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Authors

Sun, Xutong
Sharma, Shruti
Fratz, Sohrab
[et al.](#)

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Disruption of Endothelial Cell Mitochondrial Bioenergetics in Lambs with Increased Pulmonary Blood Flow

Xutong Sun,^{1,*} Shruti Sharma,^{1,*} Sohrab Fratz,^{2,*} Sanjiv Kumar,¹ Ruslan Rafikov,¹ Saurabh Aggarwal,¹ Olga Rafikova,¹ Qing Lu,¹ Tiantiana Burns,¹ Sridevi Dasarathy,¹ Johnny Wright,¹ Christian Schreiber,² Monique Radman,³ Jeffrey R. Fineman,^{3,4} and Stephen M. Black¹

Abstract

Aims: The mitochondrial dysfunction in our lamb model of congenital heart disease with increased pulmonary blood flow (PBF) (Shunt) is associated with disrupted carnitine metabolism. Our recent studies have also shown that asymmetric dimethylarginine (ADMA) levels are increased in Shunt lambs and ADMA increases the nitration of mitochondrial proteins in lamb pulmonary arterial endothelial cells (PAEC) in a nitric oxide synthase (NOS)-dependent manner. Thus, we determined whether there was a mechanistic link between endothelial nitric oxide synthase (eNOS), ADMA, and the disruption of carnitine homeostasis in PAEC. **Results:** Exposure of PAEC to ADMA induced the redistribution of eNOS to the mitochondria, resulting in an increase in carnitine acetyl transferase (CrAT) nitration and decreased CrAT activity. The resulting increase in acyl-carnitine levels resulted in mitochondrial dysfunction and the disruption of mitochondrial bioenergetics. Since the addition of L-arginine prevented these pathologic changes, we examined the effect of L-arginine supplementation on carnitine homeostasis, mitochondrial function, and nitric oxide (NO) signaling in Shunt lambs. We found that the treatment of Shunt lambs with L-arginine prevented the ADMA-mediated mitochondrial redistribution of eNOS, the nitration-mediated inhibition of CrAT, and maintained carnitine homeostasis. In turn, adenosine-5'-triphosphate levels and eNOS/heat shock protein 90 interactions were preserved, and this decreased NOS uncoupling and enhanced NO generation. **Innovation:** Our data link alterations in cellular L-arginine metabolism with the disruption of mitochondrial bioenergetics and implicate altered carnitine homeostasis as a key player in this process. **Conclusion:** L-arginine supplementation may be a useful therapy to prevent the mitochondrial dysfunction involved in the pulmonary vascular alterations secondary to increased PBF. *Antioxid. Redox Signal.* 18, 1739–1752.

Introduction

DISRUPTION OF MITOCHONDRIAL FUNCTION is a critical event in a number of pathologic conditions, including hypoxia-ischemic injuries (5), stroke (54), diabetes (15), and hypertension (34). Under conditions of metabolic stress, mitochondria accumulate acyl-coenzyme A (acyl-CoA), which can inhibit oxidative phosphorylation (12). There is a decline in mitochondrial function associated with aging (30, 31), and

oxidative damage to the mitochondrial enzymes regulating carnitine homeostasis is an important mediator in this process (30, 31). The principal enzyme affected has been identified as carnitine acetyltransferase (CrAT), which catalyzes a reversible equilibrium reaction between acyl-CoA and CoA, and acylcarnitine and carnitine (59). Pulmonary mitochondrial function is attenuated in our lamb model of a congenital heart defect with increased pulmonary blood flow (PBF) (Shunt), and this correlates with a disruption of carnitine metabolism

¹Pulmonary Disease Program, Vascular Biology Center, Georgia Health Sciences University, Augusta, Georgia.

²The German Heart Center, Munich, Germany.

³Department of Pediatrics, University of California San Francisco, San Francisco, California.

⁴Cardiovascular Research Institute, University of California San Francisco, San Francisco, California.

*These three authors contributed equally.

Innovation

Our study provides a novel insight into the role of endothelial nitric oxide synthase mitochondrial targeting and the disruption of endothelial mitochondrial bioenergetics in pulmonary hypertension. Further, our data implicate increases in asymmetric dimethylarginine and the disruption of carnitine homeostasis as key players in this process. We speculate that L-arginine supplementation may have therapeutic potential in the treatment of the pulmonary endothelial dysfunction in patients with increased pulmonary blood flow.

(42). One of the major correlations with the disrupted carnitine homeostasis was a reduction in CrAT activity associated with increased nitration (42). However, the mechanism by which CrAT becomes nitrated was not elucidated. Asymmetric dimethylarginine (ADMA) is an endogenous competitive inhibitor of nitric oxide synthase (NOS). Increased ADMA levels are implicated in a number of conditions affecting the cardiovascular system. Our recent studies have shown that the ADMA levels are increased in Shunt lambs secondary to a decrease in dimethylarginine hydrolases (DDAH) activity (47) and that ADMA increases the nitration of mitochondrial proteins in cultured lamb pulmonary arterial endothelial cells (PAEC) (46). Thus, the purpose of this study was to determine whether there was a mechanistic link between increases in ADMA and the disruption of carnitine metabolism; and if so, whether L-arginine supplementation could prevent the mitochondrial dysfunction in Shunt lambs. In cultured PAEC, we found that ADMA increased CrAT nitration and decreased CrAT activity *via* the redistribution of endothelial nitric oxide synthase (eNOS) from the plasma membrane to the mitochondria, which resulted in a disruption in carnitine metabolism and mitochondrial bioenergetics. In Shunt lambs, we found that L-arginine supplementation prevented the ADMA-mediated translocation of eNOS to the mitochondria and this attenuated the nitration-mediated inhibition of CrAT associated with increased PBF. This, in turn, preserved carnitine homeostasis, adenosine-5'-triphosphate (ATP) levels, and eNOS/heat shock protein 90 (Hsp90) interactions. This resulted in a decrease in NOS uncoupling and enhanced nitric oxide (NO) generation in L-arginine supplemented Shunt lambs. Taken together, our data suggest that there is a link between cellular arginine metabolism and mitochondrial dysfunction through the disruption of carnitine homeostasis, indicating that L-arginine supplementation may be a useful therapy for the endothelial dysfunction associated with various cardiovascular disorders, including pulmonary hypertension.

Results

The ADMA-induced redistribution of eNOS to the mitochondria disrupts mitochondrial bioenergetics

PAEC were transiently transfected with an eNOS-green fluorescent protein (GFP) construct (48), treated with Mitotracker, and then exposed to ADMA (5 μ M) in the presence or absence of L-arginine (75 μ M). The co-localization of the GFP-tagged eNOS with the red-labeled mitochondria was determined over a 60-min period, and the Pearson product-

moment correlation coefficient was determined to estimate the redistribution of eNOS to the mitochondria. ADMA induced a time-dependent increase in the localization of eNOS to the mitochondria (Fig. 1A). Western blotting of isolated mitochondria was also used to confirm the ADMA-mediated increase in eNOS localized to the mitochondria (Fig. 1B). The redistribution of eNOS to the mitochondria correlated with a decrease in mitochondrial membrane potential (Fig. 1C, D), a decrease in superoxide dismutase (SOD) 2 (Fig. 1E), and an increase in uncoupling protein 2-protein levels (Fig. 1F). Consistent with these results, the NOS inhibitors, N^G-methyl-L-arginine acetate (L-NMMA) and N^ω-nitro-L-arginine methyl ester hydrochloride (L-NAME) increased protein nitration levels (Fig. 1G) and decreased mitochondrial membrane potential (Fig. 1H, I). Further, ADMA treatment increased both the level of uncoupled NOS associated with the mitochondria (Fig. 1J) and the levels of peroxynitrite within the mitochondria (Fig. 1K). Although basal respiration was unaffected (Fig. 2A, B), both reserve respiratory capacity (Fig. 2A, C) and maximal respiratory capacity (Fig. 2A, D) were significantly reduced by ADMA. In all cases, changes were prevented by the addition of L-arginine (Figs. 1 and 2). Treatment of PAEC with ADMA in the presence or absence of L-arginine did not alter either DDAH1- (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/ars) or DDAH2-protein levels (Supplementary Fig. S1B). These treatments did not alter cellular DDAH activity (Supplementary Fig. S1C) or appear to induce apoptotic events in PAEC (Supplementary Fig. S2).

