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[^{11}C]Ascorbic and [^{11}C]Dehydroascorbic Acid, An Endogenous Redox Pair for Sensing Reactive Oxygen Species Using Positron Emission Tomography

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General Methods

L-Xylosone was purchased from Omicron Biochemicals (South Bend, IN). All other chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO). Activity was obtained using a GE PETtrace cyclotron with a GE [^{11}C]HCN production unit. HPLC purification of radiolabeled compounds was performed using a Waters 600 system (Waters, Milford, MA) with a Shimadzu-10A UV-vis detector (Shimadzu, Koyoto, Japan) and an in line CsI radiation detector (Carroll & Ramsey, Berkeley, CA) with a Synergi Hydro-RP semiprep column (Phenomenex, 80A, 250 x 10 mm). A Perkin Elmer 1480 wizard 3" automatic gamma counter (Perkin Elmer, Waltham, MA) was used to count activity for *in vitro* studies. PET imaging was preformed using a Siemens Inveon microPET/CT scanner.

Synthetic Procedures

Radiosynthesis of [^{11}C]VitC

[^{11}C]HCN precursor was obtained from using a GE PETtrace cyclotron using a GE [^{11}C]HCN Production Unit. 50 – 100 mCi (1850 – 3700 MBq) [^{11}C]VitC was trapped on a 1 mL length of Teflon tube (OD = 1.35 mm) coated with a minimal volume of 0.5 M KOH. To coat the trap 2 mL of KOH was passed through the tube followed by 2 mL of air. Next vacuum (~630 Torr) was applied for 5 minutes to remove excess NH_3 from production and the line was flushed with N_2. The activity was eluted with 1 mL water (carrier free) or aqueous KCN (carrier added) into a vial containing 200 µL of 0.2 M L-xylosone. The resulting imine intermediate was then hydrolyzed with addition of 15 µL 12 M HCl and heated for 10 min at 150°C to yield the desired product. [^{11}C]VitC was isolated in >99% radiochemical purity by HPLC using a semiprep Hydro-RP column with 5 mL/min 0.1% H_3PO_4 isocratic with a t_r = 4.5 min. The radio peak was collected and confirmed by co-injection with a vitamin C standard (Figure S1). Specific activity was calculated using a standard curve with triplicate injections of the radiolabeled product. Table 1 (main text) summarizes decay corrected radiochemical yields and decay corrected specific activities for varying levels of carrier added preparations.

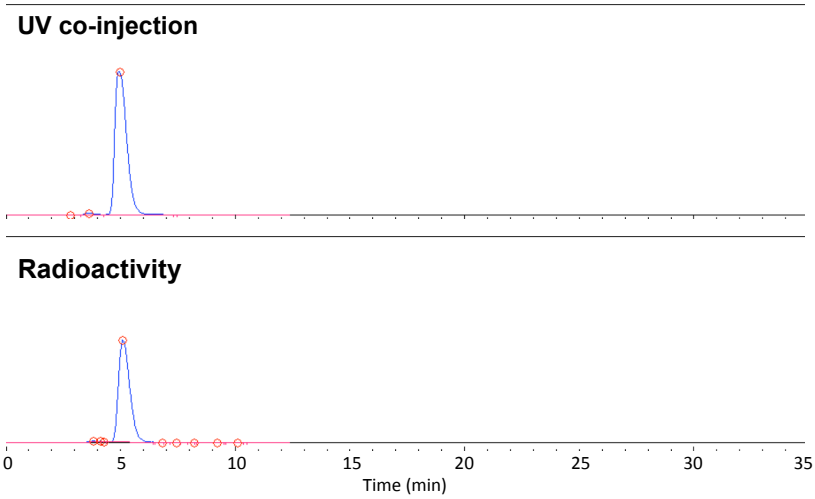

Figure S1: Analytical HPLC of collected [^{11}C]VitC showing co-injection with non-radioactive standard t_r = 5 min.

Radiosynthesis of [^{11}C]DHA

1 mL of HPLC purified [^{11}C]VitC was added to a vial containing 1.0 mg activated charcoal. [^{11}C]VitC was oxidized with rapid bubbling of O_2 using a balloon and septa setup. The reaction was monitored by HPLC using an analytical Hydro-RP column with isocratic elution using 1 mL/min 0.1% H_3PO_4 isocratic (Figure S2). Oxidation was complete within 10 min. The mixture was filtered and used

without further purification. Formation of [^{11}C]DHA was confirmed by co-injection with weakly UV active DHA standard (Figure S3). DHA standards were freshly prepared day of the experiment as per Bohndiek *et. Al.*[1] A 100 µL co-injection was preformed using a mixture of 100 µL non-radioactive DHA (10 mg/mL) with 50 µL [^{11}C]DHA.

