L-Carnitine preserves endothelial function in a lamb model of increased pulmonary blood flow

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BACKGROUND: In our model of a congenital heart defect (CHD) with increased pulmonary blood flow (PBF; shunt), we have recently shown a disruption in carnitine homeostasis, associated with mitochondrial dysfunction and decreased endothelial nitric oxide synthase (eNOS)/heat shock protein (Hsp)90 interactions that contribute to eNOS uncoupling, increased superoxide levels, and decreased bioavailable nitric oxide (NO). Therefore, we undertook this study to test the hypothesis that L-carnitine therapy would maintain mitochondrial function and NO signaling.

METHODS: Thirteen fetal lambs underwent *in utero* placement of an aortopulmonary graft. Immediately after delivery, lambs received daily treatment with oral L-carnitine or its vehicle.

RESULTS: L-Carnitine–treated lambs had decreased levels of acylcarnitine and a reduced acylcarnitine:free carnitine ratio as compared with vehicle-treated shunt lambs. These changes correlated with increased carnitine acetyl transferase (CrAT) protein and enzyme activity and decreased levels of nitrated CrAT. The lactate:pyruvate ratio was also decreased in L-carnitine–treated lambs. Hsp70 protein levels were significantly decreased, and this correlated with increases in eNOS/Hsp90 interactions, NOS activity, and NO_x levels, and a significant decrease in eNOS-derived superoxide. Furthermore, acetylcholine significantly decreased left pulmonary vascular resistance only in L-carnitine–treated lambs.

CONCLUSION: L-Carnitine therapy may improve the endothelial dysfunction noted in children with CHDs and has important clinical implications that warrant further investigation.

Children with congenital heart defects (CHDs) that result in increased pulmonary blood flow (PBF) develop early and progressive alterations in pulmonary vascular function that cause significant morbidity (1). The mechanisms involved in this pulmonary vascular disease are not fully understood; however, secondary endothelial injury is thought to be an early hallmark. The most important consequence of endothelial injury is a decrease in bioavailable nitric oxide (NO), with subsequent endothelial dysfunction, or impaired ability of the endothelium to mediate vasodilation (2). Compelling evidence suggests that impaired NO signaling and oxidative stress play a key role in these events (3).

Oxidative stress occurs when generation of reactive oxygen species (ROS) overwhelms the cells' natural antioxidant defenses, resulting in cellular damage and impaired function of vulnerable tissues. Four enzyme systems are thought to predominate in vascular endothelial ROS generation: nicotinamide adenine dinucleotide phosphate oxidase, xanthine oxidase, uncoupled endothelial nitric oxide synthase (eNOS), and mitochondrial electron leakage. Whereas the former three have been extensively studied, the role of mitochondrial-derived ROS in the vascular endothelium has received less attention (4). Mitochondria, through oxidative phosphorylation, are considered the major source of ROS in most mammalian cells. At the same time, mitochondria are potential targets of ROS action. Therefore, increased ROS can damage DNA, proteins, and lipids within the mitochondria, leading to alterations in the respiratory chain resulting in decreased energy production and a further increase in ROS generation ("ROS-induced ROS release") (5,6). In recent years, it has become clear that mitochondrial dysfunction is a critical event in numerous pathologic conditions associated with oxidative stress, including diabetes mellitus, chronic renal failure, and neurodegenerative or cardiovascular diseases (4,7–9). The contribution of the mitochondria, however, to the pathogenesis of pulmonary vascular disease remains poorly understood.

Previously, we have established a clinically relevant animal model of a CHD with increased PBF, by placing a large aortopulmonary vascular graft (shunt) into the late-gestation fetal lamb (10). This allows the study of early mechanisms of pulmonary vascular disease. In this model, we have shown a selective impairment of endothelium-mediated pulmonary vasodilation (11), associated with decreased NO signaling and increased oxidative stress (3,12,13). Recently,

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we also demonstrated a disruption in carnitine homeostasis in shunt lambs, correlated with mitochondrial dysfunction and decreased eNOS/Hsp90 interactions, which contributed to eNOS uncoupling and decreased NO signaling (14). Carnitine plays an important role in cellular energy metabolism and is essential for mitochondrial health (15). However, whether carnitine supplementation can modify the course of pulmonary vascular disease secondary to increased PBF is unknown. Therefore, the purpose of this study was to determine if chronic supplementation with L-carnitine would attenuate oxidative stress and preserve carnitine homeostasis, mitochondrial homeostasis, and NO signaling in our lamb model of CHD with increased PBF and thereby result in improved endothelial function.

Table 1. Baseline hemodynamic variables and blood gases

Hemodynamic variable	Control ($n=5$)	Shunt ($n=6$)	Shunt + carnitine ($n=6$)
mPAP (mm Hg)	15.3 ± 3.8	23.1 ± 4.3*	21.2 ± 3.6
mSAP (mm Hg)	77.6 ± 7.7	70.7 ± 8.9	60.0 ± 6.8*
mRAP (mm Hg)	3.5 ± 2	3.8 ± 1.4	4.2 ± 1.3
mLAP (mm Hg)	4.1 ± 1.6	7.1 ± 1.7	9.0 ± 3.1*
HR	126 ± 13	174 ± 26*	144 ± 40
Q_{lpa} (ml/min/kg)	0.8 ± 0.2	1.8 ± 0.6	2.1 ± 0.8*
LPVR (mm Hg/ml/min/kg)	0.24 ± 0.10	0.14 ± 0.06	0.08 ± 0.02*
Qp:Qs		2.8 ± 0.9	2.8 ± 0.4
pH (units)	7.36 ± 0.03	7.40 ± 0.02	7.41 ± 0.02
pCO_2 (torr)	42.3 ± 2.1	40.0 ± 3.7	42.0 ± 2.0
pO_2 (torr)	78.3 ± 12.2	70.7 ± 11.2	75.4 ± 14.3
Hb concentration	9.0 ± 1.7	9.8 ± 1.6	8.7 ± 1.1
Weight (kg)	15.7 ± 2.5	13.9 ± 2.1	13.4 ± 2.7

Values are mean ± SD.