ADMA disrupts carnitine metabolism in PAEC

Our previous studies demonstrated that increased levels of ADMA in Shunt lambs (47) correlated with a disruption in carnitine homeostasis and a loss of carnitine acetyltransferase (CrAT) activity (42). Carnitine homeostasis is of critical importance for maintaining oxidative phosphorylation (41). To determine whether ADMA disrupts mitochondrial bioenergetics in PAEC *via* the disruption of carnitine homeostasis, we first examined the effect of ADMA (5 μ M, 2 h) on CrAT activity and found it to be significantly reduced (Fig. 3A), although protein levels were unaffected (Fig. 3B). The reduction in CrAT activity correlated with an increase in CrAT nitration (Fig. 3C), and increased levels of acyl carnitine (Fig. 3D) and the acyl carnitine:free carnitine ratio (Fig. 3E), indicating that carnitine homeostasis was disrupted. Again, the addition of L-arginine preserved CrAT activity and maintained carnitine homeostasis (Fig. 3). No changes in the protein levels of the other carnitine homeostasis enzymes (carnitine palmitoyltransferase I [CPT1] and carnitine palmitoyltransferase II [CPT2]) were observed (data not shown).

L-Arginine supplementation prevents the mitochondrial redistribution of eNOS in Shunt lambs

Our previous studies have demonstrated that in Shunt lambs, ADMA levels are increased due to a reduction in DDAH activity (47). Interestingly, this reduction in DDAH activity does not correlate with a reduction in either DDAH1- (Fig. 4A) or DDAH2- (Fig. 4B) protein levels. However, as in PAEC exposed to ADMA, there is an increase in eNOS in the mitochondrial fraction of Shunt lambs (Fig. 4C). This increase is not observed in Shunt lambs supplemented with L-arginine

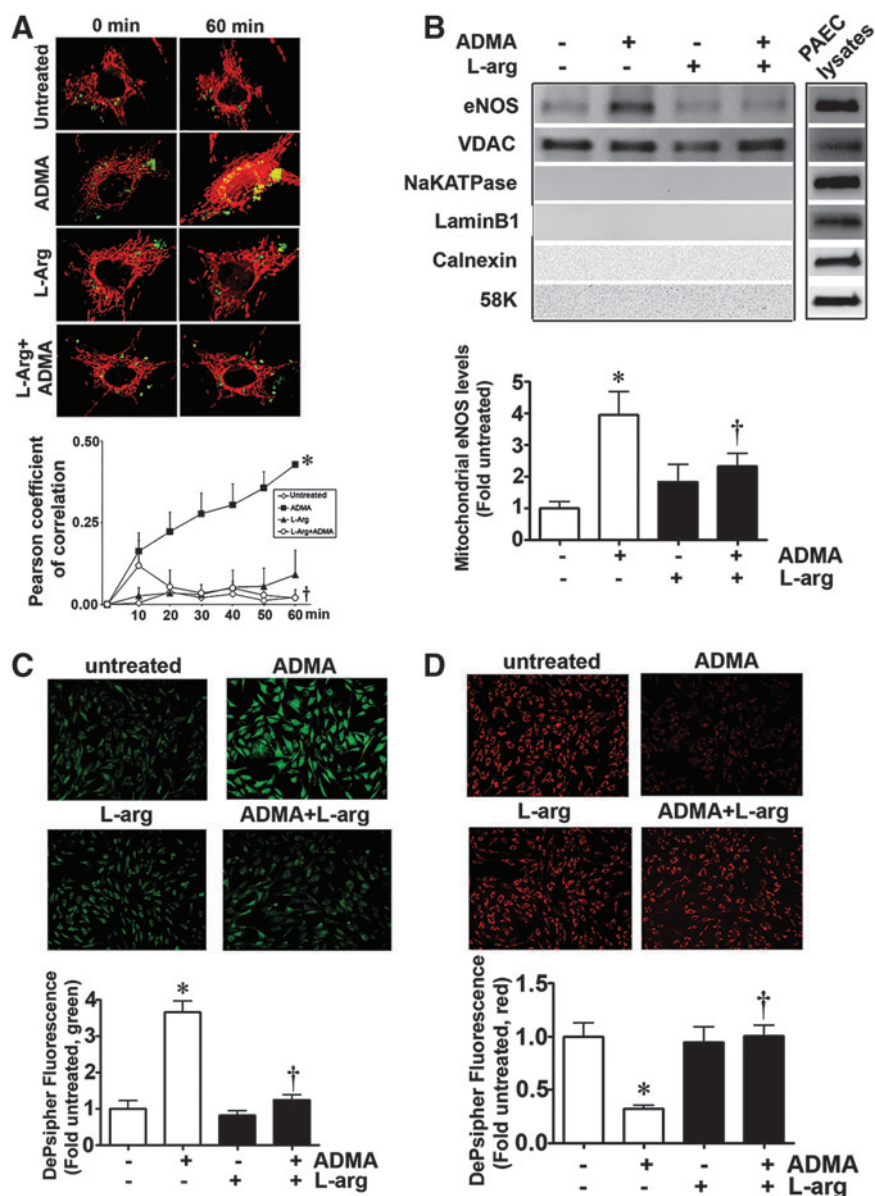


FIG. 1. L-Arginine attenuates the ADMA-mediated eNOS mitochondrial translocation and mitochondrial dysfunction in PAEC. Cells were transfected with eNOS-GFP. The mitochondria of these cells were then labeled with MitoTracker (red), and the cells were exposed to ADMA ($5 \mu\text{M}$) in the presence or absence of L-arginine ($75 \mu\text{M}$). The extent of mitochondrial localization of eNOS was then determined by measuring the intensity of yellow fluorescence (overlap of red fluorescence of MitoTracker and green fluorescence of eNOS-GFP). There is a time-dependent increase in yellow fluorescence in the ADMA treated cells, and this is blocked by the addition of L-arginine (A). Mitochondrial fractions were also isolated, and the level of eNOS protein accumulation in mitochondria was determined by Western blotting (B). ADMA increases eNOS accumulation in the mitochondria, and this is blocked by L-arginine (B). Loading was normalized by reprobings with the mitochondrial protein, VDAC and reprobed with antibodies raised against NaKATPase, Calnexin, 58K, or laminB1 to demonstrate no cross-contamination with the plasma membrane, ER membrane, Golgi, or nuclear fractions, respectively (B). A separate gel was run using a PAEC cell lysate ($20 \mu\text{g}$) to demonstrate that each antibody recognizes the ovine protein (B). ADMA also reduces the mitochondrial membrane potential as estimated by the increase in the green, monomeric form and the decrease in the aggregated red form of the DePsipher compound (C, D). Western blot analysis also revealed that ADMA decreased the levels of SOD2 (E) and increased the levels of UCP2 (F). L-Arginine treatment prevented these changes (E, F). The NOS inhibitors, L-NMMA ($100 \mu\text{M}$) and L-NAME ($100 \mu\text{M}$) also increased PAEC protein nitration (G) and reduced the mitochondrial membrane potential (H, I). The translocation of eNOS to the mitochondria was associated with NOS uncoupling as shown by an increase in NOS-derived superoxide (J) as well as an increase in mitochondrial peroxynitrite levels (K). Values are means \pm SEM; $n = 4-8$. * $p < 0.05$ versus untreated, † $p < 0.05$ versus ADMA alone. 3-NT, 3-nitrotyrosine; ADMA, asymmetric dimethylarginine; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; GFP, green fluorescent protein; L-NAME, N^{ω} -nitro-L-arginine methyl ester hydrochloride; L-NMMA, N^G -methyl-L-arginine acetate; NOS, nitric oxide synthase; PAEC, pulmonary arterial endothelial cells; SEM, standard error of the mean; SOD, superoxide dismutase; UCP2, uncoupling protein 2; VDAC, voltage-dependent anion channel. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars.)