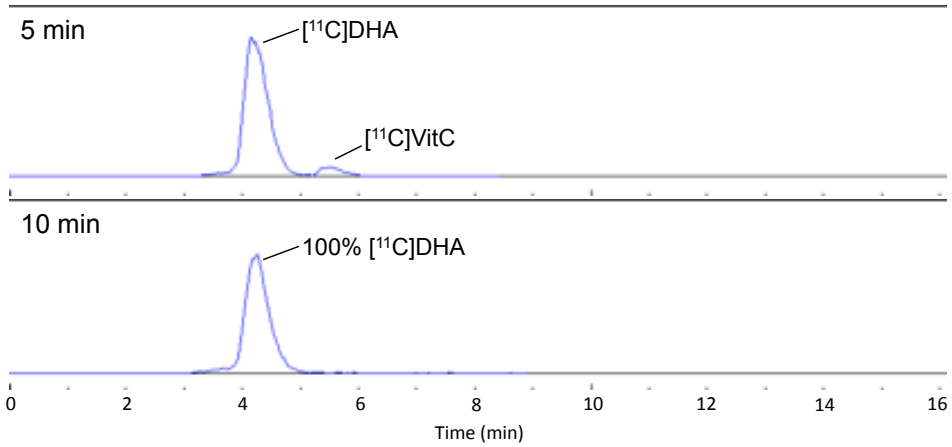

Figure S2: HPLC radio chromatogram monitoring oxidation of [^{11}C]VitC to [^{11}C]DHA.

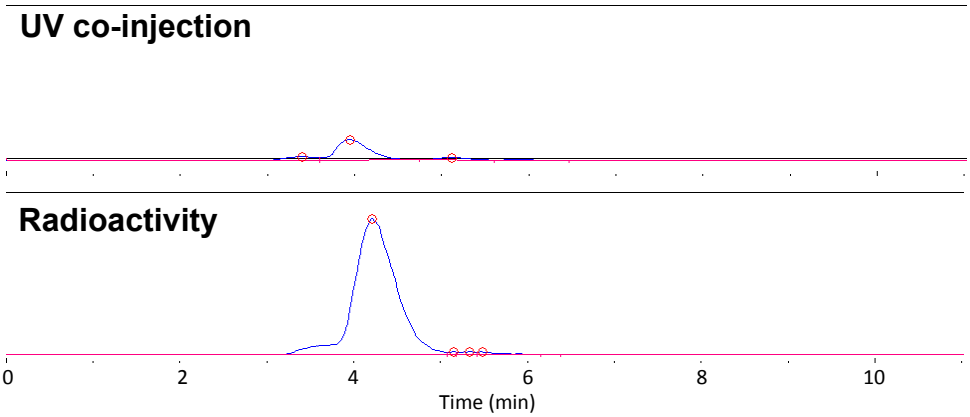

Figure S3: Analytical HPLC showing co-injection of [^{11}C]DHA with non-radioactive standard.

Carrier Free [^{11}C]VitC and Stability considerations

With no carrier added [^{11}C]VitC instability toward oxidation was noted at pH 7 (Figure S4). This may be due to acceleration of oxidation due to radiation effects, as previously described.[2] Stability of the radiotracer was monitored over time using varying concentrations of carrier added vitamin C (Figure S5). With the addition of ~1.0 mM carrier vitamin C (SA = 3.7 mCi/µmol; 137 MBq/µmol) >90% stability was observed at all time points tested.