Hb, hemoglobin; HR, heart rate; LPVR, left pulmonary vascular resistance; mLAP, mean left atrial pressure; mPAP, mean pulmonary arterial pressure; mRAP, mean right atrial pressure; mSAP, mean systemic arterial pressure; Q_{lpa}, blood flow through the left pulmonary artery; Qp:Qs, ratio of pulmonary to systemic blood flow.

*$P < 0.05$ control vs. vehicle-treated shunt. *$P < 0.05$ control vs. shunt + carnitine.

RESULTS

Hemodynamics

The baseline hemodynamic data, hemoglobin concentration, and systemic arterial blood gases for carnitine-treated shunt lambs, vehicle-treated shunt lambs, and age-matched control lambs at 4 wk of age are shown in **Table 1**. There were no statistically significant differences in baseline hemodynamic indexes between the two shunt groups. There were also no differences in hemoglobin concentrations, arterial blood gases, or ventilator parameters among the three groups. The shunt fraction (Qp/Qs) was 2.8 in each group, demonstrating the large aortopulmonary shunt. However, as compared with age-matched control lambs, the significant increases in mean pulmonary arterial pressure and heart rates in vehicle-treated shunt lambs was not observed in carnitine-treated shunt lambs. Carnitine treatment also decreased mean systemic arterial pressure and left pulmonary vascular resistance as compared with age-matched control lambs. Blood flow through the left pulmonary artery and mean left atrial pressure were also significantly increased in carnitine-treated shunt lambs as compared with age-matched controls.

Evaluation of the Proteins Responsible for Maintaining Carnitine Homeostasis

As compared with vehicle-treated shunt lambs, carnitine-treated shunt lambs displayed an increase in the peripheral lung protein levels of carnitine acetyl transferase (CrAT) (**Figure 1a**). Furthermore, CrAT activity was significantly higher in carnitine-treated shunt lambs as compared with vehicle-treated shunt lambs (**Figure 1b**). The level of nitrated

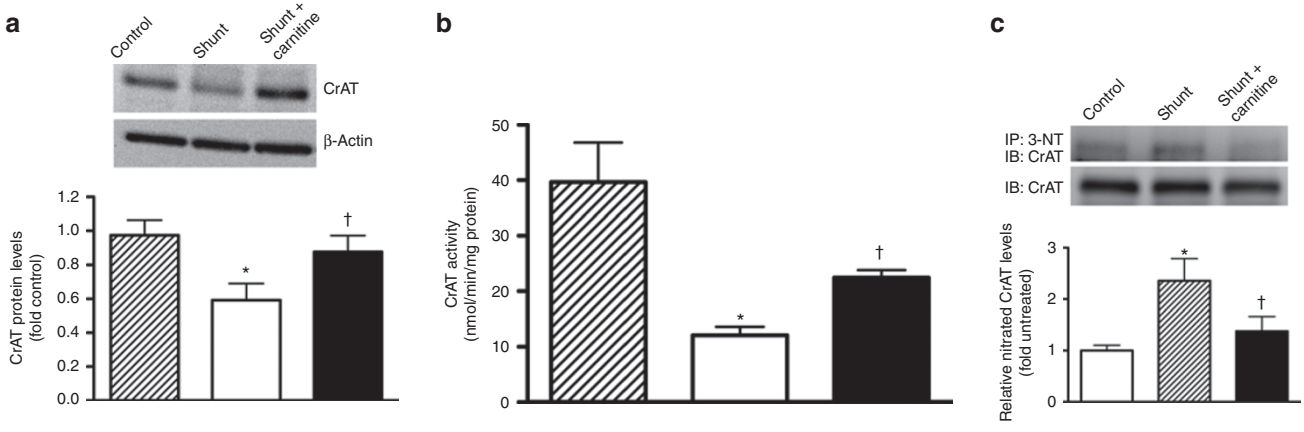

Figure 1. Carnitine acetyltransferase (CrAT) protein levels and activity in the lamb lung. (**a**) Protein extracts (50 μg), prepared from peripheral lung of vehicle- (white) and carnitine-treated (black) shunt lambs as well as age-matched control lambs (hatched) were analyzed by western blot analysis using a specific antiserum raised against CrAT protein. Blots were also normalized for loading using β-actin. A representative blot is shown. (**b**) CrAT activity was determined in protein extracts (40 μg) prepared from peripheral lung tissue from all three groups of lambs. (**c**) Protein extracts (1 mg) were also subjected to immunoprecipitation using an antibody specific to 3-NT and then analyzed by western blot analysis using a specific antiserum raised against CrAT protein. A representative blot is shown. Values are mean ± SEM; $n = 6$ vehicle-treated shunt lambs, $n = 7$ carnitine-treated shunt lambs, and $n = 4$ age-matched control lambs. *$P < 0.05$ vs. control; †$P < 0.05$ vs. vehicle-treated shunt lambs. IB, immunoblot; IP, immunoprecipitation.

