Supplementary materials for:

Ceramide initiated protein phosphatase 2A activation contributes to arterial dysfunction in vivo

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Supplementary Figure 1.
Ceramide assessment via immunofluorescence (A-D). These data are supplementary to Figure 1 of the main manuscript. After the respective treatments, cells were fixed with 4% paraformaldehyde, permeabilized in 0.1% triton in PBS, blocked with 5% BSA PBST, and incubated overnight with 1º antibody to ceramide (Enzo Life Science, Farmington, NY). Next, cells were incubated with a fluorophore Alexa-488 tagged 2º antibody. 8 wells x 4 fields / well x 12 cells / field were imaged at 4X or 60X magnification using confocal microscopy. (1;2) (A) Endogenous ceramide in cells incubated with vehicle (BSA), 500 or 700 uM palmitate (pal), 100 uM synthetic C2 ceramide (C2), or control (no antibody) for 3 h. (B) ECs incubated for 3 h with 500 uM palmitate, 250 µM N-oleolyethanolamine (NOE) - an inhibitor of ceramidase, or control. (C) ECs incubated with 500 uM palmitate, 1 mM L-cycloserine (LC) - an inhibitor of serine palmitoyl transferase activity, or control. (D) Compared to vehicle-treated cells, none of these treatments increased cell death.