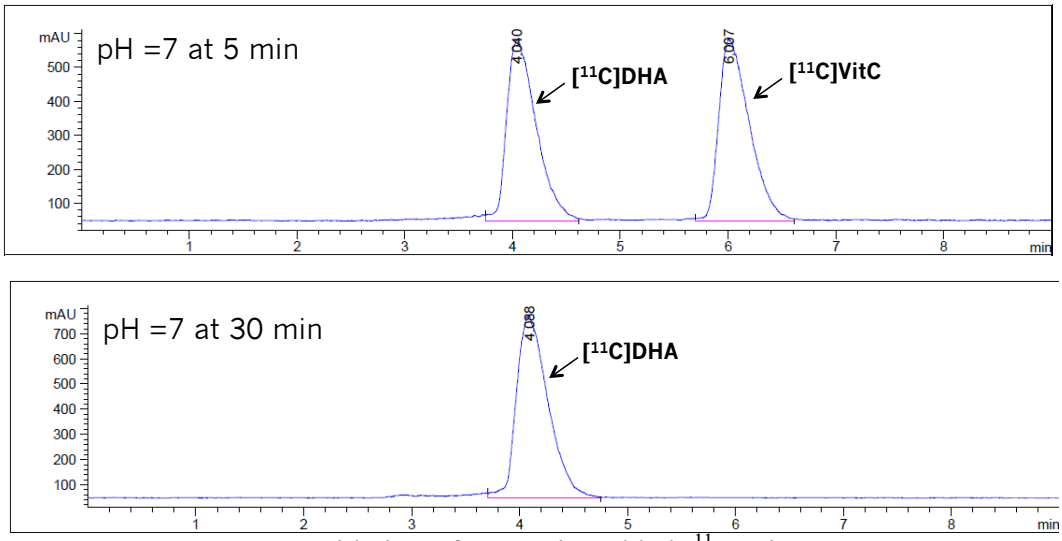

Figure S4: Oxidation of no carrier added [^{11}C]VitC at pH 7.

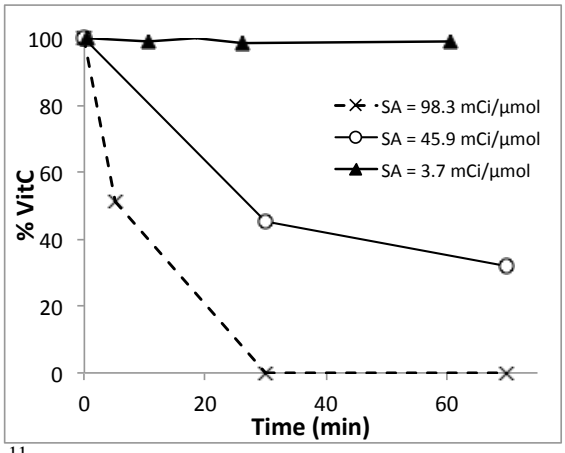

Figure S5: Stability of [^{11}C]VitC over time at pH 7.0 with the addition of carrier vitamin C.

[^{18}F]FDG Glucose Assay

Clinical samples of [^{18}F]FDG were obtained from the University of California Cyclotron Facility and allowed to decay for 24 h. Once the samples were no longer radioactive the amount of glucose present was determined using a colorimetric Glucose (GO) Assay Kit (Sigma Aldrich) measured at 540 nM.

ROS Sensitivity Assay

Sensitivity studies were preformed in the presence of ROS species known to be relevant in activated neutrophils (Figure S6). [^{11}C]VitC shows sensitivity toward oxidation in the presence of H_2O_2, O_2^- and $^{\bullet}OCl^-$ – All ROS species were tested at a concentration of 5 mM with 1 mM [^{11}C]VitC spiked vitamin C except for O_2^-, which was generated enzymatically at a rate of 24 µmol/min. Samples were analyzed at the given time points by HPLC. Percent of DHA was established by integration of the radiochemical peak at (t_r = 4.1 min).

H_2O_2 – 11.4 µL of 3% wt (0.88 M) H_2O_2 was added to 2 mL of 1 mM vitamin C in 20 mM pH 7.4 phosphate buffer.

O_2^- – Superoxide was generated enzymatically according to the method of Lippert *et al.*[3] in the presence of 1 mM vitamin C.

$^{\bullet}OCl^-$ – 6 µL 6.15% NaOCl was added to a 1.0 mL solution of 1.0 mM vitamin C in pH 7 phosphate buffer.

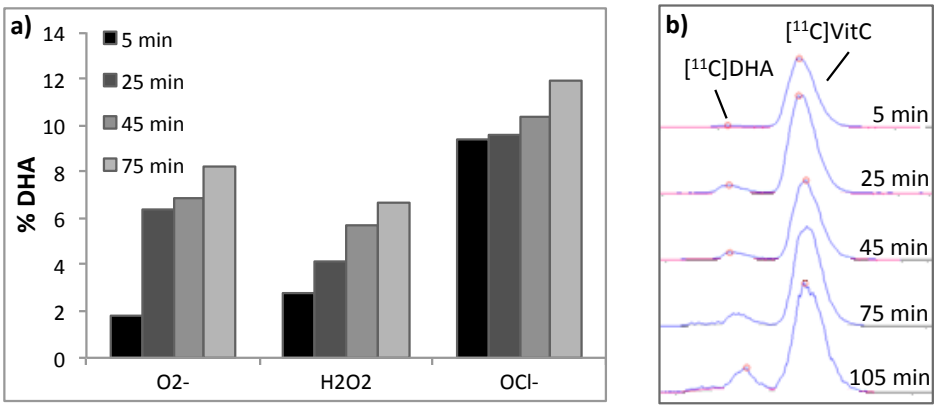

Figure S6: a) Response of 1mM vitamin C to various ROS. b) HPLC evaluation of ROS sensitivity for O_2^-.