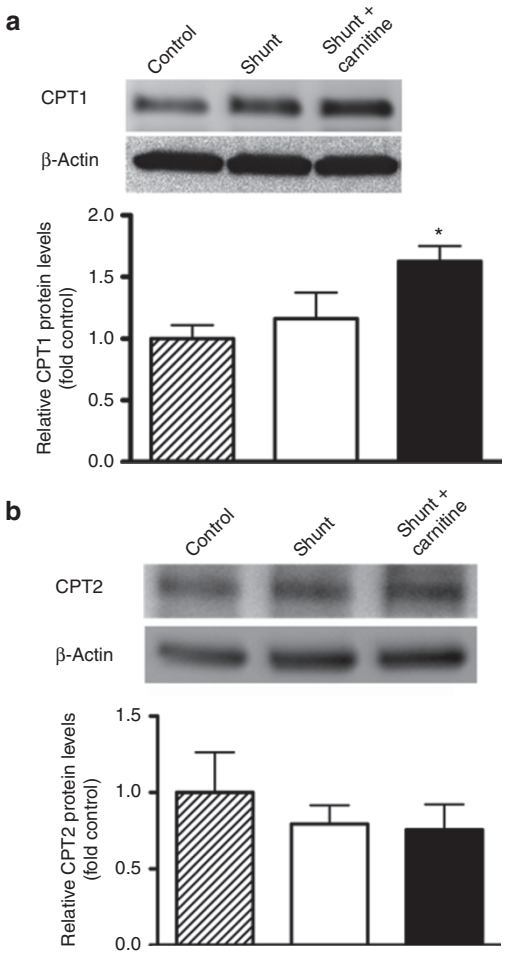

Figure 2. Carnitine palmitoyltransferase 1B (CPT1) and carnitine palmitoyltransferase 2 (CPT2) protein levels in the lamb lung. (**a**,**b**) Protein extracts (50 µg), prepared from peripheral lung of vehicle- (white) and carnitine-treated (black) shunt lambs as well as age-matched control lambs (hatched) were analyzed by western blot analysis using specific antisera raised against CPT1 and CPT2. Blots were also normalized for loading using β-actin. Representative blots are shown. Values are mean ± SEM; $n = 6$ vehicle-treated shunt lambs, $n = 7$ carnitine-treated shunt lambs, and $n = 5$ age-matched control lambs. *$P < 0.05$ vs. control.

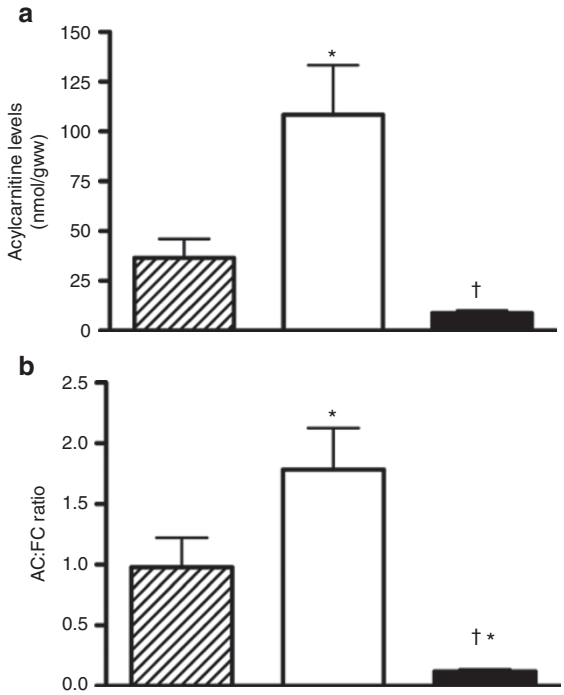

Figure 3. Carnitine homeostasis in the lamb lung. (**a**) Acylcarnitines and (**b**) the acylcarnitine:free carnitine (AC:FC) ratio were determined in peripheral lung of vehicle- (white) and carnitine-treated (black) shunt lambs as well as age-matched control lambs (hatched). Values are mean ± SEM; $n = 6$ vehicle-treated shunt lambs, $n = 7$ carnitine-treated shunt lambs, and $n = 4$ age-matched control lambs. *$P < 0.05$ vs. control; †$P < 0.05$ vs. vehicle-treated shunt lambs.

CrAT was also significantly reduced in carnitine-treated shunt lambs as compared with vehicle-treated shunt lambs (**Figure 1c**). Furthermore, although CrAT protein levels (**Figure 1a**) and nitrated CrAT (**Figure 1c**) were not significantly different from those of age-matched control lambs with normal PBF, CrAT activity was still significantly reduced (**Figure 1b**). Furthermore, the peripheral lung protein levels of carnitine palmitoyltransferase 1 (CPT1) was significantly higher in carnitine-treated shunt lambs as compared with age-matched control lambs with normal PBF (**Figure 2a**), whereas CPT2 levels were unchanged in all three groups (**Figure 2b**).

Evaluation of Carnitine Homeostasis

In the current study, we determined peripheral lung carnitine levels in carnitine- and vehicle-treated shunt lambs as well as age-matched control lambs with normal PBF. We found that acyl carnitine levels were significantly higher in vehicle-treated shunt lambs than in either carnitine-treated shunt lambs or age-matched control lambs with normal PBF (**Figure 3a**). Similarly, the peripheral lung acylcarnitine:free carnitine ratio was significantly higher in vehicle-treated shunt lambs than in either carnitine-treated shunt lambs or age-matched control lambs with normal PBF (**Figure 3b**). In addition, although the acylcarnitine levels were unchanged between carnitine-treated shunt lambs and age-matched control lambs with normal PBF, the acylcarnitine:free carnitine ratio was significantly lower (**Figure 3b**).