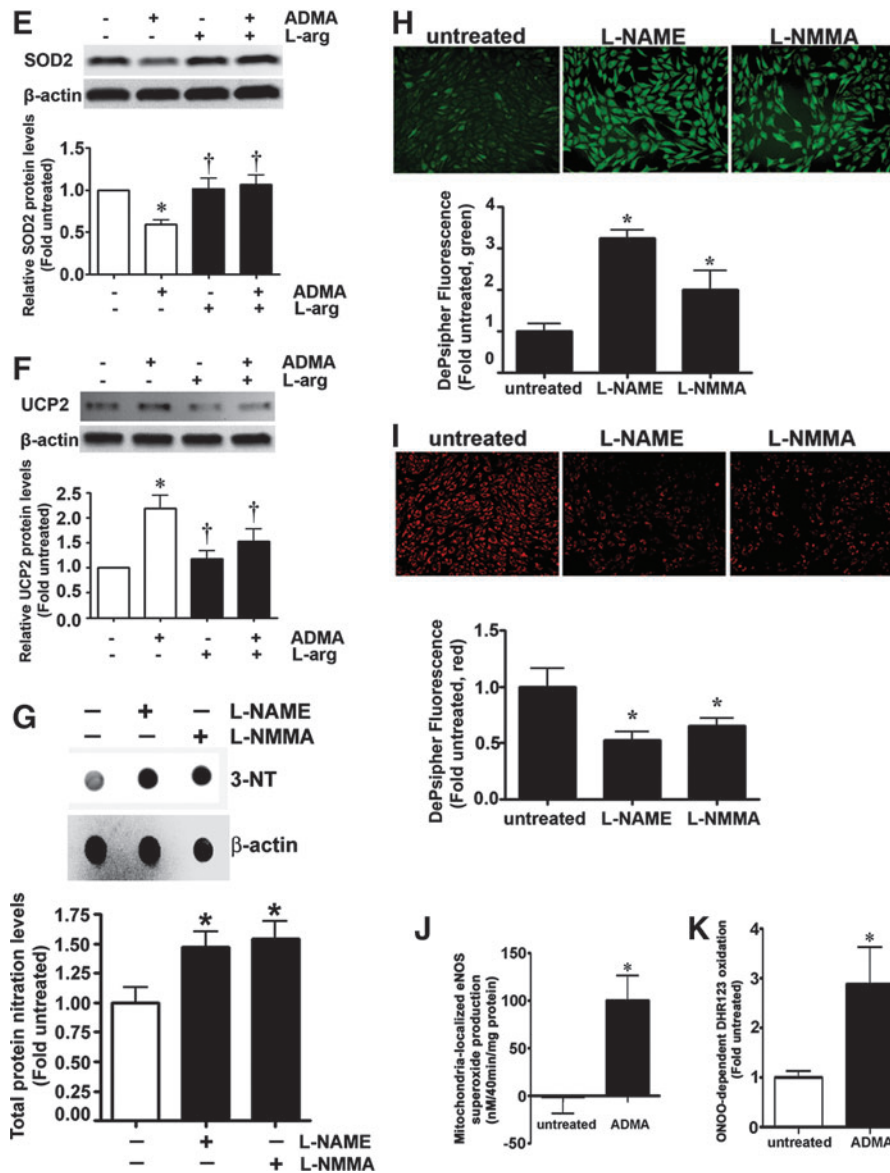


FIG. 1. (Continued)

(500 mg/kg/day, Fig. 4C). L-Arginine had no effect on the mitochondrial distribution of eNOS in control lambs (Fig. 4C).

L-Arginine supplementation preserves carnitine homeostasis and lung ATP levels in Shunt lambs

Our recent studies have shown that increased CrAT nitration and decreased CrAT activity correlates with a disruption in carnitine homeostasis and NO signaling in Shunt lambs (42). To determine whether L-arginine supplementation could preserve carnitine homeostasis and mitochondrial function in Shunt lambs, we examined the effect of L-arginine supplementation on the levels of total protein nitration, CrAT activity, and CrAT nitration in control and Shunt lambs exposed to L-arginine (500 mg/kg/day) or vehicle for 3 weeks. We found that SOD2 activity is decreased in Shunt lambs (Fig. 5A) but even though there is a trend toward preservation, L-arginine supplementation did not prevent the decrease in SOD2 activity (Fig. 5A). L-Arginine had no effect on SOD2 activity in control lambs (Fig. 5A). Our data also indicate that in Shunt

lambs, total protein nitration (Fig. 5B) as well as CrAT nitration (Fig. 5C) are increased whereas CrAT activity (Fig. 5D) is decreased compared with age-matched control lambs. L-Arginine supplementation decreased the levels of both total protein- (Fig. 5B) and CrAT-nitration (Fig. 5C) and preserved CrAT activity (Fig. 5D) without altering CrAT protein levels (Fig. 5E). L-Arginine supplementation had no effect on total protein- (Fig. 5B) or CrAT nitration (Fig. 5C) or CrAT activity (Fig. 5D) in control lambs. L-Arginine supplementation in either control or Shunt lambs did not significantly change the levels of CPT1 or CPT2 (data not shown). Consistent with our *in vitro* data, in Shunt lambs, both acylcarnitine levels and the acylcarnitine:free carnitine ratios were significantly increased compared with control lambs (Fig. 5F, G), while L-arginine supplementation completely abolished these increases (Fig. 5F, G). L-Arginine supplementation had no effect on carnitine homeostasis in control lambs (Fig. 5F, G). Since carnitine homeostasis is of critical importance for maintaining oxidative phosphorylation (41) and ATP generation (59), we determined ATP levels in control and Shunt lambs with and

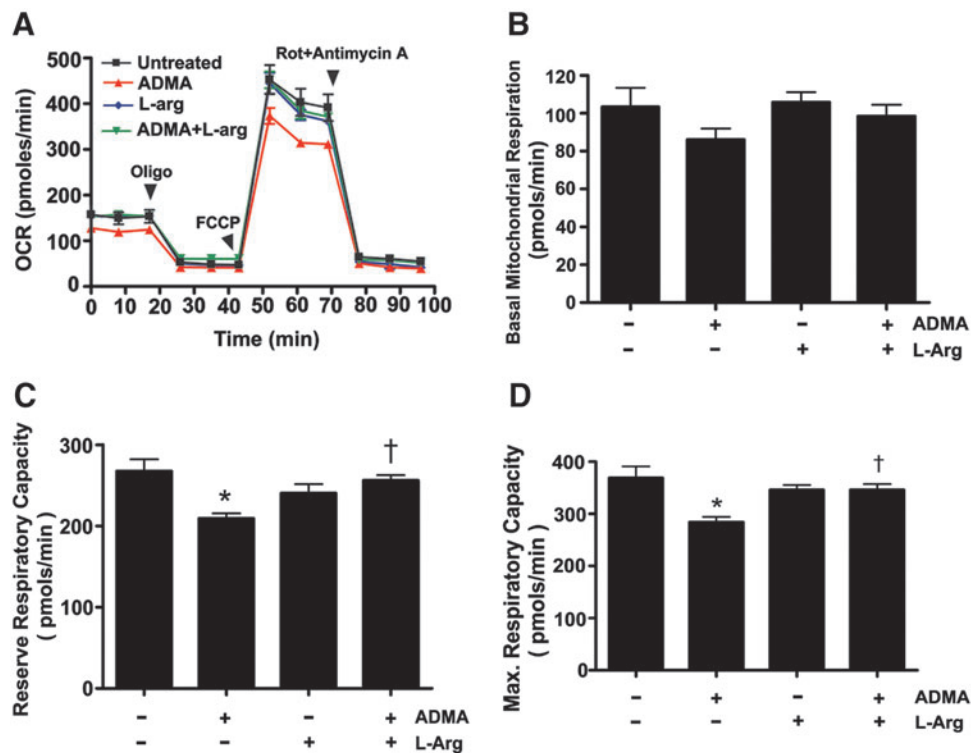


FIG. 2. ADMA inhibits mitochondrial respiration in PAEC. PAEC ($75,000$ cells/ 0.32 cm²) were exposed to ADMA (5 μ M, 2 h) in the presence or absence of L-arginine (75 μ M); then, the Seahorse XF24 analyzer was used to take measurements. Oligomycin (1 μ M), FCCP (1 μ M), and rotenone and antimycin A (1 μ M each) were added at the indicated points (A). ADMA did not significantly alter basal mitochondrial respiration as estimated by measuring the rate of OCR (B). However, both the reserve respiratory capacity (C) and the maximal respiratory capacity (D) in PAEC were significantly attenuated by ADMA, and these changes were prevented by L-arginine (C, D). Values are means \pm SEM; $n = 5-6$. * $p < 0.05$ versus untreated; † $p < 0.05$ versus ADMA only. FCCP, carbonilcyanide p-trifluoromethoxyphenylhydrazone; OCR, oxygen consumption rate. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars.)

without L-arginine administration. L-Arginine supplementation prevented the reduction of ATP levels observed in Shunt lambs (Fig. 6) but had no effect on control lambs (Fig. 6).