Protein colocalization following pharmacological inhibition of ceramide biosynthesis (E-J). Protein colocalization experiments were performed in the absence and presence (i.e., ±) of palmitate and myriocin. Images were captured using an Olympus FV1000 confocal microscope (X60), and data from 8 wells x 10 fields / well x 10 cells / field were quantified and used for analysis. (1;2) 1º antibody to eNOS (Cell Signaling Technology, Boston, MA), PP2A (EMD Millipore, Billerica, MA), I2PP2A (Santa Cruz Biotechnology, Dallas, Texas) and ceramide (Enzo Life Science, Farmingdale, NY) were used according to guidelines provided by the manufacturer. The membrane stain wheat germ agglutinin (WGA) was used to differentiate the membrane from the cytosol. Palmitate increased ceramide and I2PP2A co-localization (E, F), decreased I2PP2A co-localization with PP2A (G,H), and increased PP2A association with eNOS (I,J). These palmitate-induced effects were negated by myriocin. Representative cell images (E,G,I) and the corresponding mean data (F,H,J) are presented. Embedded scale bar = 50 µm. Histograms represent mean ± SE of 8 wells x 4 fields per well x 12 cells per field. For A-C n=8. *p<0.05 vs. veh; # vs. 500 uM pal (A) or vs. pal (B, C). For D, n=3 each “n” refers to 1 well of a 6-well plate. For E-J n=8 per treatment.
Supplementary Figure 2.
Protein colocalization following genetic inhibition of ceramide biosynthesis (A-J). These data are supplementary to Figure 1 of the main manuscript. A genetic approach to limit ceramide accumulation was used. Serine palmitoyl transferase (SPT) contains 2 subunits: long chain base 1 (SPTLC1) and base 2 (SPTLC2). Interactions between both are required for optimal SPT activity in vivo (3). siRNA-mediated knockdown of SPTLC1 protein expression was performed using Lipofectamine 2000® and standard procedures (4). siRNA sequences are provided in Supplementary Table 1. Cells transfected with SPTLC1 siRNA had > 75% knockdown of SPTLC1 protein content vs. cells transfected with scrambled (control) siRNA (n=8-10 per treatment) (A). Representative image (B) and mean data (C) indicate that palmitate-induced ceramide accumulation occurs in control ECs but not in ECs after siRNA-mediated reduction of SPTLC1. Palmitate promoted ceramide colocalization with I2PP2A (D, E), disrupted I2PP2A and PP2A association (F, G), and increased PP2A interaction with eNOS (H, I) in control ECs but not in cells after SPTLC1 knockdown. (J) Cell viability was similar between groups. A and B (n=8-10 per treatment), D-I (n=8 wells x 4 fields per well x 12 cells per field. Representative images (D, F, H) and mean data (E, G, I) are shown. Embedded scale bar = 50 µm. Data are mean ± SE. *p<0.05 vs. all treatments.
Supplementary Figure 3.
Signaling kinase activation of eNOS in ECs ± palmitate. These data are supplementary to Figure 2 of the main manuscript. Inhibiting PP2A with LB1 has the potential to alter kinase signaling to eNOS. To address this, ECs were incubated ± palmitate ± LB1. For the last 10-min of the 180-min protocol, ECs were stimulated with 100 nM insulin (ins) or vehicle (veh; saline). (5;6) Compared to veh-treated cells i.e., (-) palmitate (-) LB1 (-) ins, 100 nM ins increased p-AktS473:Akt (A), p-AktT308:Akt (B) and p-ERK1/2 : ERK1/2 (D), whereas p-AMPK T172 : AMPK (C) was unaltered. Neither treatment with palmitate nor treatment with LB1 affected these responses. The positive regulatory site (p-eNOS S617 ) and negative regulatory site (p-eNOS T495 ) on eNOS also were assessed. (E) Palmitate-induced suppression of ins-stimulated p-eNOS S617 :eNOS was restored by LB1, whereas p-eNOS T495 was not altered by palmitate or insulin (F). Images above are representative of the histograms below. For panels A-F n=4, each “n” refers to 1X10 cm Petri dish. For each treatment shown in panel A-D, F, representative blots are shown with lines above the images, indicating equivalently treated samples. Data are mean ± SE. *p<0.05 vs. veh treated cells.
Supplementary Figure 4. Assessment of NO generation using EPR. These data are supplementary to Figure 2 of the main manuscript. Measures of NO production were made using EPR spectroscopy. (A) BAECs were grown to 70-80% confluency in six-well plates and exposed for 60-min to vehicle (buffer only) and 1, 10, or 100 µM of the NO donor diethylammonium (DEA) NONOate to serve as a positive control. DEA NONOate was dissolved in 1mM sodium hydroxide. This experiment was used to establish a dose-response relationship and a representative image is shown in Panel B. (C). BAECs were treated with 600 µM of the NO agonist insulin, 10 µM of the NO synthase inhibitor N\textsuperscript{G}-monomethyl-L-arginine acetate salt (L-NMMA), or insulin + L-NMMA. This experiment was performed to establish the sensitivity and specificity of our measurement technique and a representative image is shown in Panel D. All treatments contained L-arginine and the spin trap FeSO\textsubscript{4} + sodium diethyldithiocarbamate trihydrate (DETC; Noxygen Science Transfer Diagnostics, Denzlingen, Germany). After the respective treatments, BAECs were dislodged from each plate, collected in a 1 ml syringe, and frozen in liquid nitrogen after an aliquot was obtained to determine protein content of the sample. At the time of analysis, frozen samples were loaded in a finger dewar with liquid nitrogen, and scanned using the EMXmicro EPR Spectrometer (Bruker Biospin Corporation, Billerica, MA) at the following settings: microwave frequency: 9.35 GHz; centerfield: 3277 G; sweep width: 80 G; sweep time: 10.0 s; receiver gain: 30 dB; modulation amplitude: 9 G; # scans: 10; attenuation: 10 dB; power: 20 mW; digital filter: manual mode with 1 point; conversion time: 112.36 ms; and time constant: 10.24 ms. All values were normalized to protein content. (E) Procedures were identical for experimental interventions described in the main text except that the following treatments were performed for 180-min: (i) vehicle (V), (ii) 500 µM palmitate, (iii) 4 µM LB1, or (iv) P + LB1. A representative image of mean data from Fig. 2 E of the main manuscript is shown in Panel E. For A, C, n=8-12, each “n” refers to 3 wells of a 6-well plate. Data are mean ± SE. *p<0.05 vs. veh treated cells.
Supplementary Figure 5.
Contribution from PP2B and PP1 to palmitate-induced reduction in p-eNOS$^{S1177}$ : eNOS. These data are supplementary to Figure 2 of the main manuscript. (A) The contribution of phosphatases PP2B and PP1 in dephosphorylating eNOS was assessed after treatment with cyclosporine A (CsA) and tautomycin (Tau). Palmitate induced decreases of p-eNOS$^{S1177}$ were refractory to treatment with either CsA or Tau. (B) No significant cell death was observed in cells treated with LB1, CsA, Tau or LNMMA vs. veh treatment. For A, n=8, each “n” refers to 1X10cm petri dish. For B, n=3, each “n” refers to 1 well of a 6- well plate. Data are mean ± SE. *p<0.05 vs. veh treated cells.
Supplementary Figure 6. Palmitate disrupts the interaction between I2PP2A and PP2A and increases PP2A colocalization with eNOS. These data are supplementary to Figure 3 of the main manuscript. Representative images (A) and mean data (B) are shown. Palmitate treatment decreased I2PP2A that coimmunoprecipitated with PP2A (A) and increased eNOS that associated with PP2A (B). These effects of palmitate were not altered by PP2A inhibition using LB1 because ceramide accumulation is refractory to LB1 treatment. Confocal microscopy images of fluorescently labeled eNOS and PP2A (C, D) support the immunoprecipitation / immunoblotting results that PP2A associates with eNOS regardless of LB1 treatment. It is noteworthy that PP2A activity is inhibited in the presence of LB1 (Fig. 2 B, C of the main manuscript). For A and B, n=4-6 each “n” refers to 4X 10 cm Petri dishes. For C and D n=8 wells of a 24 well plate per treatment. Embedded scale bar = 50 µm. Data are mean ± SE. *p<0.05 vs. veh treated cells.
SUPPLEMENTARY DATA