Evaluation of Mitochondrial Function

In the current study, we determined and compared the pulmonary levels of lactate and pyruvate to estimate lung mitochondrial activity. As shown in **Figure 4a**, the lactate:pyruvate ratio was significantly higher in vehicle-treated shunt lambs than in either carnitine-treated shunt lambs or age-matched control lambs with normal PBF (**Figure 4a**). The lactate:pyruvate ratio was unchanged in age-matched control lambs with normal PBF as compared with shunt lambs treated with L-carnitine (**Figure 4a**). In addition, our data indicate that Hsp70 protein levels were significantly higher in vehicle-treated shunt lambs than in either carnitine-treated shunt lambs or age-matched control lambs with normal PBF (**Figure 4b**). The levels of Hsp70 were not different between carnitine-treated shunt lambs and age-matched control lambs with normal PBF (**Figure 4b**).

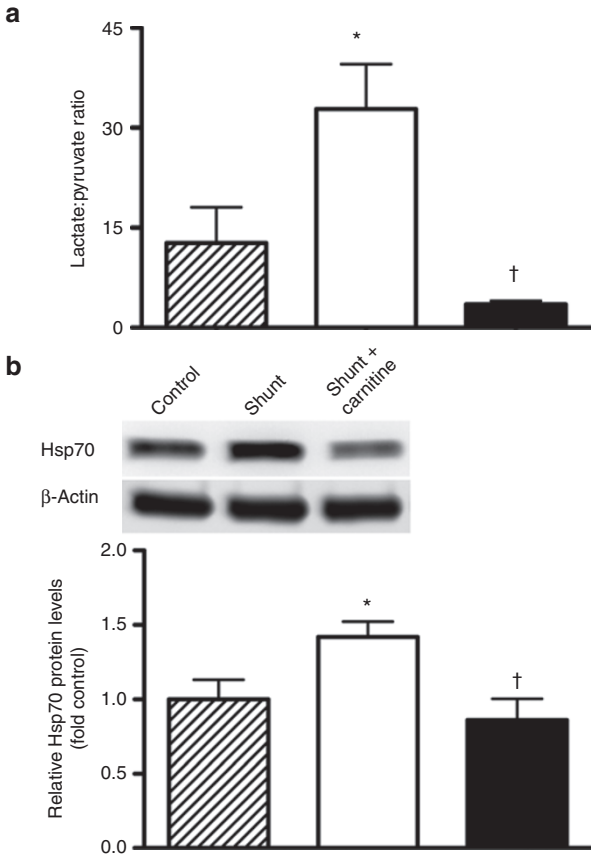

Figure 4. Hsp90–eNOS interactions in the lamb lung. (**a**) The lactate:pyruvate ratio was determined in peripheral lung of vehicle (white)- and carnitine (black)-treated shunt lambs as well as age-matched control lambs (hatched). (**b**) Protein extracts (50 µg), prepared from all three groups were also analyzed by western blot analysis using a specific antisera raised against Hsp70. Blots were also normalized for loading using β-actin. A representative blot is shown. Values are mean ± SEM; $n = 6$ vehicle-treated shunt lambs, $n = 7$ carnitine-treated shunt lambs, and $n = 4$ age-matched control lambs. *$P < 0.05$ vs. control; †$P < 0.05$ vs. vehicle-treated shunt lambs. eNOS, endothelial nitric oxide synthase; Hsp, heat shock protein.

Determinations of eNOS–Hsp90 Interactions, Superoxide Levels, and NO Signaling

In the current study, we determined and compared these parameters in carnitine and vehicle-treated shunt lambs as well as age-matched control lambs with normal PBF. We found that eNOS-bound Hsp90 was significantly lower in vehicle-treated shunt lambs as compared with either carnitine-treated shunt lambs or age-matched control lambs with normal PBF (Figure 5a). Hsp90–eNOS interactions were not significantly different between carnitine-treated shunt lambs and age-matched control lambs with normal PBF (Figure 5a). In addition, we found a decrease in NOS-dependent superoxide levels, indicative of decreased eNOS uncoupling, in carnitine-treated shunt lambs as compared with vehicle-treated shunt lambs (Figure 5b). Again, NOS-derived superoxide levels were not significantly different between carnitine-treated shunt lambs and age-matched control lambs with normal PBF (Figure 5b). The maximum rate of the reaction for total NOS activity in the peripheral lung was also significantly higher in carnitine-treated shunt lambs as compared with both vehicle-treated shunt lambs and age-matched control lambs with normal PBF (Figure 5c). Finally, the increased eNOS–Hsp90 interactions, decreased eNOS-derived superoxide production, and enhanced maximal NOS activity in carnitine-treated shunt lambs were associated with improved NO signaling, as demonstrated by increased plasma NO_x levels as compared with those of vehicle-treated shunt lambs (Figure 5d).

Pulmonary Vascular Reactivity

In the current study, we found that the endothelium-dependent vasodilator acetylcholine (Ach) chloride (1 µg/kg) did not decrease pulmonary vascular resistance (PVR) in vehicle-treated shunt lambs (Figure 6). However, in carnitine-treated shunt lambs, PVR decreased significantly in response to acetylcholine (Figure 6). The Ach-mediated decrease in PVR was not significantly different between carnitine-treated shunt lambs and age-matched control lambs with normal PBF (Figure 6). By contrast, in response to the endothelium-independent vasodilator inhaled NO (40 ppm), calculated PVR decreased similarly in all groups (Figure 6). Figure 6 shows percentage change. These data are identical when analyzed for absolute change over time (data not shown).

