Protein Modification by Endogenously Generated Lipid Electrophiles: Mitochondria as the Source and Target

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Supporting Methods

Materials. All chemicals were purchased from Sigma-Aldrich, St. Louis, MO, unless otherwise stated. *a*LA and *a*AA were synthesized as previously described[1].

KLA Preparation. KLA (Avanti Polar Lipids, Alabaster, AL) was stored at -20 °C in aliquots dissolved in sterile Dulbecco's Phosphate Buffered Saline (DPBS) at 1 mg/mL. Aliquots were diluted 1:10 in sterile DPBS and sonicated 15 min before being diluted to 100 ng/mL in culture medium[2].

Cell Culture. RAW264.7 macrophages (ATCC, Manassas, VA) were passaged in Dulbecco's Modified Eagle Medium + Glutamax (DMEM) (Invitrogen, Grand Island, NY) containing 10% fetal bovine serum (FBS, Atlas Biologicals, Fort Collins, CO). Cells were plated in the passaging medium to keep them near 50% confluence at the time of harvesting. After 24 h at 37 °C, the medium was replaced with serum-free DMEM with or without 15 μM *a*LA or 15 μM *a*AA. After 24 h at 37 °C, the cells were washed with serum-free DMEM to remove any *a*LA or *a*AA not incorporated into the cell membranes. The cells were then treated with serum-free DMEM with or without 100 ng/mL KLA for 24 h at 37 °C.

aLA Elongation to aAA. RAW264.7 macrophages were incorporated with *a*LA as described above. Cells were scraped into DPBS containing 1 μM butylated hydroxytoluene (BHT) and 1 mM tris(2-carboxyethyl)phosphine (TCEP), and counted on BioRad TC10 automated cell counter. Heptadecanoic acid was added to each cell pellet as an internal standard, and the pellets were taken up in 5% HCl. A 2:1 mixture of chloroform:methanol containing 50 mg/L BHT was added to each sample. Samples were vortexed, then centrifuged for 10 min at 15,000 rpm. The organic layer was removed and blown dry under stream of N_2, and dissolved in MeOH with 1.0 M KOH. Samples were vortexed, then incubated for 1 h at 37 °C. After incubation, samples

were immediately placed on ice and acidified with 1.0 M HCl. BHT/triphenyl phosphine (PPh_3) in ethanol was added to the samples, which were then extracted with a 4:1 chloroform:ethyl acetate mixture. The organic layer was dried under a stream of N_2, dissolved in methanol, and stored at -80 °C until analysis.

Reverse-phase high-performance liquid chromatography mass spectrometry analysis was performed on a Supelco Analytical Discovery C_{18} column (150 x 2.1 mm, 5 μm), which was eluted at 0.2 mL/min with methanol and 0.1% acetic acid. The effluent of the column was introduced by atmospheric-pressure chemical ionization (APCI) into a triple quadrupole mass spectrometer (TSQ, Thermo). The transfer capillary was heated to 300 °C, discharge current was set to 22.0 V, and the ion isolation width was set to 1. The collision energy was set at 10 V. Scans were recorded at 0.25 s intervals and utilized the Gaussian algorithm for peak smoothing (Xcalibur software).

Quantification of *a*LA or *a*AA-incorporated phospholipids. After incorporation with *a*LA or *a*AA and activation with KLA, the plates were placed on ice to stop the reaction and washed with 2 ml of ice-cold PBS. Cells were scraped in 1 ml cold PBS, split in two, and transferred into cold 1.5-ml microfuge tubes (Laboratory Product Sales, Rochester, NY, USA), pelleted and supernatant discarded. Half of each sample was lysed and protein quantified as described above for normalization after quantification. Glycerophospholipids were extracted using a modified Bligh and Dyer procedure[9]. Briefly, 800 μL of ice-cold 0.1 N HCl:CH_3OH (1:1) was added to the cell pellet, samples were homogenized by vortexing at 4 °C and 400 μL of cold $CHCl_3$ was added to the suspension. After vortexing for 1 min at 4 °C, the extraction proceeded with centrifugation (5 min, 4 °C, 18,000 x g) to separate the two phases. Lower organic layer was collected and solvent evaporated. The resulting lipid film was dissolved in 100 μL of

isopropanol:hexane:100 mM NH_4CO_2H(aq) 58:40:2 (mobile phase A). Quantification of glycerophospholipids was achieved by the use of an LC-MS technique employing synthetic (non-naturally occurring) diacyl and lysophospholipid standards. Typically, 200 ng of each odd-carbon standard was added per sample. Glycerophospholipids were analyzed on an Applied Biosystems/MDS SCIEX 4000 Q TRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) and a Shimadzu high pressure liquid chromatography system with a Phenomenex Luna Silica column (2 × 250 mm, 5-μm particle size) using a gradient elution as previously described[4]. The identification of the individual species, achieved by LC/MS/MS, was based on their chromatographic and mass spectral characteristic. This analysis allows identification of the two fatty acid moieties but does not determine their position on the glycerol backbone (*sn-1* versus *sn-2*)[4,5]. Standard curve slopes for alkyne-AA species were based the comparison of representative alkyne and natural glycerophospholipids, which were found to differ by ~30-50%.