L-arginine supplementation preserves NO signaling in Shunt lambs

Consistent with our previous studies (3), we found that eNOS protein levels were significantly increased in Shunt, compared with control lambs (Fig. 7A). However, this increase was ablated by L-arginine supplementation (Fig. 7A). L-Arginine alone had no effect on eNOS protein levels in control lambs (Fig. 7A). The eNOS dimer:monomer ratio is also decreased in Shunt lambs (Fig. 7B), and this was again prevented by L-arginine supplementation (Fig. 7B). L-Arginine had no effect on the eNOS dimer:monomer ratio in control lambs (Fig. 7B). Since ATP levels are decreased in Shunt lambs (Fig. 6) and Hsp90 chaperone activity is ATP dependent (42), we also determined the affect on Hsp90/eNOS interactions in control and Shunt lambs with and without L-arginine supplementation. Consistent with our previous studies (42), our data indicate that, when normalized by total eNOS protein levels, Hsp90/eNOS interactions are disrupted in Shunt lambs (Fig. 7C), while L-arginine treatment prevented the decrease in Hsp90/eNOS interactions in Shunt lambs (Fig. 7C). Again, L-arginine supplementation alone had no affect on Hsp90/eNOS interactions in control lambs (Fig.

7C). Finally, we tested the effect of L-arginine on NOS uncoupling and nitric oxide metabolites (NO_x) generation. Our data indicate that NOS uncoupling, as estimated by an increase in NOS-derived superoxide, is significantly increased in Shunt lambs, while L-arginine treatment completely abolished this increase (Fig. 7D). We also found that L-arginine supplementation preserved NO_x generation in Shunt lambs (Fig. 7E). Again, L-arginine supplementation alone had no effect on NOS-derived superoxide (Fig. 7D) or NO_x generation in control lambs (Fig. 7E).

Discussion

The carnitine "shuttle" is critical for maintaining normal mitochondrial function, and its disruption is associated with disrupted mitochondrial bioenergetics (9, 41). CrAT plays a central role in this process. CrAT reconverts the short- and medium-chain acyl-CoAs into acylcarnitines. Through this mechanism of reversible acylation, the cell is able to modulate the intracellular concentrations of free-CoA and acyl-CoA (50). Oxidative damage to CrAT can result in the disruption of carnitine homeostasis (30, 31), indicating that the activity of CrAT may be more important than changes in its expression. Indeed, in 2-week-old Shunt lambs, the decrease in CrAT activity is greater than the overall decrease in CrAT protein (42). This decrease in CrAT activity is associated with increased CrAT nitration (42). Consistent with these results, we

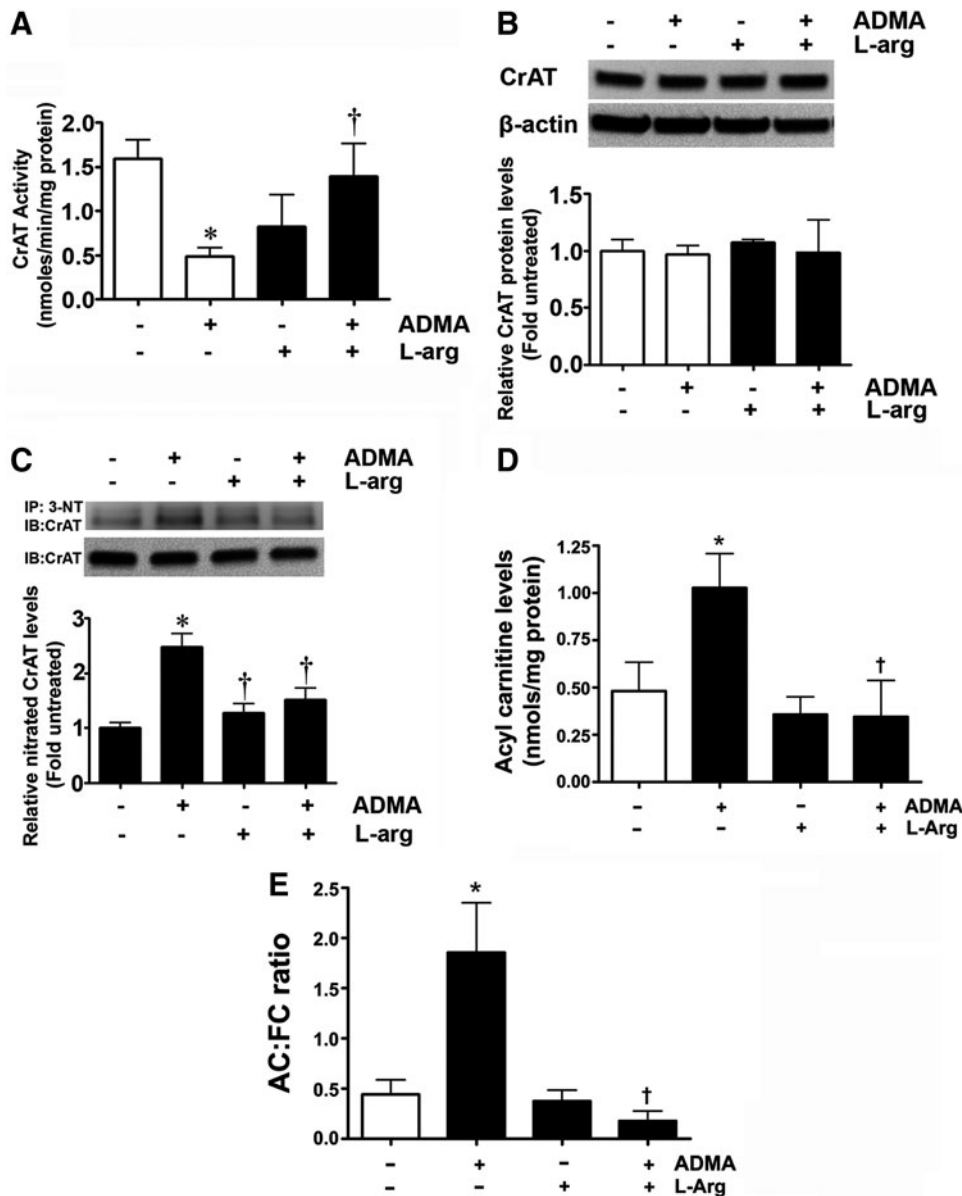


FIG. 3. ADMA disrupts carnitine homeostasis in PAEC. PAEC were treated with ADMA (5 μ M, 2 h) in the presence or absence of L-arginine (75 μ M), and CrAT activity was determined. ADMA decreases CrAT activity in PAEC, and this is prevented by L-arginine (A). Western blot analysis indicates that ADMA does not change CrAT protein levels (B). Protein extracts (1 mg) were also subjected to IP using an antibody that was specific to 3-NT and then analyzed using a specific antiserum raised against CrAT. A representative IB is shown. Data were normalized by carrying out Western blot analysis on IP input (20 μ g) of the protein extracts probed with an antibody to CrAT. ADMA significantly increases CrAT nitration (C), and this is prevented by L-arginine (C). The affect of ADMA on acylcarnitine levels (D) and the acylcarnitine:free carnitine ratio (E) was also determined. ADMA significantly increases acylcarnitine levels and the acylcarnitine:free carnitine ratio. Again, L-arginine treatment prevented these changes (D, E). Values are means \pm SEM; $n=4-6$. * $p < 0.05$ versus untreated; † $p < 0.05$ versus ADMA alone. CrAT, carnitine acetyl transferase; IB, immunoblot; IP, immunoprecipitation.