A. Graph showing fold change for I2PP2A and PP2A with ins, pal, and +LB1 conditions.

B. Graph showing fold change for eNOS and PP2A with ins, pal, and +LB1 conditions.

C. Images showing eNOS, PP2A, WGA, and eNOS + PP2A with veh, pal, LB1, and pal + LB1 conditions.

D. Graph showing fold change for eNOS:PP2A with pal and LB1 conditions.
Supplementary Figure 7. I2PP2A, PP2A, and p-eNOS in the cytosolic component of the cell. These data are supplementary to Figure 4 of the main manuscript. In ECs incubated ± palmitate ± myriocin ± LB1, the cytosolic fraction was obtained, and immunoblotting was performed to verify successful fractionation. Cytosolic I2PP2A (A) was similar among treatments. Palmitate decreased cytosolic PP2A in the absence and presence of LB1, whereas myriocin negated this effect (B). Insulin-decreased cytosolic p-eNOSS1177:eNOS, an effect that was negated by palmitate, and restored by concurrent treatment with myriocin or LB1 (C). For panels A-C n=4, each “n” refers to 10X10 cm Petri dish. *p<0.05 vs. veh treated cells, † p=0.06 vs. veh treated cells.
**Supplementary Figure 8.**
**Body composition of lean and obese mice.** These data are supplementary to Figure 6E in the main manuscript. Time-domain, nuclear magnetic resonance (TD-NMR) imaging (Bruker, Minispec) was used to assess fat mass, lean mass, and fluid mass in conscious mice fed Con or HF chow ± LB1. Results are expressed as a percentage of total body weight. Fat mass was elevated in fat-fed mice regardless of LB1 treatment. Data are mean ± SE for 8-10 mice per group. *p<0.05 vs. respective Con mice.
Supplementary Figure 9.
Control data for IP experiments. These data are supplementary to Figures 1, 3, 5, 7 in the main manuscript. ECs were grown and IP experiments were performed after vehicle treatment as described. (A) Lane 1. After eNOS IP, IB was performed to quantify p-AktS473, Hsp90, eNOS, and PP2A. Lane 2. The eNOS antibody was directly added to lysate buffer (no sample) and IB was performed. Lane 3. IB was performed using whole cell lysate buffer (no sample) and IB was performed. Lane 4. IB was performed using supernatant of whole cell lysate after eNOS IP. (B) All procedures were similar to those described in panel A after Akt IP(data not shown). (C) HA tag IP was performed followed by HA-IB in cells transfected with either HA-tagged wt PP2AC (Lane 1, 2) or catalytic site mutant PP2AC (Lane 3, 4). The anti HA antibody was directly added to lysate buffer (no sample) and IB was performed (Lane 5). IB was completed using whole cell lysate (Lane 6) or the supernatant of the whole cell lysate after HA IP (Lane 7).
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Supplementary Table 1. Primers and siRNA sequences

| Primer                   | Primer sequences                      |
|-------------------------|---------------------------------------|
| Genotyping of Des \(1^{+/+}\) |                                       |
| F 86                    | GGACAAATTGTTGGGTATG                   |
| LTR                     | AAATGGCGTTACTTAAGCTAGCTTG            |
| R466                    | GCTAGTCACTCTGATTTTG                  |
| SPTLC1 siRNA            | Sense- 5’-GCUCCUGCUUAUCAUCUUA5T-3’   |
|                         | Anti sense- 5’UAAGAUGAUAGCAGGAGGC5T-3’|
| Non-targeting control siRNA | Sense 5’-GCUUCGUCUAUCAUCUUA5T-3’    |
|                         | Antisense 5’-UAAGGUAGAUAGACGAAGC5T-3’|
| I2PP2A siRNA            | Sense 5’-CAAGCCAGUGAGGAGAUUU-3’      |
|                         | Anti sense 5’-AAAUCUCCUCACUGCUUG-3’  |