Several steps were used to modify the quantitation method previously described[6] to allow for simultaneous estimation of phospholipid amounts for species containing labeled AA and naturally occurring species. First, a background subtraction of isobaric natural lipids was performed using the non-LA or non-AA treated control samples. This removes the influence of large natural peaks of other saturation levels at those *m/z*'s where *a*LA or *a*AA containing species also occur.

Generation of Samples for Click-blots. RAW264.7 macrophages were incorporated with *a*LA and activated with KLA as described above. TEMPOL or MitoTEMPO were both added to 10 μM, when present, at the same time as KLA. Cells were scraped, pelleted, and lysed in 1% IGEPAL (MP Biomedicals), 150 mM NaCl, 50 mM HEPES, and 0.5% mammalian protease

inhibitor cocktail. Electrophile adducts were stabilized to the proteome with 5 mM $NaBH_4$ for 1 h, and the reduction was quenched with acetone. Samples were diluted to 2 mg/mL total protein and precleared overnight at 4 °C with streptavidin sepharose beads (GE Healthcare). After preclearing, samples were diluted to 1 mg/mL, click reagents (1 mM $CuSO_4$, 1 mM TCEP, 0.1 mM tris[(1-benzyl-1H-1,2,3-triazole-4-yl)methyl]amine (TBTA), and 0.2 mM N_3-biotin) were added, and the samples were turned end over end for 2 h[7]. Proteomes were separated by SDS-PAGE (Bio-Rad), transferred to 0.45 μm nitrocellulose (Bio-Rad), and probed with goat anti-actin (Santa Cruz) primary and Streptavidin IRDye 800CW (LI-COR) overnight at 4 °C. Secondary anti-goat IGG IRDye 680LT antibody was from LI-COR and incubated with the blots for 1 h at 25 °C. Blots were visualized on a LI-COR Odyssey system scanning at 800 nm emission for streptavidin and 700 nm emission for actin.

SILAC RAW264.7 Macrophages Line. All SILAC reagents were from Pierce, Rockford, IL. RAW264.7 macrophages were passaged in SILAC DMEM containing 10% SILAC FBS. Heavy medium contained 0.1 mg/mL $^{13}C_6{}^{15}N_2$-lysine and $^{13}C_6{}^{15}N_4$-arginine, while the light medium contained 0.1 mg/mL $^{12}C_6{}^{14}N_2$-lysine and $^{12}C_6{}^{14}N_4$-arginine. After four passages, the cell lines were harvested, lysed, proteins separated by SDS-PAGE, and stained with SimplyBlue (Invitrogen). Similar molecular weight areas of the stained gel were excised for both the light and heavy lines, digested with trypsin, and analyzed by LC-MS/MS. The resulting comparison of heavy/light peptide ratios for each sample individually confirmed that the heavy and light samples have greater than 99% incorporation of the respectively labeled lysine and arginine[8].

SILAC Sample Preparation. Incorporation of SILAC samples with *a*LA was performed as above on both the heavy and light lines individually. Light cells were treated with vehicle, and represent the unactivated state, while heavy cells were treated with KLA, and represent the

activated state. After harvesting, the cells were lysed, and proteins quantified by BCA assay (Pierce). The heavy and light lines were combined in equal weights of protein, and adducts were stabilized with $NaBH_4$ and attached to 0.2 mM UV-biotin[9] (instead of N_3-biotin) via click chemistry as described above.

Streptavidin Affinity Purification. After attaching UV-biotin, the sample was dialyzed against PBS to remove any excess click reagents using 2,000 Da molecular weight cutoff dialysis cassettes (Pierce). After dialysis, the sample was added to streptavidin beads (GE Healthcare), and turned end over end overnight at 4 °C in the dark. The beads were washed twice each with 1% SDS, 4 M urea, 1 M NaCl in PBS, PBS, and water. Adducted proteins were eluted into water by stirring the beads under 365 nm light for 2 h[7].

SILAC Proteomic Analysis. Proteome (input) and adductome (eluate) samples were precipitated with 25% trichloroacetic acid on ice for 1 h. Following incubation, samples were centrifuged at 18,000 x *g* at 4 °C, and precipitates were washed with cold acetone, dried, and reconstituted in 50 mM Tris, pH 8.0, containing 50% 2,2,2-trifluoroethanol (TFE). Samples were reduced with TCEP, carbamidomethylated with iodoacetamide, diluted 5-fold with 100 mM Tris, pH 8, (to obtain a final solution containing 10% TFE), and digested with sequencing-grade trypsin overnight (Promega).