found that at 3 weeks of age, total protein nitration and CrAT nitration levels were also significantly increased in Shunt lambs, which corresponded with reduced CrAT activity. Further, the increase in CrAT nitration appears to be driven by the redistribution of eNOS to the mitochondria. Although the mechanism for this translocation has not been resolved, it requires the presence of the mitochondrial targeting sequence previously identified in eNOS (17). However, it has been proposed that eNOS redistribution from the plasma membrane to other sub-cellular compartments may be a mechanism for regulating the enzymatic activity of eNOS (16), and plasma membrane-bound eNOS and Golgi-bound eNOS generate more NO than cytosolic eNOS (10, 22, 60). Alternatively, since NO has been shown to regulate the electron transport chain (8) in the mitochondria, it may be involved in regulating ATP generation. However, further studies will be required to evaluate these possibilities.

Recent data have shown that in pulmonary hypertension there is a phenotypic switch in the smooth muscle cell (SMC)

that results in the appearance of a glycolytic phenotype or "Warburg effect" to generate the ATP required to maintain cell viability (40). In SMC, this reduction in oxidative phosphorylation correlates with an increase in mitochondrial membrane potential, a reduction in mitochondrial-derived reactive oxygen species (ROS), and an anti-apoptotic phenotype (14). In PAEC, we observe a similar reduction in oxygen consumption and a decrease in both reserve and maximal respiratory capacity, indicative of an attenuation of oxidative phosphorylation. However, we also observe a reduction in mitochondrial membrane potential and, as we have, previously published, an increase in mitochondrial-derived ROS and a reduction in ATP levels (46). Thus, unlike SMC, PAEC exposed to a "stress milieu" do not appear to be able to maintain their ATP levels, suggesting that they are incapable of enhancing their glycolytic rate. Thus, our data suggest that strategies which target the glycolytic phenotype in SMC (49) could result in even greater injury to the endothelium and further attenuate NO signaling.

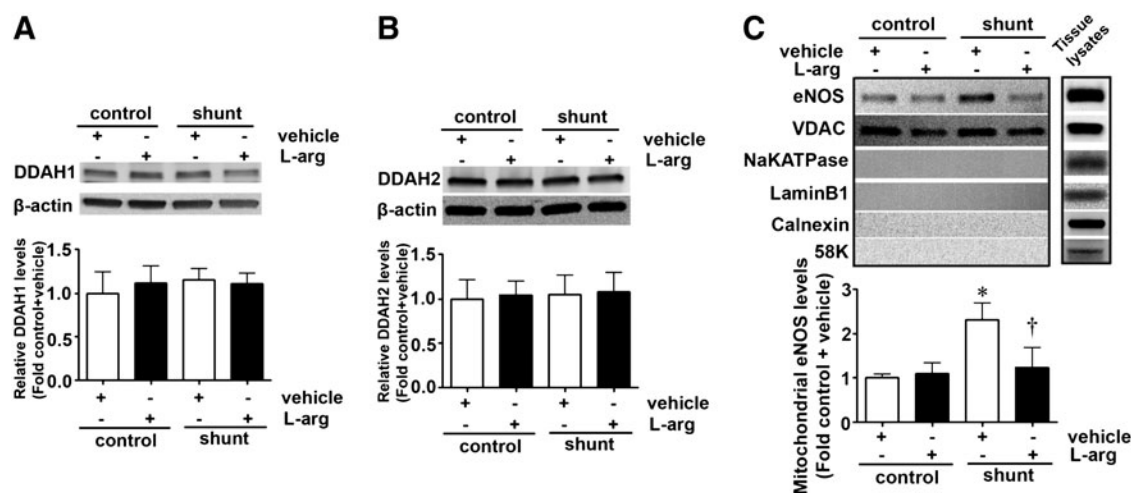


FIG. 4. L-Arginine supplementation prevents eNOS mitochondrial translocation in lambs with increased PBF. Total protein extracts prepared from the peripheral lung of Shunt or age-matched control lambs supplemented or not with L-arginine at 3 weeks of age and subjected to Western blotting using antibodies raised against DDAH1 (**A**) or DDAH2 (**B**). Representative images are shown. Blots were reprobated with β -actin to normalize loading. No changes in DDAH1- or DDAH2-protein levels are observed between the four groups. Mitochondrial protein extracts (5 μ g) were also prepared from each of the four groups and subjected to Western blotting using an antibody raised against eNOS. Loading was normalized by reprobating the membranes with an antibody raised against the mitochondrial protein, VDAC and reprobated with antibodies raised against NaKATPase, Calnexin, 58K, or laminB1 to demonstrate no cross-contamination with the plasma membrane, ER membrane, Golgi, or nuclear fractions, respectively. A representative image is shown. The levels of eNOS in the mitochondria are significantly increased in Shunt lambs, and this was prevented by L-arginine supplementation (**C**). Values are means \pm SEM; $n = 4-9$. * $p < 0.05$ versus control + vehicle; † $p < 0.05$ versus Shunt + vehicle. PBF, pulmonary blood flow.

There also appears to be developmental regulation of the carnitine homeostasis enzymes in lambs exposed to increased PBF. At 2 weeks of age, we have previously shown that CPT1, CPT2, and CrAT protein levels are decreased in Shunt lambs (42). However, our Western blot analyses indicate that these differences are lost in 3-week-old Shunt lambs, suggesting that there are compensatory signaling pathways which normalize the expression of the carnitine homeostasis enzymes. Although this is possible, as we are examining protein levels in peripheral lung tissue containing multiple cell types, we are missing changes in CPT1, CPT2, and CrAT that are occurring only in the endothelium. However, in support of our data, we have observed similar developmental changes in the expression of a number of genes in Shunt lambs over the first 2 months of life, including eNOS (3), prepro-endothelin-1 (2, 36), endothelin type A receptor and endothelin type B receptors (4), and transforming growth factor β 1 (33). Further, we speculate that it is the inhibition of CrAT activity which is key to the mitochondrial dysfunction we observe in Shunt lambs. Indeed, our data demonstrate that the level of a protein does not necessarily reflect its actual activity and emphasizes the need to evaluate the role of post-translational protein modifications in the disease process. Examining post-translational modifications is a complex process, as our data indicate that CrAT activity is decreased $\sim 2-3$ -fold at 3 weeks of age, at 2 weeks of age relative CrAT activity (normalized to changes in CrAT protein levels) are decreased $\sim 8-10$ -fold, suggesting that in addition to modifications which inhibit CrAT activity (nitration), there are also potential post-translational events that may stimulate activity. Although these events are unresolved, carnitine homeostasis can be regulated by malonyl-CoA binding to the N-terminal domain

of CPT-1 (25, 43, 44). In turn, changes in the levels of malonyl-CoA are regulated by the activity of acetyl-CoA carboxylase mediated by 5'-adenosine 5'-monophosphate-activated protein kinase (26, 32, 56). Direct phosphorylation of the carnitine homeostasis enzymes by Ca^{2+} /calmodulin-dependent protein kinase II (51, 53) or alterations in the interactions of cytoskeletal components with the mitochondrial outer membrane have also been proposed (52). However, future studies will be required to elucidate these events.

