Rosiglitazone preserves pulmonary vascular function in lambs with increased pulmonary blood flow

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BACKGROUND: Pulmonary vascular function is impaired with increased pulmonary blood flow (PBF). We hypothesized that a peroxisome proliferator-activated receptor- γ (PPAR- γ) agonist would mitigate this effect.

METHODS: An aorta-to-pulmonary-artery shunt was placed in 11 fetal lambs. Lambs received the PPAR- γ agonist rosiglitazone (RG, 3 mg/kg/d, $n = 6$) or vehicle ($n = 5$) for 4 wk. Lung tissue from five normal 4-wk-old lambs was used for comparisons.

RESULTS: At 4 wk, pulmonary artery pressure (PAP) and vascular resistance (PVR) decreased with inhaled nitric oxide (NO) in RG- and vehicle-treated shunt lambs. PAP and PVR decreased with acetylcholine (ACh) in RG-treated, but not vehicle-treated, shunt lambs. In vehicle-treated shunt lambs, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, rac1, superoxide, and 3-nitrotyrosine (3-NT) levels were increased, and Ser1177 endothelial NO synthase (eNOS) protein was decreased as compared with normal lambs. In RG-treated shunt lambs, NOx, Ser1177 eNOS protein, and eNOS activity were increased, and NADPH activity, rac1, superoxide levels, and 3-NT levels were decreased, as compared with vehicle-treated shunt lambs. PPAR- γ protein expression was lower in vehicle-treated shunt lambs than in normal and RG-treated shunt lambs.

CONCLUSION: The PPAR- γ agonist RG prevents the loss of agonist-induced endothelium-dependent pulmonary vascular relaxation in lambs with increased PBF, in part, due to decreased oxidative stress and/or increased NO production.

Infants and children with congenital cardiac defects that cause increased pulmonary blood flow (PBF) are at risk for developing pulmonary vascular disease. In fact, even early pulmonary vascular dysfunction, with abnormal vascular reactivity, causes significant morbidity and mortality in these patients. A decrease in bioavailable nitric oxide (NO), in part, due to oxidative stress, is known to contribute to this pathology (1–3).

Peroxisome proliferator-activated receptors (PPARs), members of a nuclear hormone receptor superfamily, are rapidly emerging as integral mediators of a wide array of disease processes, including vascular disorders (4). Although investigations of the vasculature have focused primarily on the systemic

circulation, one study found that PPAR- γ , one of the three PPAR subclasses, was decreased in lung tissue taken from patients with severe pulmonary arterial hypertension (5). The mechanisms by which PPAR- γ may affect the development of pulmonary vascular disease are not fully understood. However, expanding data indicate that the loss of PPAR- γ may result in perturbations of a number of factors that affect the pulmonary vasculature (6). For example, in recent studies, PPAR- γ ligands have been shown to increase NO production by endothelial NO synthase (eNOS), in part, by increasing eNOS activity by a promotion of eNOS phosphorylation at Ser1177, and to decrease reactive oxygen species production through alterations of NADPH oxidase (7–11).

In earlier studies, we investigated alterations in NO and reactive oxygen species production in the development of pulmonary vascular disease under conditions of increased PBF, using our ovine model of a congenital cardiac defect with increased PBF, created by the *in utero* placement of an aortopulmonary vascular graft (shunt) (1–3). In the postnatal period, these shunt lambs demonstrated impaired endothelium-dependent relaxation, which was associated with a decrease in bioavailable NO, increased NADPH oxidase subunit expression, increased superoxide production, and increased protein nitration (1–3). Recently, we found that PPAR- γ protein expression was decreased in lung tissue taken from 2-wk shunt lambs as compared with healthy age-matched controls, raising the possibility that impaired PPAR- γ signaling could contribute to the observed alterations in pulmonary vascular function and NO signaling (12).