DISCUSSION

The results of this study show that chronic L-carnitine treatment prevents the disruption of carnitine homeostasis, reduces oxidative stress, and improves pulmonary mitochondrial function, NO signaling, and endothelial function in our lamb model of CHD with increased PBF. Carnitine is present in the organism as FC or as AC (esterified form), which along with carnitine-dependent enzymes and plasma membrane transporters constitute the carnitine system. Adequate carnitine levels, as well as optimal activities of carnitine-dependent enzymes, are needed to allow the carnitine system to function. The main function of L-carnitine is the transport of long-chain fatty acids from the cytosol to the mitochondrial matrix for β-oxidation and adenosine triphosphate production. However, L-carnitine also plays a key regulatory role in intermediary metabolism by modulating cellular acyl-coenzyme A (CoA)/CoA ratio. This function is mostly dependent on the freely reversible conversion of short-chain acyl-CoA and carnitine to free CoA and acylcarnitine by the intramitochondrial enzyme CrAT. CoA is an obligate cofactor for many enzymes involved in intermediary metabolism. It remains compartmentalized in limited pools within the cell, mainly in the mitochondria, and is normally kept in homeostasis with carnitine. The reversible transfer of acyl groups from CoA to carnitine ensures the vital maintenance of free CoA pools within the mitochondria and prevents the accumulation of poorly metabolized short-chain acyl-CoA compounds, which are exported out of the mitochondria as carnitine esters. Therefore, the carnitine system is crucial for normal mitochondrial function, as the accumulation of acyl groups and the unavailability of

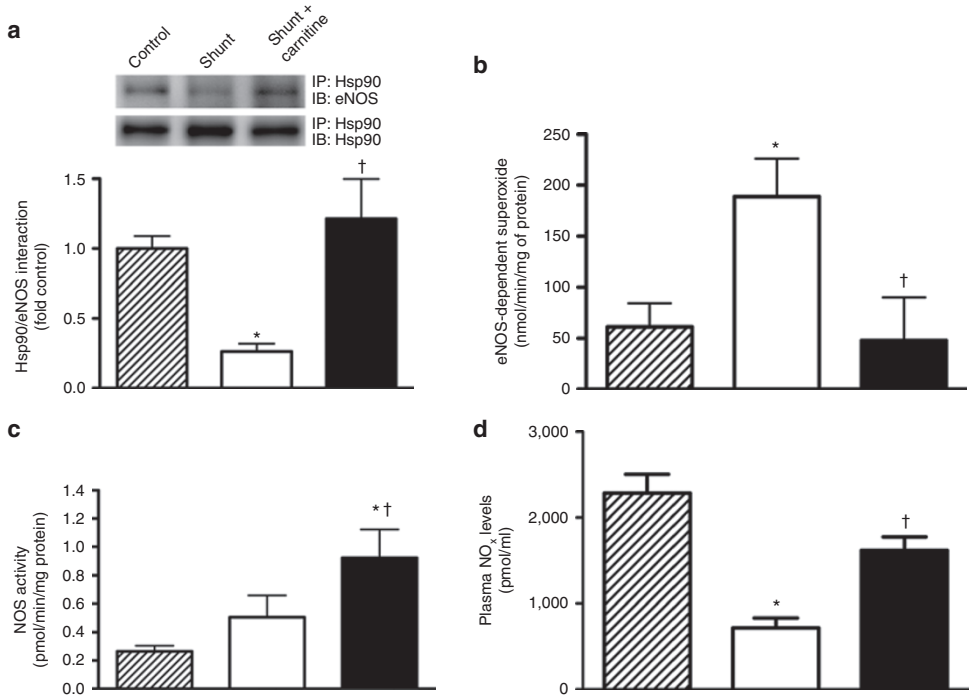

Figure 5. NO signaling in the lamb lung. (**a**) The interaction of eNOS with Hsp90 was determined by immunoprecipitation using specific antiserum raised against eNOS in the peripheral lung of vehicle- (white) and carnitine-treated (black) shunt lambs as well as age-matched control lambs (hatched). Immunoprecipitated extracts were analyzed using antisera against either eNOS or Hsp90 (to normalize for immunoprecipitation efficiency). A representative image is shown. (**b**) Superoxide anion levels were also determined by electronic paramagnetic resonance in snap-frozen lung tissue in each group of lambs, in the presence and absence of the NOS inhibitor, 2-ethyl-2-thiopseudourea (100 µmol/l). (**c**) Total NOS activity and (**d**) NO_x levels were also determined in peripheral lung in all three groups. Values are mean ± SEM; $n = 5$ vehicle-treated shunt lambs, $n = 6$ carnitine-treated shunt lambs, and $n = 6$ age-matched control lambs. *$P < 0.05$ vs. control; †$P < 0.05$ vs. vehicle-treated shunt lambs. eNOS, endothelial nitric oxide synthase; Hsp, heat shock protein; IB, immunoblot; IP, immunoprecipitation.

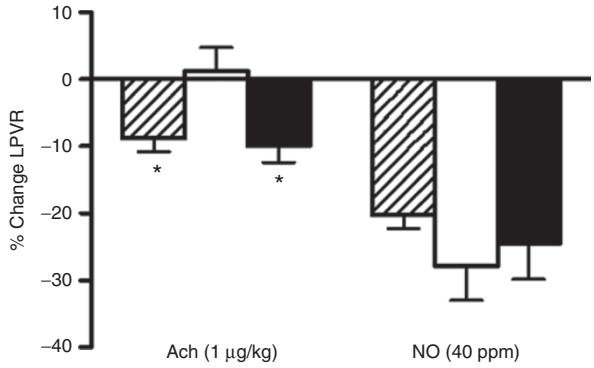

Figure 6. Measurements of left pulmonary vascular resistance (LPVR). Changes in LPVR, expressed as percentage change from baseline, in response to acetylcholine (Ach; 1 µg/kg), an endothelium-dependent agent, and inhaled nitric oxide (40 ppm), an endothelium-independent agent, in carnitine-treated ($n = 7$, black) or vehicle-treated ($n = 5$, white) shunt lambs and age-matched control lambs ($n = 5$, hatched). Acetylcholine significantly decreased LPVR in the carnitine-treated but not in the vehicle-treated shunt lambs. The decrease in PVR was not significantly different between carnitine-treated shunt lambs and age-matched control lambs. All groups experienced a similar drop in PVR in response to inhaled NO (40 ppm). Values are mean ± SD. *$P < 0.05$ compared with baseline.

free CoA result in a metabolic roadblock within the mitochondria, with subsequent impaired oxidative metabolism, increased mitochondrial ROS generation, and decreased energy production (15–17).

