Absorbance spectra of (a) 1 and 5 mM DHA and (b) 0.02 and 0.1 mM ASC in the HPLC mobile phase (0.1% formic acid and 2 mM ammonium acetate in water) for the range of 200 nm to 290 nm. c; Overlay of a and b (5 mM DHA and 0.1 mM ASC). All data in this figure were collected by UV-visible spectrophotometer (V-650 spectorometer, Jasco International Co. Ltd., Tokyo, Japan).
Fig. S3 Overlay of HPLC-DAD chromatograms for DHA (red) and its degraded products (purple). 50 mM of DHA was degraded by treating with 65 mM NaOH aqueous solution at 20 °C for 6 min following the procedure previously reported. According to this report, degraded products contain 2,3-Diketo-L-gulonate (DKG), L-Erythroascorbate, 2,3-Enediol-DKG, 3,4-Enediol-DKGL, 2-Furoic acid, 5-Methyl-3,4-dihydroxytetrone, L-Erythrulose, Oxalate and Succinate. Chromatograms of blank solutions (without DHA or ASC) are shown with gray colour lines.
Fig. S4 DHA-uptake kinetics by erythrocytes as determined by “reduction method” involving MPA-extraction. Rabbit erythrocytes were exposed to 10 mM DHA for various periods of time and the extract (based on MPA treatment) were reduced by DTT according to a previous report. Then, the total vitamin C (calculated as ASC peak) at each time point was measured by HPLC-DAD. Data are presented as means ± SD, n=3.
Fig. S5 Oxidation of ASC to DHA as analyzed on HPLC-DAD chromatogram. The oxidation was carried out by treating 10mM of ASC in the milli-Q water with ascorbate oxidase (10 U/mL) for 5 min at 22°C, according to a previous report by Yun et al.,3 Before the treatment, ASC peak at 9.11 min is observed (blue chromatogram), which completely disappears and a new peak at 6.95 min attributable to DHA appears after the treatment (red chromatogram), consistent with the peak assignments in Fig. 1.
Fig. S6 Representative HPLC-DAD chromatograms for (a) a standard ASC-DHA mixture and (b) an extract from MIA PaCa-2 cells. (a) HPLC-DAD chromatogram of 50 nmol DHA (red chromatogram) and 10 pmol ASC standard (blue chromatogram). DHA standard was dissolved in 5% MPA (as a stabilizer of DHA and ASC) solution and analysed by HPLC-DAD. Mobile phase of HPLC was adjusted to 0.03% formic acid and 2 mM ammonium acetate to avoid the cell-derived background noise. The solution without DHA is shown as gray chromatogram. Monitored by UV absorbance at 240 nm (b) HPLC-DAD chromatogram of MIA PaCa-2 cells extract after 1 hour exposure to 10 mM DHA in DMEM medium with no glucose. Monitored by UV absorbance at 240 nm. (c) HPLC-DAD chromatogram of MIA PaCa-2 cells extract after 1 hour exposure to 10 mM DHA in DMEM medium with no glucose. Monitored by UV absorbance at 210 nm. The extract from cells without DHA exposure is shown as gray chromatogram.
Table S1
Effect of incubation time and DTT concentration on the reduction rate of ASC.

| Incubation time (min) | Concentration of DTT (mM) | Reduction rate of ASC (%) |
|-----------------------|---------------------------|---------------------------|
| 5                     | 10                        | 74.61 ± 1.02              |
| 30                    | 10                        | 76.67 ± 2.29              |
| 60                    | 10                        | 79.62 ± 0.77              |
| 60                    | 100                       | 89.68 ± 3.27              |
| 60                    | 1000                      | 89.18 ± 1.43              |
| 180                   | 10                        | 80.35 ± 1.15              |
| 360                   | 10                        | 77.07 ± 1.67              |

Means ± SD, n=3
## Table S2
Comparison of “direct” and “reduction” method in quantification of standards.

| DHA and ASC in the solution | Direct method | Reduction method |
|-----------------------------|---------------|------------------|
| DHA (mM) | ASC (mM) | DHA (mM) | ASC (mM) | SUM of DHA and ASC (mM) | Total vitamin C (mM) |
|-----------------|-------------|-------------|-------------|-------------------------|--------------------------|
| 10 | 0 | 0.83 ± 0.02 | 6.11 ± 0.34 | 11.13 ± 0.24 | 10.53 ± 0.21 |
| 5 | 5 | 5.02 ± 0.19 | 0.49 ± 0.03 | 0.99 ± 0.03 | 0.88 ± 0.02 |
| 0.5 | 0.5 | 0.50 ± 0.09 | 0.49 ± 0.03 | 0.99 ± 0.03 | 0.88 ± 0.02 |
| 0 | 1 | 1.12 ± 0.02 | 0.99 ± 0.11 | 10.61 ± 0.25 | 10.99 ± 0.11 |
| 0 | 10 | 10.22 ± 0.03 | 0.99 ± 0.11 | 10.61 ± 0.25 | 10.99 ± 0.11 |

Means ± SD, n=3
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