The non-targeting siRNA was obtained from Dharmacon Inc, (Lafayette, CO)
Supplementary Table 2. V, vehicle, 3 d pretreatment with saline; Con, control, a combination of wild-type (WT) mice and des1+/+ mice pre-treated with V and infused with glycerol (G) for 6h; LO, 20% lard-oil infusion for 6h; HET, mice heterozygous for des1 i.e., des1+/− mice; KCL, potassium chloride; ACh, acetylcholine; SNP, sodium nitroprusside; LB1, PP2A inhibitor Lixte Biotechnology 1. These vessels were used for data shown in the main manuscript, Fig. 5 G-J. Mice were anesthetized with 2-5% isoflurane. After opening the chest and obtaining a blood sample via a cardiac puncture, the entire aorta, both iliac arteries, and the femoral arteries were dissected free from adherent tissue. Femoral arteries were isolated distal to the bifurcation of the internal iliac artery. During dissection, tissues were bathed in ice-cold, oxygenated normal physiological saline solution (NPSS). Aorta and iliac arteries were frozen in liquid nitrogen and used for immunoblotting (Figure 5 D-F), and segments of femoral artery were mounted on a wire-type myograph while immersed in a temperature-controlled, 8 ml tissue bath containing NPSS. After the arteries were mounted, the tissue bath was warmed over 30-min to 37°C with vessels at 0 mg tension. Throughout each experiment, pH and temperature of all buffer solutions were checked at 20-30-min intervals, and contents of the tissue bath were exchanged at 15-min intervals. When the vessel chamber reached 37°C, tension on femoral arteries was increased manually over 8-min to 200 mg (i.e., 50 mg every 2-min). Final tensions did not differ among groups. Thirty-min later, a series of internal circumference-active tension curves was constructed to determine the vessel diameter that evoked the greatest tension development (L_max) to 100 mM KCl. L_max tension did not differ among groups. Thirty-min later, vasocontractile responses to potassium chloride (KCl, 10-100 mM) and phenylephrine (PE, 10^-8-10^-5 M) were assessed. Next, after arteries were precontracted with PE to approximately 65% of maximal developed tension and stability was achieved, responses to: acetylcholine (ACh, 10^-8-10^-4 M; to determine endothelium-dependent vasorelaxation); and sodium nitroprusside (SNP, 10^-9-10^-4 M; to determine endothelium-independent vasorelaxation) were evaluated. Each experimental protocol was separated by at least 30-min. All tension data were recorded continuously by a computer through an analog-to-digital interface card (Biopac Systems Inc., Santa Barbara, CA) that allowed for subsequent off-line quantitative analyses.(4-6)