The ATP-dependent molecular chaperone, Hsp90 interacts with proteins that are required for efficient NO biosynthesis, including eNOS (23), soluble guanylate cyclase (58), guanosine-5'-triphosphate cyclohydrolase I (47), 3-phosphoinositide-dependent kinase-1 (55), and Akt1 (7). Reductions in Hsp90-eNOS attenuate NO production (18, 19, 42, 46), leading to eNOS uncoupling (37, 42, 46). eNOS uncoupling is a complex process that is mediated by decreases in the availability of the substrate, L-arginine, the co-factor tetrahydrobiopterin or, as we have recently shown, increases in the cellular levels of ADMA (46). Consistent with these studies, we demonstrate that decreased ATP levels in Shunt lambs correlate with disturbed Hsp90-eNOS interactions and increased eNOS uncoupling, while L-arginine supplementation preserves Hsp90-eNOS and NO_x production. L-Arginine supplementation did not enhance NO_x levels in control lambs. The reasons for this are unclear. However, ADMA inhibits NO generation by competing with L-arginine for the substrate-binding site of NOS. Thus, the eNOS coupling/uncoupling decision is a competition between L-arginine and ADMA for the eNOS active site. Thus, the preservation of NO generation Shunt lambs supplemented with L-arginine may be due, in part, to an increased likelihood that L-arginine rather

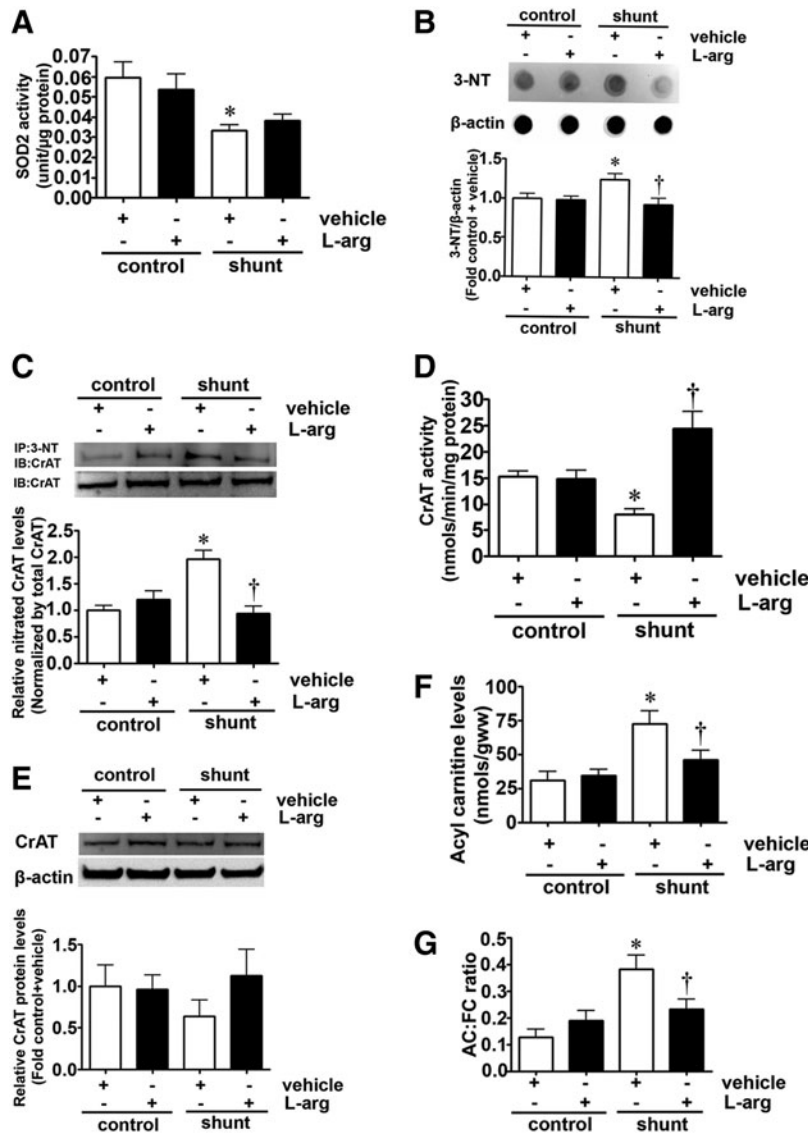


FIG. 5. L-Arginine supplementation preserves CrAT activity in lambs with increased PBF. SOD2 activity is decreased in Shunt lambs (A), but L-arginine supplementation failed to preserve SOD2 activity (A). Protein extracts (30 μ g) prepared from peripheral lungs of Shunt lambs or age-matched controls with either L-arginine or vehicle administration were subjected to dot-blot analysis using an antibody raised against 3-NT. Protein levels were normalized by re-probing with an antibody that was specific to β -actin. There is an increase in total protein nitration in Shunt lambs that is attenuated by L-arginine supplementation (B). Protein extracts (1 mg) prepared from peripheral lungs of control and Shunt lambs with L-arginine or vehicle administration were subjected to IP using an antibody that was specific to 3-NT and then analyzed by Western blot analysis using a specific antiserum raised against CrAT. A representative IB is shown. Data were normalized by carrying out Western blot analysis on IP input (20 μ g) of the protein extracts probed with an antibody to CrAT. CrAT nitration is significantly increased in Shunt lambs, and this is prevented by L-arginine supplementation (C). CrAT activity (D) and CrAT protein levels (E) were also determined in peripheral lung lysates. The decrease in CrAT activity in Shunt lambs is prevented by L-arginine supplementation (D). L-Arginine supplementation does not alter CrAT protein levels in either Shunt or control lambs (E). The affect of L-arginine supplementation on acylcarnitine levels (F) and the acylcarnitine:free carnitine ratio (G) was also determined. The increase in acylcarnitine levels (F) and the acylcarnitine:free carnitine ratio (G) in Shunt lambs is prevented by L-arginine supplementation. L-Arginine supplementation did not alter SOD2 activity (A), total CrAT or CrAT nitration levels (B, C), acylcarnitine levels (F) or the acylcarnitine:free carnitine ratio (G) in control lambs. Values are means \pm SEM; $n = 6-9$. * $p < 0.05$ versus control + vehicle; † $p < 0.05$ versus Shunt + vehicle.

than ADMA will be in the active site. However, under physiologic conditions, there is already sufficient L-arginine present to keep eNOS tightly coupled so that increasing its levels has no significant effect on its ability to generate NO. It is also unclear why L-arginine supplementation prevents the in-

crease in eNOS expression in Shunt lambs. However, it is possible that the increase in NO signaling observed in L-arginine-treated Shunt lambs induces vasodilation and reduces the exposure of pulmonary endothelium to shear stress. Since shear stress is a major stimulus of increased eNOS gene

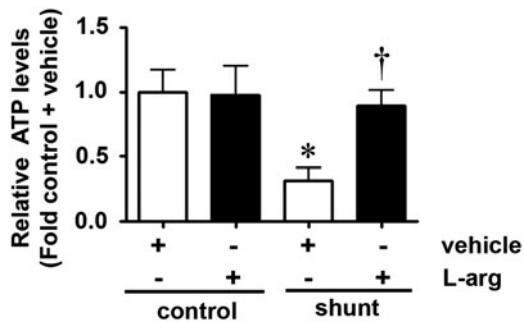


FIG. 6. L-Arginine supplementation preserves ATP levels in lambs with increased PBF. ATP levels were determined in peripheral lung lysates prepared from Shunt or age-matched control lamb with either L-arginine or vehicle administration. The decrease in ATP levels in Shunt lambs is prevented by L-arginine supplementation. L-Arginine supplementation does not alter ATP levels in control lambs. Values are means \pm SEM; $n=6-9$. * $p<0.05$ versus control+vehicle; † $p<0.05$ versus Shunt+vehicle. ATP, adenosine-5'-triphosphate.

expression, this would result in less eNOS transcription and a reduction in eNOS protein levels as observed. Alternatively, it is possible that L-arginine is exerting a positive effect independent of its effects on the ADMA:L-arginine ratio and NO signaling in Shunt lambs. This possibility is supported by a recent work in which L-arginine was shown to prevent D-glucose-mediated increases in arginase expression, oxidative stress, endothelial dysfunction, and advanced glycation end-products (AGE) formation in an NO-independent manner (13).