Cell Culture

U87 MG transport of [^{11}C]VitC and [^{11}C]DHA

Grade IV glioblastoma multiforme cells (U87 MG) were cultured in DMEM (Invitrogen) with 10% FBS, penicillin/streptomycin in a humidified incubator at 37 °C and 5 % CO_2. U87MG were plated at 5 x 10^5 cells/well in the top well of a 12 wells plate (Corning, USA). U87 MG cells were washed and incubated in 1.0 mL buffer (15 mM pH 7.4 HEPES, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$). Alternatively in the case of Na (-) studies 135 mM LiCl was used in place of the NaCl as per Vera *et al*.[4] GLUT blocking was preformed by administration of 10 µg/mL cytochalasin B CB, EMD Millipore Biosciences) dissolved in DMSO. CB solutions were prepared from a stock solution of 1 mg/ml, which was stored frozen and protected from light. Control wells instead received the equal volume of DMSO (0.25% to 1% final DMSO concentration). ~10 µCi (0.37 MBq) of either [^{11}C]VitC, [^{11}C]DHA or ~5 µCi (0.185 MBq) of [^{18}F]FDG was added to each vial by bath application. Samples were incubated at 37^{o}C C and 5 % CO_2. Viability of cells was determined by MTT cell proliferation assay as described below. After 1h cells were centrifuged and the media was removed. Each sample was washed 3 times with 1 mL cold PBS. The cells were lysed with 1ml of 1M NaOH and collected. The remaining cell pellets were then counted using a Perkin Elmer gamma detector. Cell viability was determined using an MTT assay. Results are displayed as % Cell Associated Activity ± standard error with n = 4 for each condition (Figure 2).

MTT Cell Proliferation Assay

The viability of the cells was assessed using an MTT dye reduction assay (Sigma-Aldrich). The cells were seeded in a 96-well plate at a density of 1 × 10^4 cells/well, cultured for 24 hours, then the media was removed and replaced with different media condition according the cell uptake experiments. At the end of the treatment, MTT, 50 µg/10 µL, was added, and the cells were incubated for another 3 hours. MTT stock solution (5 mg/ml) is added to each culture being assayed to equal one-tenth the original culture volume and incubated for 3 to 4 hours. At the end of the incubation period the resultant formazan crystals were dissolved in dimethyl sulfoxide (100 µl) and the absorbance intensity measured by a microplate reader (TECAN Infinite M200) at 570 nm with a reference wavelength of 620 nm; all measurements were performed six times.

Neutrophil Studies

The human promyelocytic leukemia HL-60 cell line (provided by the core facility of UCSF) was routinely maintained in dishes incubated at 37 °C under 5% CO_2-balance air. HL-60 cells were maintained in RPMI 1640 medium (Life Technologies Inc.) supplemented with 10% heat-inactivated fetal bovine serum.

Freshly isolated neutrophils from human blood were obtained from AllCells LLC (Alameda, CA) and used same day. 1x106neutrophil cells or HL60 cells were suspended in 1 mL of Hanks media. Neutrophils were activated with the addition of 2 µm PMA and GLUT blocking was preformed with 20 µg/mL cytochalasin B. ~10 µCi (0.37 MBq) of either [^{11}C]VitC or [^{11}C]DHA was added by bath application. Samples were incubated at 37^{o}C. After 30 min cells were pelleted and the media was removed. Each sample was washed 3 times with 1 mL cold PBS. The remaining cell pellets were then counted using a Perkin Elmer gamma detector. Cell viability was estimated using a trypan blue assay. Results are displayed as % Cell Associated Activity ± standard error with n = 4 for each condition (Figure 4 and Figure S7).