Digests were acidified to 0.1% formic acid, and peptides were loaded onto a self-packed biphasic C_{18}/strong cation exchange (SCX) MudPIT column using a helium-pressurized cell (pressure bomb). The MudPIT column consisted of 360 x 150 μm fused silica, which was fritted with a filter-end fitting (IDEX Health & Science) and packed with 6 cm of Luna SCX material (5 μm, 100 Å) followed by 4 cm of Jupiter C_{18} material (5 μm, 300 Å, Phenomenex). The MudPIT column was connected using an M-520 microfilter union (IDEX Health & Science) to an

analytical column (360 x 100 μm), equipped with a laser-pulled emitter tip and packed with 20 cm of C_{18} reverse phase material (Jupiter, 3 μm beads, 300 Å, Phenomenex). Using an Eksigent NanoLC Ultra HPLC and Autosampler, MudPIT analysis was performed with an 11-step salt pulse gradient (25, 50, 75, 100, 150, 200, 250, 300, 500, 750, and 1000 mM ammonium acetate). Following each salt pulse, peptides were gradient-eluted from the reverse analytical column at a flow rate of 500 nL/min, and the mobile phase solvents consisted of 0.1% formic acid, 99.9% water (solvent A) and 0.1% formic acid, 99.9% acetonitrile (solvent B). For the peptides from the first 10 SCX fractions, the reverse phase gradient consisted of 2–40% B in 90 min, followed by a 15 min equilibration at 2% B. For the last SCX-eluted peptide fraction, the peptides were eluted from the reverse phase analytical column using a gradient of 2-98% B in 100 min, followed by a 10 min equilibration at 2% B. Peptides were introduced via nano-electrospray into an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific), and the data were collected using a 17-scan event data-dependent method. Full scan (*m/z* 350-2000) spectra were acquired with the Orbitrap as the mass analyzer (resolution 60,000), and the sixteen most abundant ions in each MS scan were selected for collision-induced dissociation in the LTQ. An isolation width of 2 *m/z*, activation time of 10 ms, and 35% normalized collision energy were used to generate MS/MS spectra. The MS^n automatic gain control target value was set to 1×10^4, and the maximum injection time was set to 100 ms. Dynamic exclusion was enabled, using a repeat count of 1 within 10 s and exclusion duration of 15 s.

For peptide and protein identification, data were analyzed using the MaxQuant software package, version 1.3.0.5[10,11]. MS/MS spectra were searched against the *Mus musculus* subset of the UniprotKB protein database (June 2012 release). Precursor mass tolerance was set to 20 ppm for the first search, and for the main search, an 8 ppm precursor mass tolerance was used. The

maximum precursor charge state was set to 6. Variable modifications included carbamidomethylation of cysteines (+57.0214), oxidation of methionines (+15.9949), and acetylation of N-termini (+42.0106). Enzyme specificity was set to Trypsin/P, and a maximum of 3 missed cleavages was allowed. The target-decoy false discovery rate (FDR) for peptide and protein identification was set to 1% for peptides and proteins. A multiplicity of 2 was used, and Arg10 and Lys8 heavy labels were selected. For SILAC protein ratios, a minimum of 1 unique peptide and a minimum ratio count of 2 were required, and the requantify option was enabled. Protein groups identified as reverse hits, or contaminants were removed from the datasets. All reported protein groups were identified with two or more distinct peptides and were quantified with two or more ratio counts.

Correlation and differential expression. All MaxQuant normalized activated:unactivated were converted to $\log_2$(activated/unactivated). Only proteins that were common across all six samples were included in this analysis. Pairwise Spearman correlations were calculated comparing each proteome and adductome to every other proteome and adductome. We applied the *limma* package[12] to estimate the significance of the heavy/light ratio across three replicates of both the proteome and adductome. This method is similar to a single-sample t-test except it uses the empirical Bayes method to adjust for variable dependence.

Time course of adduction. RAW264.7 macrophages were incorporated with *a*LA and activated with KLA as described above. At 0, 3, 6, 9, 12, and 24 h post activation, adducted proteins were isolated using UV-biotin as described for the affinity enrichment for the SILAC experiments. Levels of individual adducted proteins were assessed by western blot using anti-Sod2 (Cayman Chemical) or anti-COX-2 (Cayman Chemical) primary antibodies both from rabbit hosts. Anti-rabbit IgG IRDye 800CW antibody from LI-COR was used as the secondary antibody.

WebGestalt pathway analysis. Analysis of adductome target location and pathway enrichment was performed in WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt/)[13]. Adductome protein lists with adjusted $P < 0.05$ were analyzed for cellular compartment enrichment. Additionally, the adductome and proteome proteins were analyzed for pathway enrichment using the WikiPathway function in WebGestalt. Uniprot accession numbers of the three proteome samples were analyzed in VENNY (http://bioinfogp.cnb.csic.es/tools/venny/index.html), an interactive tool for comparing lists with Venn Diagrams, and used as the reference proteome. Settings in WebGestalt were as follows, Statistical Method: Hypergeometric, Multiple Test Adjustment: Benjamini Hochberg (BH), Significance Level (adjusted P value): 0.05, Minimum Number of Genes for a Category: 3.

MitoTEMPO modulation of adduction. RAW264.7 macrophages were incorporated with *a*LA and activated with KLA as described above. MitoTEMPO was added to both vehicle- and KLA-treated samples at the same time as KLA, when present, as describe above. Cells were isolated, lipid electrophile adducts stabilized, and attached to UV-biotin by click chemistry as described. Adducted proteins were affinity purified with streptavidin beads and UV-elutes as described above. Total recovered protein was assessed by SDS-PAGE separation of the recovered adductome followed by Simply Blue staining. Levels of individual adducted proteins were assessed by western blot using anti-Sod2 or anti-COX-2 primary antibodies both from rabbit hosts. Anti-rabbit IgG IRDye 800CW antibody was used as the secondary antibody.