We have previously demonstrated disrupted carnitine homeostasis in shunt lambs, and this leads to mitochondrial dysfunction and attenuated NO signaling (14). These lambs showed high AC/FC ratios, reflecting an imbalance in mitochondrial acyl-CoA/CoA, as well as a decreased expression of three important carnitine-dependent enzymes (CPT1, CPT2, and CrAT) and a nitration-dependent decrease in CrAT activity. Despite compelling evidence that oxidative stress plays a causal role in the development of pulmonary vascular disease secondary to increased PBF (13), this study was the first to suggest a mitochondrial component linked to alterations in the carnitine system in its pathogenesis. Previously, lung mitochondrial dysfunction had only been reported in the pulmonary hypertension syndrome observed in fast-growing broilers, which was interestingly attenuated by antioxidant therapy with vitamin E (18). Different mechanisms probably explain this disrupted carnitine homeostasis in our lamb model of increased PBF. It has been proposed that under conditions of metabolic stress such as ischemia–reperfusion injury, the endogenous pool of carnitine can become insufficient for the acyl transfer demand, leading to a carnitine insufficiency state resulting in an increased mitochondrial acyl-CoA/CoA ratio and impaired mitochondrial function (19,20). Other evidence suggests that mitochondrial oxidative stress damages CrAT, decreasing its binding affinity for substrates and resulting in mitochondrial dysfunction and further oxidative stress (21). However, adequate exogenous

carnitine supplementation can overcome this oxidative inhibition (15,19,21). Endothelial NOS is uncoupled in shunt lambs (14). Uncoupled eNOS, through mechanisms not fully elucidated, redistributes from the plasma membrane to the mitochondria, where it induces nitrosative stress by increasing the nitration of mitochondrial proteins (22); our prior *in vivo* data indicate that eNOS-dependent CrAT nitration contributes to the disruption of carnitine homeostasis, which results in mitochondrial dysfunction and subsequent impaired adenosine triphosphate production (14). Previous studies have shown the importance of adenosine triphosphate in pulmonary endothelial function, as demonstrated by its key role in the birth-related pulmonary vasodilation in fetal lambs, a role likely due to its ability to stimulate NO release via the activation of eNOS (23). eNOS activity is tightly controlled through multiple mechanisms that include phosphorylation and protein–protein interactions. Hsp90, a member of a molecular chaperone family, is among the proteins that increase eNOS activity. Therefore, it is plausible that if, as suggested by our data, disruption in carnitine homeostasis decreases Hsp90/eNOS interactions and attenuates NO production (14), L-carnitine supplementation would result in improved endothelial function. It is important to note that as well as eNOS, GTP cyclohydrolase I, the rate-limiting enzyme in tetrahydrobiopterin biosynthesis, is also chaperoned by Hsp90 (24,25). Tetrahydrobiopterin levels are reduced in shunt lambs and also preserved by L-carnitine supplementation (24,25). Therefore, the preservation of NO signaling and endothelial function in L-carnitine–supplemented shunt lambs probably involves increases of GTP cyclohydrolase I /Hsp90 as well as eNOS/hsp90 interactions.

In the current study, we show how chronic L-carnitine supplementation preserved lung carnitine homeostasis in shunt lambs, decreasing the AC:FC ratio. In addition, L-carnitine reduced levels of nitrated CrAT, and this improved the activity of the enzyme. Furthermore, these alterations in the carnitine system were associated with improved mitochondrial function, as demonstrated by a significantly lower lactate/pyruvate ratio and improved NO signaling. Data indicating this improvement include a significant decrease in Hsp70 protein levels, an increase in eNOS-bound Hsp90, and enhanced NO_x levels, as well as reduced eNOS-derived superoxide in carnitine-treated shunt lambs. Of note, all these changes translated functionally into enhanced endothelial function, as demonstrated by a conserved reduction of PVR in response to Ach. The physiological improvement appears to be selective to the endothelium because the response to inhaled NO was unchanged.

Prior studies have evaluated L-carnitine as a therapeutic tool in other conditions characterized by mitochondrial dysfunction and oxidative stress. In addition to reducing the toxicity resulting from excess acyl-CoA, exogenous L-carnitine has been shown to have antioxidant and antiapoptotic properties (26–28). The mechanisms by which L-carnitine protect cells against ROS is not completely clear but may include direct free-radical scavenging and inhibition and/or repair of peroxidized biomolecules (27,29). L-Carnitine supplementation has also been shown to enhance NO production and attenuate oxidative stress and endothelial dysfunction in systemic hypertensive rats (30,31). With respect to pulmonary vascular disease, in a recent study in cold-exposed broilers with pulmonary hypertension syndrome, L-carnitine supplementation showed beneficial effects on lipid peroxidation and pulmonary vascular remodeling and postponed the occurrence of pulmonary hypertension syndrome for 1 wk. Nevertheless, it did not reduce cumulative pulmonary hypertension syndrome mortality (32). In addition, propionyl-L-carnitine increased eNOS protein expression in the same animal model (33). Finally, two recent small studies on children with sickle cell disease and β-thalassemia–associated pulmonary arterial hypertension suggested a benefit of L-carnitine therapy in decreasing pulmonary artery systolic pressure (34,35).