Materials and Methods

Surgical preparations and care

This is described in the online Supplementary Data. All protocols and procedures were approved by the Committees on Animal Research at University of California, San Francisco, Georgia Health Sciences University, and the German Heart Center.

Antibodies and chemicals

Anti-CrAT antibody was purchased from Proteintech; anti-Hsp90 and anti-eNOS antibodies were purchased from BD Transduction laboratories. The anti-nitrotyrosine antibody was obtained from Calbiochem. sodium potassium ATPase (NaKATPase; cat# ab7671), LaminB1 (cat# ab8982), Calnexin (cat# ab119202), and 58K (ab115832) were purchased from Abcam, and an antibody that specifically recognizes cleaved, but not full length, caspase-3 (Cell signaling Technology; cat#9661S). DDAH1 and DDAH2 rabbit polyclonal antibodies were purchased from Biosynthesis, Inc. For the cleaved caspase Western blots, cell extracts (5 μ l) prepared from Jurkat cells exposed or not to etoposide (25 μ M, 5 h) were purchased from Cell Signaling Technology (cat#2043S) and used as positive and negative controls, respectively. β -Actin was purchased from Sigma, and voltage-dependent anion channel was bought from Cell Signaling. ADMA was purchased from Calbiochem. L-NMMA, and L-NAME was purchased from Sigma. L-[4,5-³H]-NMMA was purchased from American Radiolabeled Chemicals, Inc.

Cell culture and treatment

Primary cultures of ovine fetal PAEC were isolated and cultured as previously described (24). Cells were utilized between passages 9 and 14. At least 24 h before ADMA treatment, standard medium was replaced with L-arginine and phenol-red-free Dulbecco's modified Eagle medium (Athena Enzyme System). Before all treatments, PAEC were serum starved for 2 h, then exposed or not to ADMA (5 μ M), L-NMMA (100 μ M), or L-NAME (100 μ M) for 2 h. Cells were also treated with L-arginine (75 μ M; Sigma) as indicated.

Live cell imaging of eNOS redistribution to the mitochondria

This was accomplished using an eNOS-tagged GFP as described in the online Supplementary Data.

Analysis of mitochondrial membrane potential

Mitochondrial membrane potential was analyzed using the lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl carbocyanine iodide as previously described (11, 57).

Mitochondrial isolation

The mitochondria from tissue or cells were isolated using Pierce Mitochondria isolation kits (Pierce) according to the manufacturer's guidelines.

Analysis of mitochondrial bioenergetics

The XF24 Analyzer (Seahorse Biosciences) was used to estimate the affect of ADMA on mitochondrial bioenergetics in PAEC as described in the online Supplementary Data.

Quantification of total and free carnitine levels by high-performance liquid chromatography

Detection of carnitines was performed using a Shimadzu UFLC system with a 5 μ m Omnispher C18 column (250 \times 4.6 mm OD) and equipped with an RF-10AXL fluorescence detector (Shimadzu USA Manufacturing Corporation). Total and free carnitine levels were quantified by fluorescence detection at 248 nm (excitation) and 418 nm (emission). The acyl carnitines were calculated by subtracting the free carnitine values from the total carnitine values for all the samples as previously described (42).

Measurement of peroxynitrite levels in isolated mitochondria

The formation of peroxynitrite was determined by the oxidation of dihydrorhodamine 123 (EMD Millipore) to rhodamine 123, as previously described (1).

Measurement of eNOS-derived superoxide production in isolated mitochondria

To detect superoxide generation in isolated mitochondria, we performed electron paramagnetic resonance (EPR) measurements in freshly isolated mitochondria as described in the online Supplementary Data.

Measurement of CrAT activity

Cells or peripheral lung tissue were lysed in 50 mM Tris-HCl, pH 7.5, 2 mM ethylenediaminetetraacetic acid,