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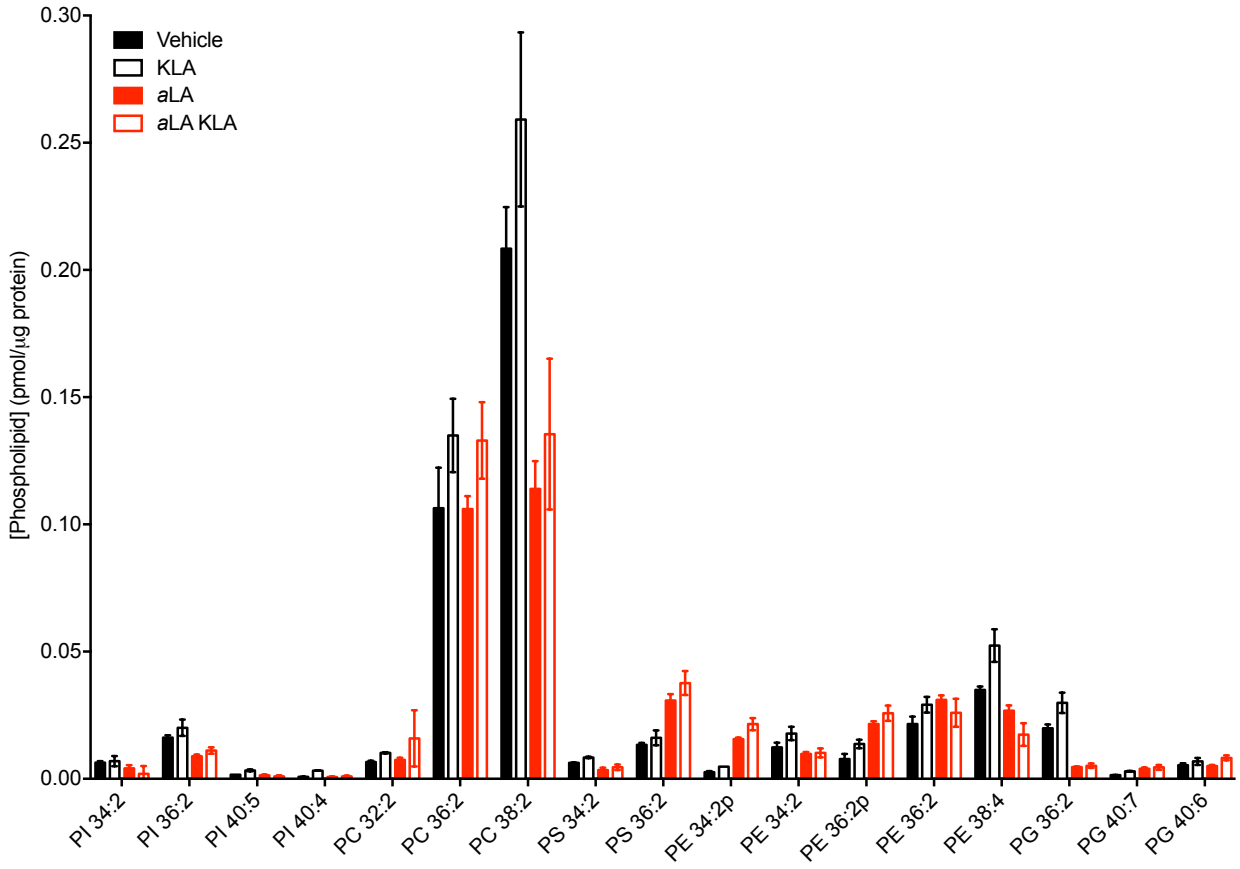

Figure S1. Incorporation of *a*LA across the phospholipid pool and changes in *a*LA and LA levels in response to KLA activation. Black bars represent LA-containing phospholipids in unenriched cells and red bars represent the corresponding *a*LA-containing phospholipid in *a*LA-enriched cells. LA and *a*LA levels reach similar amounts in the phospholipid pools, and do not decrease upon KLA activation. PI = phosphatidyl inositol, PC = phosphatidyl choline, PS = phosphatidyl serine, PE = phosphatidyl ethanolamine, and PG = phosphatidyl glycerol. Data are mean ± standard deviation of triplicate determinations normalized to total cellular protein.