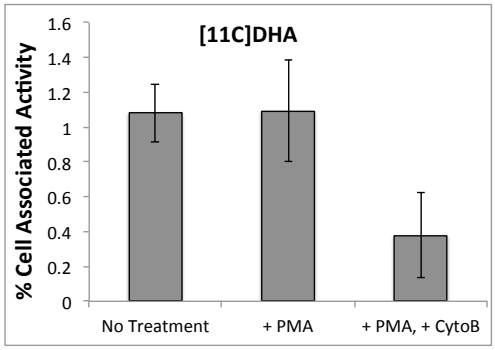

Figure S7: Uptake of [^{11}C]DHA in human neutrophils (+)/(-) 2 µm PMA and (+)/(-) 20 µg/mL cytochalasin B.

Animal Imaging Studies

Sprague Dawley rats (n = 3) were placed under isofluorane anesthesia and administered 250 ± 107 µCi (9.3 ± 4 MBq) [^{11}C]DHA each (SA = 9.2 ± 4.9 mCi/µmol; 340 ± 180 MBq/µmol) via tail vein catheter. A 30 min dynamic scan was preformed immediately upon injection using a Siemens Inveon microPET/CT scanner. Anesthesia was maintained during imaging using isofluorane. 15 days later, 170 ± 39 µCi (6.3 ± 1.4MBq) [^{11}C]VitC (SA = 2.7 ± 1.5 mCi/µmol; 100 ± 55 MBq/µmol) was then administered to the same animals and 40 min dynamic scans were preformed. Images shown (Figure 3 and Figure S8) are for entire scan time (t = 0 -1800 s). Ellipsoid regions of interest were drawn in the center portion of the brain and ROI analysis was preformed for all images using open source Amide software v1.0.5 (amide.sourceforge.net). Brain uptake (Figure 3) is expressed as mean % injected dose for brain ROI's ± standard deviation. At 1500 – 1800 seconds the median brain uptake for [^{11}C]VitC was 0.058 ± 0.006% ID/g while the median brain uptake for [^{11}C]DHA was 0.32 ± 0.05% ID/g. Organ distribution was determined by ROI analysis at 30 min post injection. All experiments were performed in compliance with the relevant laws and institutional guidelines. Experiments were approved by UCSF Institutional Animal Care and Use Committee (IACUC).

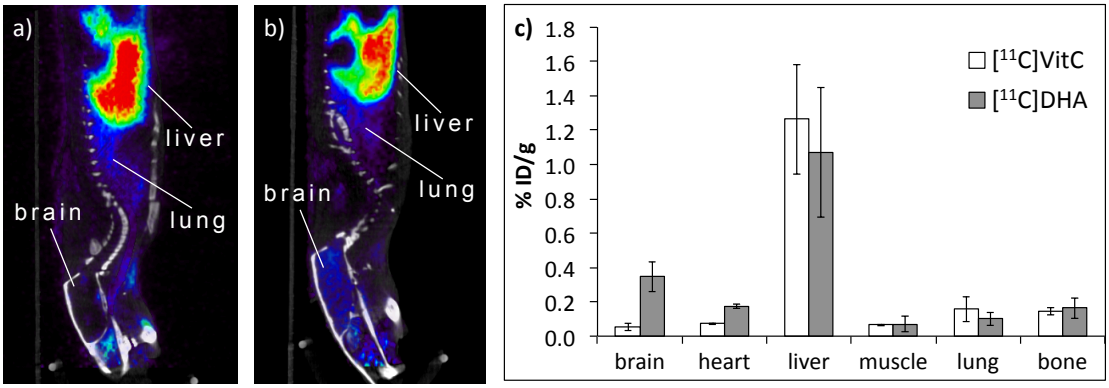

Figure S8: a) Whole body animal image for [^{11}C]VitC, b) Whole body animal image for [^{11}C]DHA and c) ROI analysis of key organs.

References:

1. Bohndiek, S. E.; Kettunen, M. I.; Hu, D.-e.; Kennedy, B. W. C.; Boren, J.; Gallagher, F. A.; Brindle, K. M., *Journal of the American Chemical Society* 2011, 133 (30), 11795.
2. Kothari, P. J.; Finn, R. D.; Bornmann, W. G.; Agus, D. B.; Vera, J. C.; Larson, S. M., *Radiochimica Acta*, 1997, 77, 87.
3. Lippert, A. R.; Keshari, K. R.; Kurhanewicz, J.; Chang, C. J., *Journal of the American Chemical Society* 2011, 133 (11), 3776.
4. Vera, J. C.; Rivas, C. I.; Velásquez, F. V.; Zhang, R. H.; Concha, I. I.; Golde, D. W., *Journal of Biological Chemistry* 1995, 270 (40), 23706.