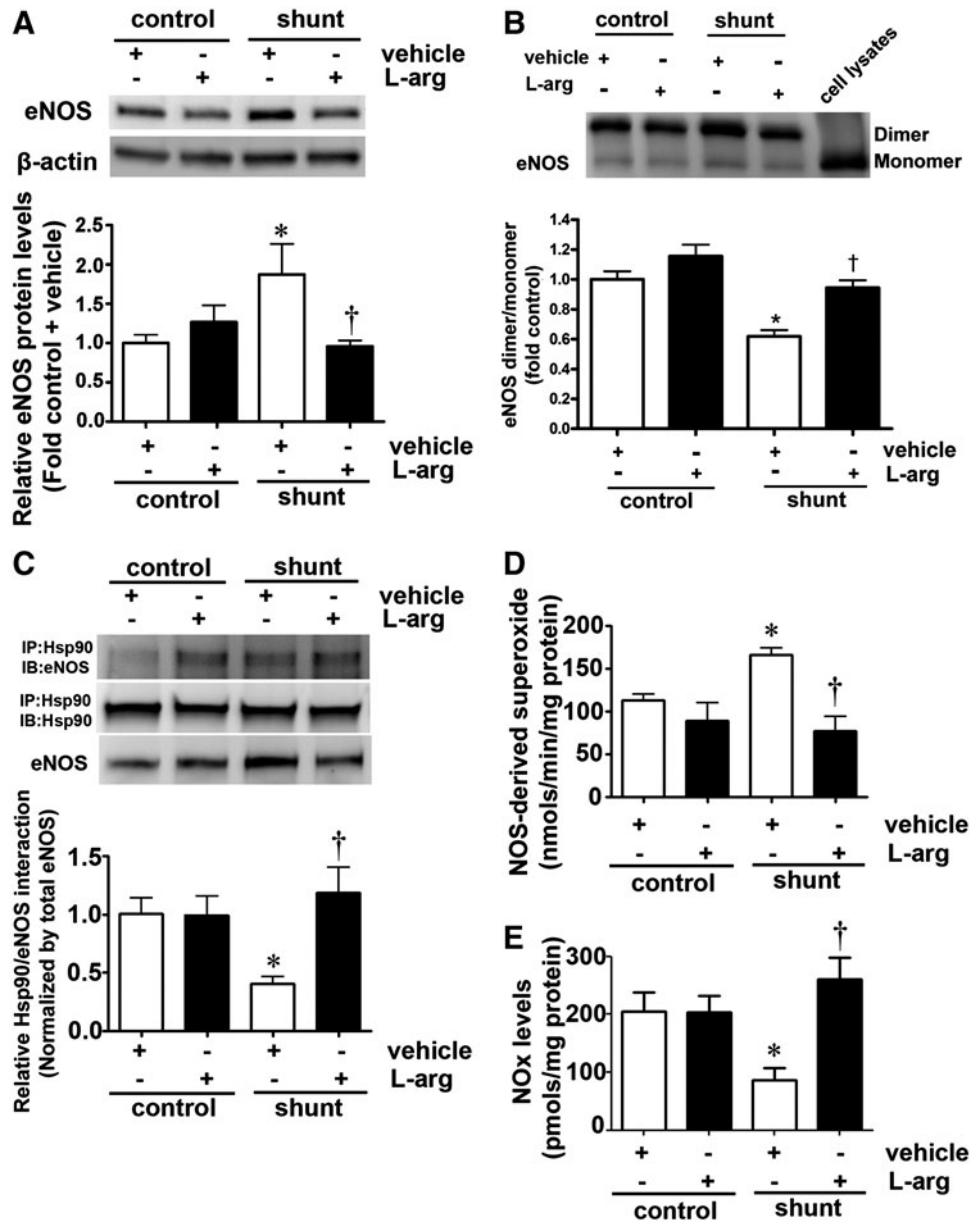


FIG. 7. L-Arginine supplementation preserves NO signaling in lambs with increased PBF. Protein extracts (30 μ g) prepared from peripheral lungs of Shunt lambs or age-matched controls with either L-arginine or vehicle administration was subjected to Western blot analysis using an antibody raised against eNOS. Loading was normalized by reprobing the membranes with an antibody specific to β -actin. A representative image is shown. eNOS protein levels are significantly increased in Shunt lambs, and this is prevented by L-arginine supplementation (A). Protein extracts (60 μ g) were also subjected to LT-PAGE to evaluate dimeric and monomeric forms of eNOS. A representative IB is shown. The eNOS dimer:monomer ratio is significantly decreased in Shunt lambs, and this is prevented by L-arginine supplementation (B). The interaction of eNOS with Hsp90 was determined by IP using a specific antiserum raised against eNOS in peripheral lung lysates (1 mg). IP extracts were analyzed using antisera against either eNOS or Hsp90. A representative image is shown (C). The levels of eNOS protein associated with Hsp90 relative to total eNOS protein were then calculated to account for the differences in the levels of eNOS protein between groups. There is a significant decrease in the association of eNOS with Hsp90 in Shunt lambs (C), while L-arginine supplementation preserves the eNOS-Hsp90 interaction (C). NOS-derived superoxide generation in the peripheral lung is increased in Shunt lambs, and this is attenuated by L-arginine supplementation (D). Peripheral lung NO_x levels are decreased in Shunt lambs and increased by L-arginine supplementation (E). L-Arginine supplementation affected eNOS protein levels (A), eNOS dimer:monomer ratio (B), eNOS-Hsp90 interaction (C), NOS-derived superoxide (D), or NO_x levels (E) in control lambs. Values are means \pm SEM; $n=6-12$. * $p < 0.05$ versus control + vehicle, † $p < 0.05$ versus Shunt + vehicle. Hsp90, heat shock protein 90; NO, nitric oxide; LT-PAGE, low-temperature polyacrylamide gel electrophoresis.

5 mM MgCl₂, 0.8 mM dithiothreitol, and protease inhibitor cocktail. Samples were briefly sonicated and centrifuged at 3000g for 5 min. CrAT activity was immediately determined using a modification of the method described by Liu *et al.* (31). The μ moles of product formed was calculated using an NADH standard curve measured at 340 nm.

Measurement of SOD2 activity

In lung tissue homogenates, the activities of SOD1 and SOD3 were inhibited by incubating samples on ice in the presence of 2 mM potassium cyanide for 1 h (27). SOD2 activity was then measured using a SOD activity kit (Enzo Life Sciences, cat #ADI-900-157). SOD2 activity was reported as units/ μ g protein.

Western blot analysis

Total protein prepared from cells (20 μ g), peripheral tissue (30 μ g) or mitochondria isolated from cells (3 μ g), or peripheral lung tissue (5 μ g) were separated on 4%–20% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (PVDF). Immunoblotting was carried out using the appropriate antibodies in Tris-base buffered saline with 0.1% Tween 20 and 5% bovine serum albumin. After washing, the membranes were probed with horseradish peroxidase-conjugated goat antiserum to rabbit or mouse. Reactive bands were visualized using chemiluminescence (Super Signal West Femto; Pierce) on a Kodak 440CF image station. Bands were quantified using Kodak Image Station software (Kodak 1D 3.6). Loading was normalized by reprobing the membranes with an antibody that was specific to β -actin.

Measurement of eNOS dimer levels

The detection of dimeric and monomeric forms of eNOS in lung homogenates was carried out using low-temperature polyacrylamide gel electrophoresis (LT-PAGE) as previously described (21). Subsequent to LT-PAGE, the gels were transferred to PVDF and probed with an anti-eNOS antibody. A cell lysate with a 5 \times Laemmli buffer, was boiled for 5 min and was used as an eNOS monomer control.

Immunoprecipitation analysis

The interactions of eNOS/Hsp90 or nitrated CrAT levels were analyzed by immunoprecipitation (IP) analysis as described (47). The efficiency of each IP was normalized by re-probing the membranes with the IP antibody (IP:Hsp90) or by Western blot analysis of 30 μ g of the IP protein extract probed using a specific antibody for CrAT (IP:3-nitrotyrosine [3-NT]).

Determination of total protein nitration

In order to examine global protein nitration cell or tissue, lysates were analyzed for 3-NT levels using a dot blot procedure as described (1).

Determination of ATP levels

ATP levels were estimated using the firefly luciferin-luciferase method utilizing a commercially available kit (Invitrogen). ATP is consumed, and light is emitted when firefly luciferase catalyzes the oxidation of luciferin. The amount of

light emitted during the reaction is proportional to the availability of ATP. Luminescence was measured using a Fluoroscanner Ascent FL luminometer (Thermo Electron).

Determination of NO_x levels

To measure NO levels in peripheral lung tissue, lysates were initially treated with cold ethanol to remove proteins; then, we utilized a Sievers 280i Nitric Oxide Analyzer (GE) to determine NO_x levels as described earlier (42).

Measurement of superoxide levels

To detect superoxide generation in peripheral lung tissue, EPR measurements were performed using the spin trap, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine .HCl as already described (35, 45). NOS-derived superoxide levels were determined by subtracting the superoxide values in the presence of the NOS inhibitor, ethylisothiourea (100 μ M, ETU) from the superoxide values in the absence of ETU.

Assay for dimethylarginine dimethylaminohydrolase activity

The dimethylarginine dimethylaminohydrolase (DDAH) activity was determined by monitoring the conversion of L-[4,5-³H]-NMMA (American Radiolabeled Chemicals, Inc.) to L-[³H]-citrulline as previously described (28) using the modifications described in the online Supplementary Data.

Statistical analyses

The mean \pm standard error of the mean were calculated, and comparisons were made by the unpaired *t*-test (for two groups) or analysis of variance followed by Newman-Keuls or Bonferroni *post-hoc* testing (for ≥ 3 groups). The statistical significance of differences was set at $p < 0.05$. Statistical analysis was performed using GraphPad Prism version 4.01 for Windows (GraphPad Software). Data > 2 standard deviation from the mean were not analyzed.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Prof. Stephen M. Black
Pulmonary Disease Program
Vascular Biology Center, CB-3211B
Georgia Health Sciences University
1459 Laney Walker Blvd.
Augusta, GA 30912

E-mail: sblack@georgiahealth.edu

Dr. Xutong Sun
Pulmonary Disease Program
Vascular Biology Center, CB-3201
Georgia Health Sciences University
1459 Laney Walker Blvd.
Augusta, GA 30912

E-mail: xsun@georgiahealth.edu

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Abbreviations Used

3-NT = 3-nitrotyrosine
 acyl-CoA = acyl-coenzyme A
 ADMA = asymmetric dimethylarginine
 ATP = adenosine-5'-triphosphate
 CC-3 = cleaved caspase-3
 CMH = 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine.HCl

CPT1 = carnitine palmitoyltransferase I
 CPT2 = carnitine palmitoyltransferase II
 CrAT = carnitine acetyl transferase
 DDAH = dimethylarginine hydrolases
 DHR = dihydrorhodamine
 DMEM = Dulbecco's modified Eagle's medium
 eNOS = endothelial nitric oxide synthase
 EPR = electron paramagnetic resonance
 ER = endoplasmic reticulum
 ETU = ethylisothiurea
 FCCP = carbonilcyanide
 p-trifluoromethoxyphenylhydrazine
 GFP = green fluorescent protein
 Hsp90 = heat shock protein 90
 IP = immunoprecipitation
 L-NAME = *N*_ω-Nitro-L-arginine methyl ester hydrochloride
 L-NMMA = *N*^G-methyl-L-arginine acetate
 LT-PAGE = low-temperature polyacrylamide gel electrophoresis
 NaCl = sodium chloride
 NAD = nicotinamide adenine dinucleotide
 NaKATPase = sodium potassium ATPase
 NO = nitric oxide
 NOS = nitric oxide synthase
 NO_x = nitric oxide metabolites
 OCR = oxygen consumption rate
 PAEC = pulmonary arterial endothelial cells
 PBF = pulmonary blood flow
 PVDF = polyvinylidene difluoride membranes
 ROS = reactive oxygen species
 SEM = standard error of the mean
 SMC = smooth muscle cell
 SOD = superoxide dismutase
 SPB = sodium phosphate buffer
 UCP2 = uncoupling protein 2
 VDAC = voltage-dependent anion channel