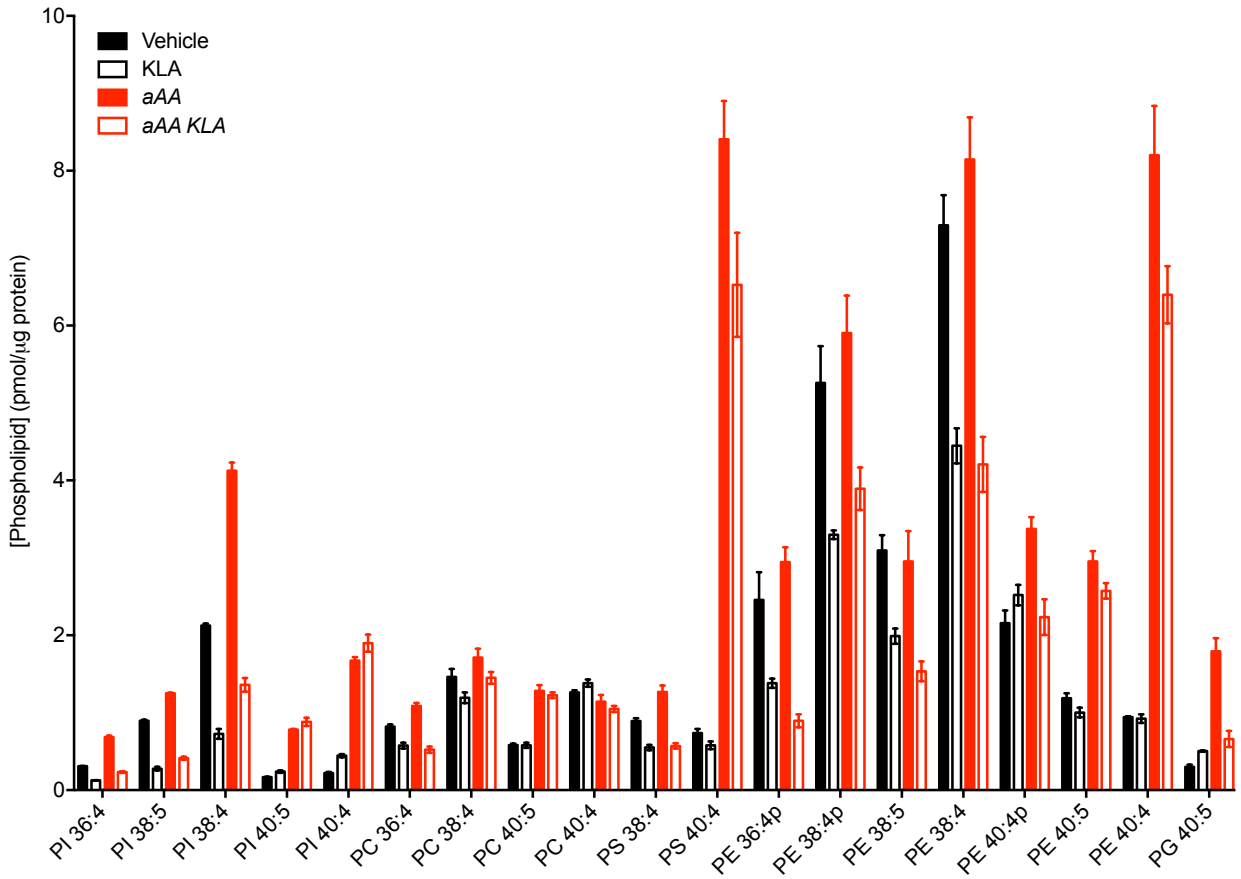

Figure S2. Incorporation of *a*AA across the phospholipid pool and changes in *a*AA and AA levels in response to KLA activation. Black bars represent AA-containing phospholipids in unenriched cells and red bars represent the corresponding *a*AA-containing phospholipid in *a*AA-enriched cells. When added to the medium, *a*AA levels reach higher amounts than native AA levels in the phospholipid pools, and decrease upon KLA activation, which is consistent with the known enrichment and release of AA by activated macrophages. PI = phosphatidyl inositol, PC = phosphatidyl choline, PS = phosphatidyl serine, PE = phosphatidyl ethanolamine, and PG = phosphatidyl glycerol. Data are mean ± standard deviation of triplicate determinations normalized to total cellular protein.