Therefore, the purpose of this study was to test the hypothesis that chronic treatment with the PPAR- γ agonist rosiglitazone (RG) would preserve endothelium-dependent pulmonary vascular relaxation, increase bioavailable NO, and decrease markers of oxidative stress in lambs with increased PBF. To this end, shunt lambs were treated from birth until 4 wk of age with enteral RG or vehicle control. At 4 wk of age, pulmonary vascular responses to acetylcholine (ACh), an endothelium-dependent vasodilator, and inhaled NO, an endothelium-independent vasodilator, were determined, and lung tissue

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was harvested for determinations of bioavailable NO (NO $_x$ levels), eNOS protein expression and activity, eNOS Ser1177 phosphorylation, NADPH subunit expression (ρ -related C3 botulinum toxin substrate (Rac1), p47^{phox} (phagocytic oxidase), and p67^{phox}) and activity, superoxide levels, and 3-nitrotyrosine (3-NT), an indirect measure of peroxynitrite.

RESULTS

There were no differences in gestational age, weight, or sex distribution between normal and vehicle- and RG-treated shunt lambs (data not shown). Likewise, baseline hemodynamic variables were not different between vehicle- and RG-treated shunt lambs (Table 1). Baseline hemodynamics and pulmonary vascular reactivity with comparisons between normal and shunt lambs at 4 wk of age have been previously reported (1).

Pulmonary Vascular Reactivity

Hemodynamic changes in response to ACh and inhaled NO in vehicle- and RG-treated shunt lambs are shown in Table 2. Pulmonary artery pressure (PAP) and calculated pulmonary vascular resistance (PVR) did not change in response to ACh in vehicle-treated shunt lambs (Table 2 and Figure 1). In contrast, PAP and calculated PVR decreased in response to ACh in RG-treated shunt lambs ($-18.6 \pm 5.4\%$ and $-18.2 \pm 7.3\%$, respectively, $P < 0.05$, Table 2 and Figure 1). In response to inhaled NO, PAP and calculated PVR decreased in both vehicle-treated ($-19.3 \pm 6.3\%$ and $-34.4 \pm 11.6\%$, respectively, $P < 0.05$) and RG-treated shunt lambs ($-16.1 \pm 4.2\%$ and $-26.4 \pm 8.2\%$, respectively, $P < 0.05$, Table 2 and Figure 1). Changes in PVR in vehicle- and RG-treated shunt lambs in response to ACh and inhaled NO are shown in Figure 1.

Lung Tissue NOS Activity, NO $_x$, eNOS Protein, and Ser117 Phosphorylation

Lung tissue NOS activity is shown in Figure 2a. Lung tissue NOS activity was greater in RG-treated shunt lambs than in vehicle-treated shunt lambs, and both were greater than in normal lambs (0.51 ± 0.28 vs. 0.22 ± 0.08 vs. 0.05 ± 0.03 pmol/min/mg protein, respectively, $P < 0.05$). Lung tissue NO $_x$ levels were greater in RG-treated shunt lambs than in vehicle-treated shunt lambs and normal lambs (16.9 ± 8.9 μ mol/l vs. 5.7 ± 3.0 vs. 4.5 ± 3.3 μ mol/l, respectively, $P < 0.05$, Figure 2b). NO $_x$ levels relative to NOS activity (NO $_x$ /NOS activity ratio) were greater in normal lambs than in vehicle-treated and RG-treated shunt lambs ($P < 0.05$). Lung tissue eNOS protein levels as determined by western blot analysis were not different between groups (data not shown). eNOS phosphorylation

at Ser1177 is shown in Figure 2c. eNOS phosphorylation at Ser1177 was lower in vehicle-treated shunt lambs than in normal lambs and RG-treated shunt lambs ($P < 0.05$).

NADPH Oxidase Subunit Expression

Lung tissue protein levels of p47^{phox}, p67^{phox}, and rac1 in normal and vehicle- and RG-treated shunt lambs are shown in Figure 3a–c). Lung tissue protein expression of p47^{phox} was $\sim 20\%$ greater and expression of p67^{phox} was twofold greater in vehicle-treated shunt lambs than in normal lambs, but differences between groups failed to reach statistical significance (ANOVA, $P = 0.08$, Figure 3a,b). Lung tissue protein expression of rac1 was twofold greater in vehicle-treated shunt lambs than in normal lambs, and levels were similar between normal lambs and RG-treated shunt lambs (Figure 3c, ANOVA, $P < 0.05$).