It is also worth noting the potential limitations of our study, especially regarding the oral delivery system we utilized (100 mg/kg/d). It has been shown that oral administration of carnitine occurs both by carrier-mediated transport and through passive diffusion. However, this process appears to be relatively inefficient because previous studies using oral doses of 1–6 g resulted in only 5–18% bioavailability as compared with the 75% bioavailability of L-carnitine ingested through dietary means. Therefore, supplemental doses of L-carnitine appear to be absorbed less efficiently (36). The dose for this study was chosen after three pilot studies in shunt lambs demonstrated that free and acylcarnitine lung levels returned to values similar to those of nonoperated controls (20). It is unclear how L-carnitine supplementation led to an increase in the expression of CPT1. However, it is possible that this may be mediated via an increase in the activity of peroxisome proliferator–activated receptor-γ (PPARγ). We have previously shown that PPARγ expression and activity are reduced in shunt lambs (37), and studies indicate that the expression of at least some of the carnitine homeostasis genes can be regulated by PPAR (38). This study also demonstrated that the promoter region of the CPT1 gene contains a PPAR response element (38). However, a PPAR response element has not been identified in the CrAT gene, and thus, it remains to be elucidated how L-carnitine preserves CrAT expression in shunt lambs. Alternatively, these genes may be downregulated in response to oxidative stress, which is reduced in the presence of L-carnitine. However, further studies will be required to elucidate these mechanisms. It is also worth noting that L-carnitine supplementation did not preserve all the parameters we measured to those observed in age-matched control lambs with normal PBF. Of note, although maximal NOS activity was enhanced in carnitine supplemented lambs, NO_x levels did not increase above those observed in age-matched control lambs with normal PBF. However, as the reduction in PVR in response to Ach was still preserved, this suggests that there is sufficient bioavailable NO produced in carnitine-supplemented shunt lambs to induce smooth muscle cell relaxation. Finally, it is unclear why, and noteworthy that, none of the mitochondrial inborn errors of metabolism associated with carnitine deficiencies have been shown to be associated with the development of pulmonary hypertension. Although it is possible that this correlation has not been investigated or alternatively, because the therapy

for carnitine homeostasis defects is high-dose L-carnitine, this could prevent the potential development of pulmonary hypertension.

In conclusion, our results indicate that chronic L-carnitine treatment attenuates the alterations in lung carnitine homeostasis previously demonstrated in our lamb model of CHD with increased PBF, reducing associated oxidative stress and improving pulmonary mitochondrial function, NO signaling, and ultimately endothelial function. Chronic L-carnitine therapy may improve and/or attenuate the decline in endothelial function noted in children with these disorders and thus has important clinical implications that warrant further investigation.

METHODS

Experimental Model

This procedure has been previously described in detail (18). A total of 24 mixed-breed western neonatal lambs were utilized in our study. These corresponded to 13 lambs with increased PBF subdivided into two experimental groups receiving daily treatment with oral L-carnitine (n = 7, 100 mg/kg/d) or its vehicle (n = 6). Eleven lambs with normal blood flow served as controls. All studies were carried out at 4 wk of age. At the end of the experimental protocol, all lambs were killed with a lethal injection of sodium pentobarbital followed by bilateral thoracotomy as described in the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The committees on animal research of the University of California, San Francisco, and Georgia Regents University approved all protocols and procedures.

Hemodynamic Measurements

Pulmonary and systemic arterial and right and left atrial pressures were measured using Sorenson Neonatal Transducers (Abbott Critical Care Systems, Chicago, IL). Mean pressures were obtained by electrical integration. Heart rates were measured by a cardiotachometer triggered from the phasic systemic arterial pressure pulse wave. Left PBF was measured on an ultrasonic flow meter (Transonic Systems, Ithaca, NY). All hemodynamic variables were measured continuously using the Gould Ponemah Physiology Platform (Version 4.2) and Acquisition Interface (model ACG-16; Gould, Cleveland, OH) and recorded with a Dell Inspiron 5160 computer (Dell, Round Rock, TX). Blood gases and pH were measured on a Radiometer ABL5 pH/blood gas analyzer (Radiometer, Copenhagen, Denmark). Hemoglobin concentration and oxyhemoglobin saturation were measured by a co-oximeter (model 682; Instrumentation Laboratory, Lexington, MA). PVR was calculated using standard formulas. Shunt fraction (Qp/Qs) was determined using the Fick principle. Body temperature was monitored continuously with a rectal temperature probe.

Pulmonary Vascular Reactivity

Pulmonary vascular responses were then assessed in response to ACh and inhaled NO. Ach chloride (1 μg/kg) followed by inhaled NO (40 ppm) was administered. Ach chloride (IOLAB, Claremont, CA) was diluted in sterile 0.9% saline and delivered by rapid injection into the pulmonary artery. Inhaled NO was delivered to the inspiratory limb of the respiratory circuit (Inovent; Ohmeda, Liberty, NJ) and continued for 15 min. The inspired concentrations of NO and nitrogen dioxide were continuously quantified by electrochemical methodology (Inovent; Ohmeda). The hemodynamic variables were monitored and recorded continuously. A minimum of 30 min separated the administration of ACh and inhaled NO, and the second agent was not given until baseline hemodynamics returned.