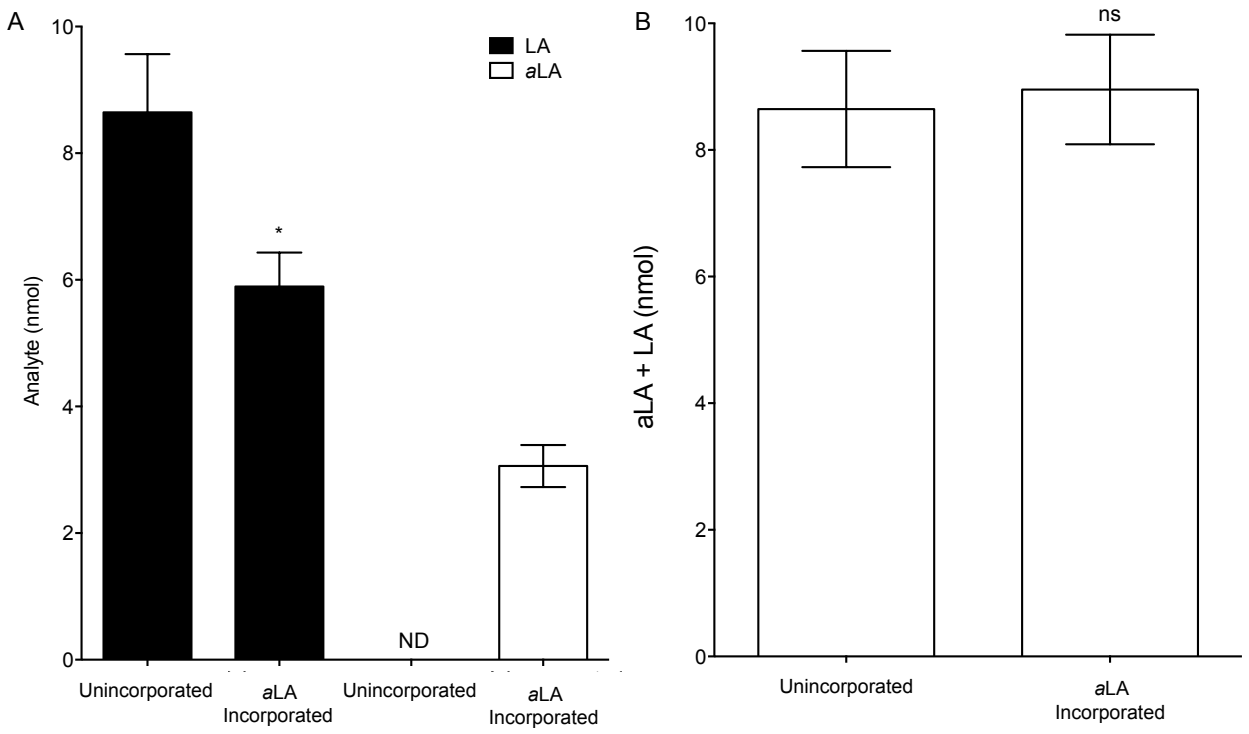

Figure S3. Total amounts of *a*LA and LA in RAW264.7 macrophages before and after *a*LA incorporation. **(A)** The total amount of LA decreases between unincorporated and *a*LA-incorporated macrophages. *a*LA is not detected in unincorporated macrophages, but is present in the *a*LA-incorporated macrophages in quantities approximately equal to the decrease in LA in those cells. **(B)** This results in no change in the total *a*LA +LA pool, indicating that *a*LA is displacing LA. Data are the mean ± standard deviation of triplicate determinations. Statistical significance was determined by an unpaired t-test where * = $P < 0.05$ and ns = $P > 0.05$.

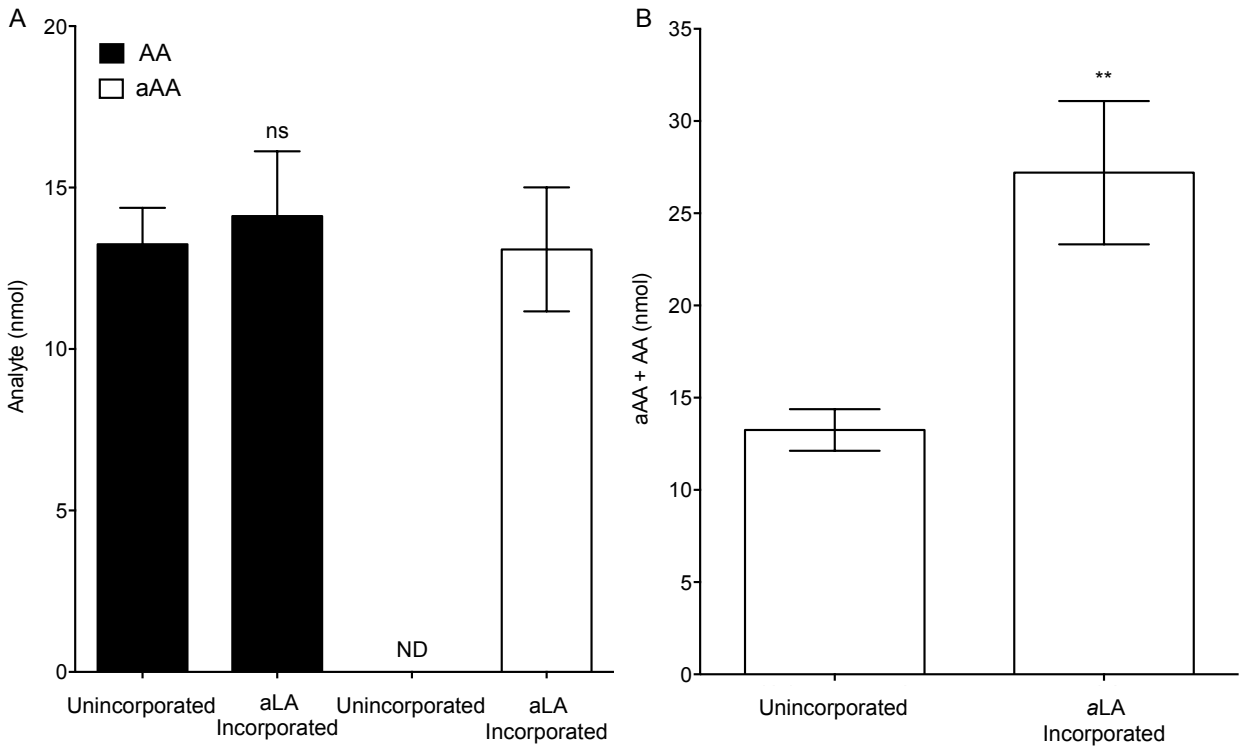

Figure S4. Total amounts of *a*AA and AA in RAW264.7 macrophages before and after *a*LA incorporation. **(A)** The total amount of AA does not change between unincorporated and *a*LA-incorporated macrophages. *a*AA is not detected in unincorporated macrophages, but is present in the *a*LA-incorporated macrophages in quantities approximately equal to the amount of AA in those cells. **(B)** This results in a significant increase in the total *a*AA + AA pool, indicating that *a*AA is not displacing AA. Data are the mean ± standard deviation of triplicate determinations. Statistical significance was determined by an unpaired t-test where ** = $P < 0.01$ and ns = $P > 0.05$.