| Table 2. Vessel characteristics: infusion study |
|-----------------------------------------------|
| Variable                                      |
| V-CON-G                                       |
| V-LO-WT                                       |
| V-LO-HET                                      |
| LB1-LO-WT                                     |
| Start width, μm                               |
| 200 ± 8                                       |
| 173 ± 7                                       |
| 184 ± 5                                       |
| 184 ± 4                                       |
| End width, μm                                 |
| 486 ± 6                                       |
| 476 ± 8                                       |
| 481 ± 5                                       |
| 476 ± 4                                       |
| Length, μm                                    |
| 1831 ± 24                                     |
| 1859 ± 29                                     |
| 1753 ± 36                                     |
| 1853 ± 17                                     |
| Tension at L_max                              |
| 704 ± 20                                      |
| 634 ± 17                                      |
| 664 ± 13                                      |
| 680 ± 6                                       |
| % precontraction - ACh                        |
| 66 ± 2                                        |
| 63 ± 3                                        |
| 66 ± 2                                        |
| 64 ± 2                                        |
| % precontraction - SNP                        |
| 66 ± 4                                        |
| 80 ± 6                                        |
| 75 ± 4                                        |
| 69 ± 2                                        |
Supplementary Table 3. Control, Con, mice that consumed standard chow for 14 weeks; Vehicle, V, daily IP treatment with saline from weeks 12-14; High-fat, HF, mice that consumed HF chow for 14 weeks; LB1, daily IP treatment with the PP2A inhibitor Lixte Biotechnology 1 (LB1) from weeks 12-14. KCL, potassium chloride; ACh, acetylcholine; SNP, sodium nitroprusside. These vessels were used for data shown in the main manuscript, Fig. 8 A-F. Mice were anesthetized with 2-5% isoflurane. After opening the chest and obtaining a blood sample via a cardiac puncture, the entire aorta, both iliac arteries, and the femoral arteries were dissected free from adherent tissue. Femoral arteries were isolated distal to the bifurcation of the internal iliac artery. During dissection, tissues were bathed in ice-cold, oxygenated NPSS. The aorta and iliac arteries were frozen in liquid nitrogen and used to obtain immunoblotting results shown in Figure 7 B-E of the main manuscript. Segments of femoral artery were mounted on a wire-type myograph while immersed in a temperature-controlled, 8 ml tissue bath containing NPSS. After the arteries were mounted, the tissue bath was warmed over 30-min to 37°C with vessels at 0 mg tension. Throughout each experiment, pH and temperature of all buffer solutions were checked at 20-30-min intervals, and contents of the tissue bath were exchanged at 15-min intervals. When the tissue bath reached 37°C, tension on femoral arteries was increased manually over 8-min to 200 mg (i.e., 50 mg every 2-min). Final tensions did not differ among groups. Thirty-min later, a series of internal circumference-active tension curves was constructed to determine the vessel diameter that evoked the greatest tension development (L$_{\text{max}}$) to 100 mM KCl. L$_{\text{max}}$ tension did not differ among groups. Thirty-min later, vasocontractile responses to potassium chloride (KCl, 10-100 mM) and (30-min later) phenylephrine (PE, 10$^{-8}$-10$^{-5}$ M) were assessed. Next, subsets of arteries were treated with N$^G$ monomethyl-L-arginine (L-NMMA, 10$^{-3}$ M; to attenuate nitric oxide synthase enzyme activity) or vehicle for 20-min, and responses to 10$^{-8}$-10$^{-5}$ M PE again were assessed (i.e. in the absence or presence of NO synthase inhibition). Next, subsets of arteries were treated with L-NMMA or vehicle for 20-min, vessels were precontracted with PE to approximately 65% of maximal developed tension, and responses to acetylcholine (ACh, 10$^{-8}$-10$^{-4}$ M; to determine endothelium-dependent vasorelaxation) were assessed. Finally, all arteries were precontracted with PE to approximately 65% of maximal developed tension, and responses to sodium nitroprusside (SNP, 10$^{-9}$-10$^{-4}$ M; to determine endothelium-independent vasorelaxation) were evaluated. All experimental protocols were separated by at least 30-min. All tension data were recorded continuously by a computer through an analog-to-digital interface card (Biopac Systems Inc., Santa Barbara, CA) that allowed for subsequent off-line quantitative analyses.(4-6)

| Table 3: Vessel characteristics: feeding study |
| Variable | CON-V | HF-V | CON-LB1 | HF-LB1 |
| Starting width, μm | 231 ± 33 | 169 ± 10 | 175 ± 5 | 164 ± 8 |
| End width, μm | 483 ± 15 | 486 ± 9 | 496 ± 19 | 484 ± 11 |
| Length, μm | 1796 ± 39 | 1768 ± 28 | 1898 ± 34 | 1804 ± 39 |
| Tension at L$_{\text{max}}$ | 708 ± 20 | 726 ± 49 | 762 ± 20 | 761 ± 14 |
| % precontraction - Ach | 69 ± 3 | 62 ± 7 | 61 ± 2 | 69 ± 3 |
| % precontraction - SNP | 66 ± 4 | 69 ± 5 | 67 ± 4 | 73 ± 4 |
Supplementary Table 4.

| Intravascular signaling kinases | CON     | HF       | CON +LB1 | HF+LB1   |
|---------------------------------|---------|----------|----------|----------|
| p-Akt 473 / Akt                 | 1.00±0.06 | 0.877±0.15 | 1.27±0.49 | 1.01±0.12 |
| p-AMPK T172 / AMPK              | 1.00±0.15 | 1.08±0.13 | 0.82±0.17 | 1.07±0.11 |
| p-ERK1/2 / ERK1/2               | 1.00±0.10 | 0.72±0.07 | 0.88±0.09 | 0.74±0.09 |

Signaling kinase activation: vessels from obese mice. This table is supplementary to Figure 7 in the main manuscript. p-eNOS<sup>S1177</sup>:eNOS was lower in arteries from HF-V mice vs. all other groups. To determine whether disrupted signaling kinase activation of eNOS contributed to this observation, p-Akt<sup>S473</sup>:Akt (A), p-AMPK<sup>T172</sup>:AMPK (B) and p-ERK1/2:ERK1/2 (C) were assessed in vascular homogenates from the same mice. Consistent with our previous investigations (4;5) none were altered in response to fat-feeding or LB1 treatment. n=3, each “n” comprised of vascular homogenates from two mice of the same treatment. Data is expressed as a fold change from the control (CON) treatment.