Preparation of Protein Extracts and Western Blot Analysis

Lung protein extracts were prepared and used for western blot analysis as previously described (3). Briefly, protein extracts (50 μg) were separated on Long-Life 4–20% Tris–sodium dodecyl sulfate–Hepes gels (Gradipore, Frenchs Forest, Australia). All gels were electrophoretically transferred to Immuno-Blot polyvinylidene fluoride membranes (Bio Rad Laboratories, Hercules, CA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20. After blocking, the membranes were probed at room temperature with antibodies to eNOS (BD Transduction Laboratories, San Jose, CA), CPT1B (Affinity Bioreagents, Rockford, IL), CPT2 (Affinity Bioreagents), CrAT (Santa Cruz Biotechnology, Santa Cruz, CA), Hsp70 (Enzo Life Sciences, Farmingdale, NY), or Hsp90 (BD Transduction Laboratories), washed with Tris-buffered saline containing 0.1% Tween, and then incubated with an appropriate IgG conjugated to horseradish peroxidase. Protein bands were then visualized with chemiluminescence (SuperSignal West Femto Substrate Kit, Pierce Laboratories, Rockford, IL) on a Kodak 440CF Image Station (Kodak, Rochester, NY). Band intensity was quantified using Kodak 1D image processing software. All captured and analyzed images were determined to be in the dynamic range of the system. To normalize for protein loading, blots were reprobed with the housekeeping protein β-actin.

Measurement of Carnitine Homeostasis

For FC (L-carnitine and acetyl-L-carnitine) determination, 100 μl samples, 300 μl water, and 100 μl of internal standard (Sigma ST 1093) were mixed. For total carnitine determination, 100-μl samples were hydrolyzed with 0.3 mol/l KOH, heated at 45 °C, and pH-neutralized using perchloric acid; the volume was made to 400 μl, and 100 μl internal standard was added. All samples were purified using solid-phase extraction columns, SAX 100 mg/ml (Varian, Harbor City, CA) and derivatized using aminoanthracene in the presence of 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide hydrochloride (EDCI) (catalyst) and kept at 30 °C for 1 h to complete reaction of carnitines. Separation was carried out with an isocratic elution in 0.1 mol/l Tris–acetate buffer (pH 3.5): acetonitrile (68:32, vol/vol) at a flow rate of 0.9 ml/min as described (39,40). ACs were calculated as total carnitine minus FC. Detection of carnitines was then performed using high-performance liquid chromatography as we have previously described (14).

Measurement of CrAT Activity

Peripheral lung tissue was homogenized in 50 mmol/l Tris–HCl (pH 7.5), 2 mmol/l EDTA, 5 mmol/l $MgCl_2$, 0.8 mmol/l dithiothreitol, and 0.25 mmol/l phenylmethylsulfonyl fluoride (PMSF) with protease inhibitor cocktail. CrAT activity was then determined as previously described (14).

Immunoprecipitation Analyses

Peripheral lung tissues were homogenized in immunoprecipitation buffer (25 mmol/l *N*-2-hydroxyethylpiperazine-*N*9-2-ethanesulfonic acid, pH 7.5, 150 mmol/l NaCl, 1% Nonidet P-40, 10 mmol/l $MgCl_2$, 1 mmol/l EDTA, and 2% glycerol supplemented with protease inhibitor cocktail (Pierce Laboratories, Rockford, IL)). Tissue homogenates (1,000 μg of protein) were precipitated either with a rabbit antibody against 3-nitrotyrosine (5 μg; Upstate Biotechnology) or to eNOS (5 μg; BD Transduction Laboratories) in 0.5 ml final volume at 4 °C overnight as previously described (14).

Determination of Lactate and Pyruvate Levels

This was carried out in peripheral lung tissue as previously described (14).

Assay for NOS Activity

NOS activity was determined using the conversion of ^{3}H-L-arginine to ^{3}H-L-citrulline as previously described (3).

Measurement of Superoxide Levels in Peripheral Lung Tissue

Approximately 0.2 g of peripheral lung tissue was sectioned from fresh-frozen tissue and immediately immersed in either normal EPR buffer (phosphate-buffered saline supplemented with 5 μmol/l diethydithiocarbamate (Sigma-Aldrich, St Louis, MO) and 25 μmol/l desferrioxamine (Sigma-Aldrich)) or EPR buffer supplemented with 100 μmol/l

3 ethylisothiourea (Sigma-Aldrich), an inhibitor of NO synthases (41). Superoxide levels were then estimated by electronic paramagnetic resonance assay using the spin-trap compound 1-hydroxy-3-methoxy-carbonyl-2,2,5,5-tetramethylpyrrolidine·HCl as we have previously described (42,43). NOS-derived superoxide levels were determined by subtracting the values in the presence of ethylisothiourea from the values in the absence of ethylisothiourea. To convert electronic paramagnetic resonance waveforms into units of superoxide, we used 1 mU of xanthine oxidase to generate 1 nmol/l/min of superoxide over a 60-min period to generate a standard curve. Using this standard curve, we were able to convert waveform amplitudes into nmol of superoxide produced/min/mg protein in each reaction condition.

Measurement of Bioavailable NO (NO_x)

Plasma samples were treated with cold ethanol for 1 h at −20 °C and then centrifuged at 20,000*g* to remove proteins that can interfere with NO measurements. Potassium iodide–acetic acid reagent was prepared fresh daily by dissolving 0.05 g of potassium iodide in 7 ml of acetic acid. KI/AcOH mixture was added into a septum-sealed purge vessel and bubbled with nitrogen gas. The gas stream was connected via a trap containing 1 N NaOH to a Sievers 280i Nitric Oxide Analyzer (GE Analytical, Boulder, CO). Samples were injected with a syringe through a silicone–Teflon septum. Results were analyzed by measuring the area under the curve of the chemiluminescence signal using the Liquid software (GE Analytical). The resultant NO_x value represents total nitric oxide and nitrite.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). The means ± SD or SEM were calculated. Statistical significance was determined either by the unpaired *t*-test (for 2 groups) or ANOVA (for ≥3 groups) with Tukey's *post hoc* testing. A value of $P < 0.05$ was considered significant.

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