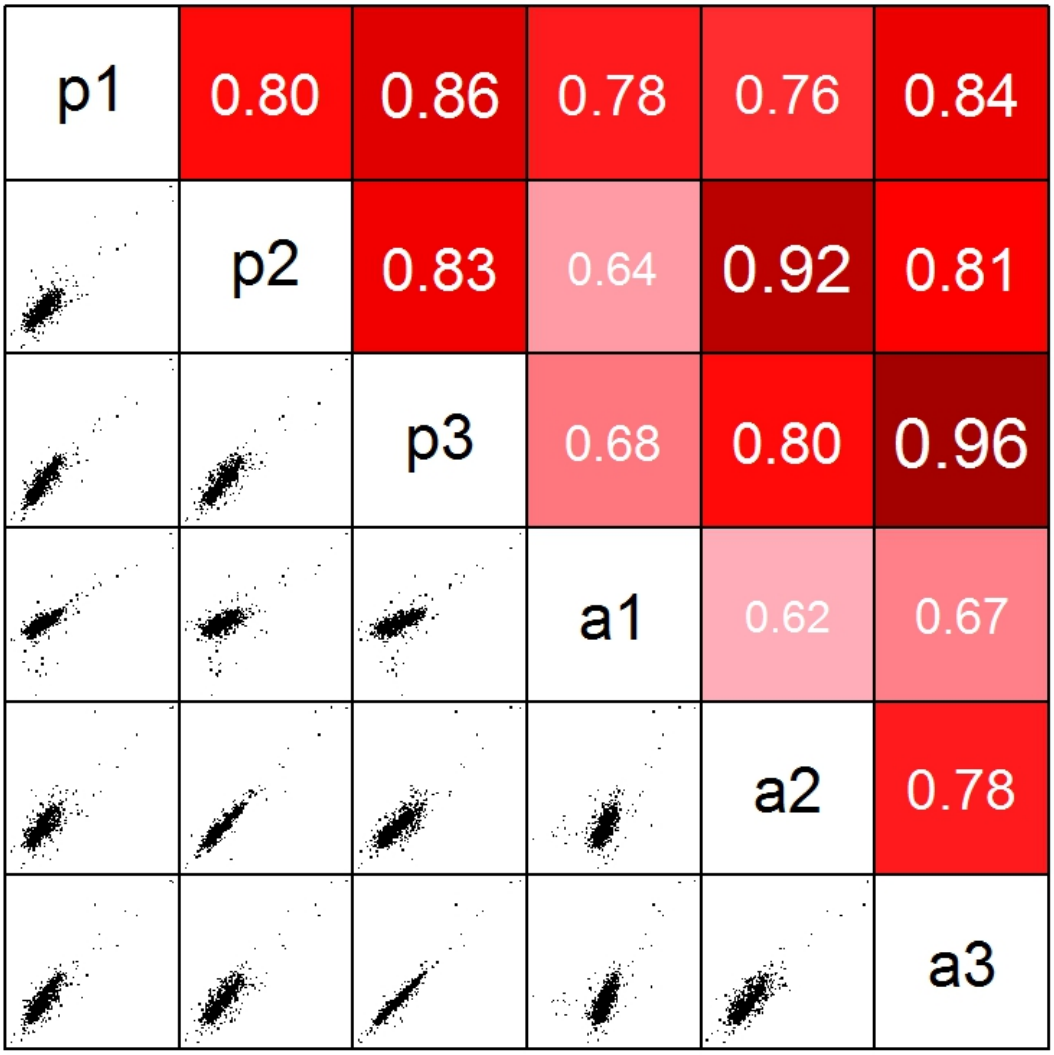

Figure S5. Spearman correlation coefficients relating the proteome and adductome replicates to each other. The heavy/light ratios were plotted for proteins common across all three proteomes (p1, p2, and p3) and adductome (a1, a2, and a3) replicates. Each replicate is labeled in the diagonal. The x-axis is the heavy/light ratio for the column replicate, and the y-axis is the heavy/light ratio for the row replicate. Each point represents a protein detected, and shows the intersection of its column and row heavy/light ratios. The Spearman correlation coefficients between replicates are given in the red squares. A ratio of 1 indicates a perfect correlation between the respective column and row. A high Spearman coefficient (> 0.75) between each proteome and adductome replicate indicates that adduction and induction are closely related for many proteins detected. However, this relationship does not necessarily indicate cause and effect for the two.

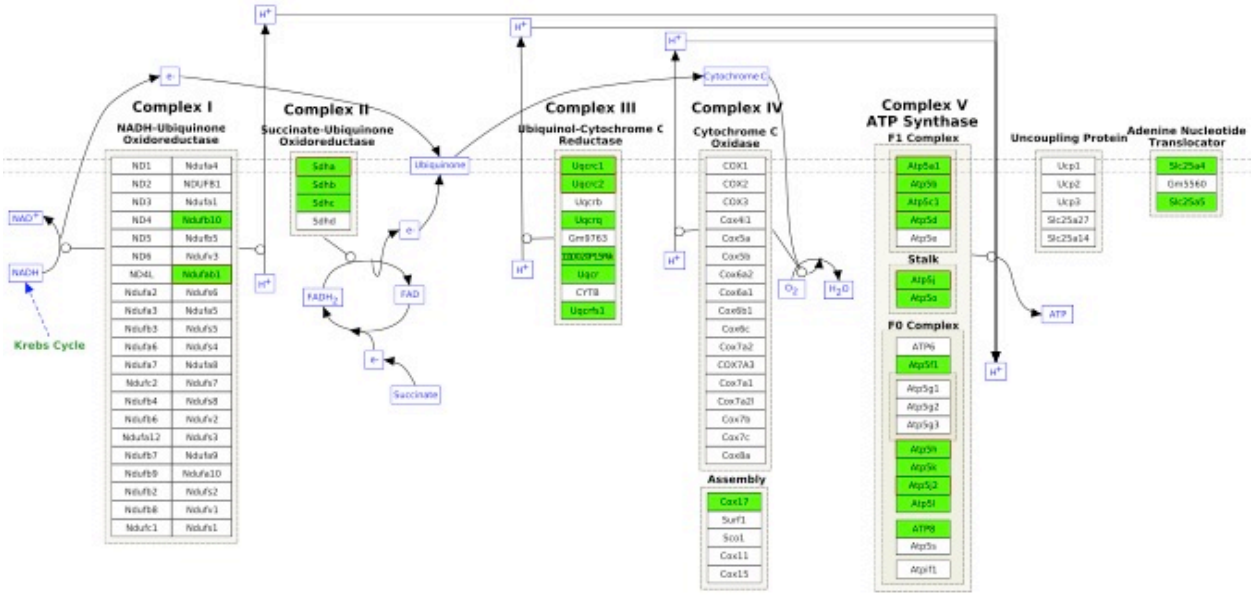

Figure S6. Complex V of the electron transport chain is the most differentially expressed pathway in activated macrophages. Electron transport chain proteins in the most differentially expressed class of protein targets are shaded in green. Expression can be seen in all of the subunits, with complex II, III, and V being the most heavily expressed. Figure modified from http://wikipathways.org/index.php/Pathway:WP295.

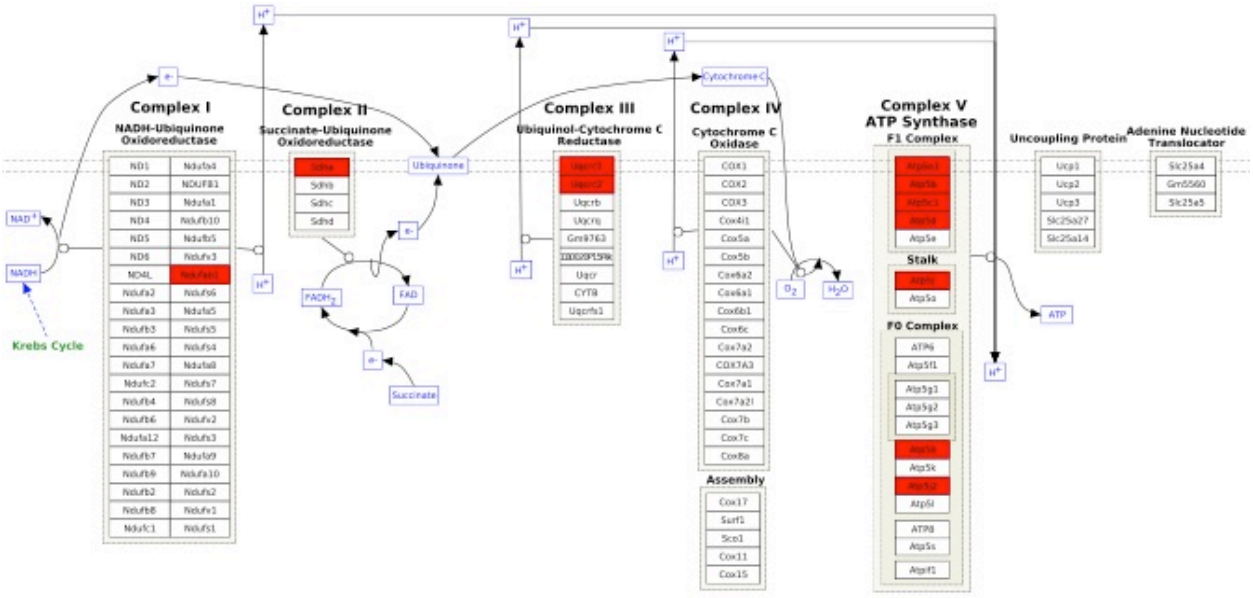

Figure S7. Complex V of the electron transport chain is the most differentially adducted pathway in activated macrophages. Electron transport chain proteins in the most differentially adducted class of protein targets are shaded in red. Adduction can be seen in all of the subunits except complex IV, with complex V (ATP synthase) being the most heavily adducted. Figure modified from http://wikipathways.org/index.php/Pathway:WP295.