NADPH Oxidase Activity

NADPH oxidase activity in lung tissue was higher in vehicle-treated shunt lambs than in normal lambs and RG-treated shunt lambs (Figure 4, ANOVA, $P < 0.05$).

Superoxide Quantitation

Relative superoxide levels, determined by electronic paramagnetic resonance (EPR) on peripheral lung taken from normal lambs and vehicle- and RG-treated shunt lambs, are shown in Figure 5. Superoxide levels were ~ 1.75 -fold greater in vehicle-treated shunt lambs than in normal lambs (Figure 5a, ANOVA, $P < 0.05$). Superoxide levels were not different between normal lambs and RG-treated shunt lambs (Figure 5a). Specificity of the EPR assay for superoxide was confirmed by a significant reduction in the waveform amplitude in vehicle-treated lambs with the addition of superoxide dismutase to the samples (13). To determine to what extent the contribution of superoxide production arose from NADPH oxidase activity, samples were incubated with apocynin (an NADPH oxidase inhibitor) for 30 min on ice before the addition of 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-HCl (CM). Apocynin-mediated inhibition of NADPH resulted in almost no change in EPR amplitude of normal lambs and RG-treated shunt lambs, whereas vehicle-treated shunt lambs showed up to $\sim 37\%$ reduction in signal (Figure 5a, $P < 0.05$).

Protein Nitration

As an indirect determination of peroxynitrite formation, lung tissue nitrotyrosine (3-NT) levels were determined and are shown in Figure 5b. Lung tissue 3-NT protein expression was greater in vehicle-treated shunt lambs than in normal

Table 1. Baseline hemodynamics

Treatment	HR (beats/min)	mPAP (mm Hg)	Syst SAP (mm Hg)	Diast SAP (mm Hg)	mSAP (mm Hg)	Q (LPA; (ml/min/kg)	RAP (mm Hg)	LAP (mm Hg)	Qp:Qs
Vehicle	155.8 $\pm$ 27.1	25.0 $\pm$ 3.7	123.0 $\pm$ 18.0	32.1 $\pm$ 7.9	60.8 $\pm$ 6.4	165.5 $\pm$ 51.7	5.4 $\pm$ 1.3	8.5 $\pm$ 2.6	2.74 $\pm$ 0.6
Rosiglitazone	124 $\pm$ 24	23.8 $\pm$ 7.4	115.7 $\pm$ 17	41.9 $\pm$ 9.2	62.6 $\pm$ 7.4	128.2 $\pm$ 41.1	5.7 $\pm$ 1.3	6.7 $\pm$ 1.8	2.8 $\pm$ 0.8

Diast SAP, diastolic systemic arterial pressure; HR, heart rate; LAP, left atrial pressure; LPA, left pulmonary artery; mPAP, mean pulmonary artery pressure; mSAP, mean systemic arterial pressure; Q, flow; Qp:Qs, ratio of pulmonary to systemic flow; RAP, right atrial pressure; Syst SAP, systolic systemic arterial pressure.

Table 2. Hemodynamic changes in response to ACh and inhaled nitric oxide

	Baseline	ACh (1 µg/kg)	Baseline	iNO (40 ppm)
Vehicle-treated lambs				
Mean PAP (mm Hg)	25.0 ± 3.7	21.9 ± 3.5	25.4 ± 3.9	20.3 ± 1.8*
LPBF (ml/min/kg)	165.5 ± 51.7	156.4 ± 39.5	171.3 ± 54.9	178.5 ± 55.8
LAP (mm Hg)	9.5 ± 2.6	8.0 ± 1.1	9.7 ± 2.5	9.6 ± 2.6
RAP (mm Hg)	5.4 ± 1.3	6.0 ± 1.5	5.4 ± 1.4	4.9 ± 1.4
HR (beats/min)	155.8 ± 27.1	141.6 ± 23.4	155.3 ± 26.7	149.6 ± 23.7
Rosiglitazone-treated lambs				
Mean PAP (mm Hg)	23.8 ± 7.4	19.4 ± 6.6*	24.5 ± 7.2	20.5 ± 6.0*
LPBF (ml/min/kg)	136.0 ± 37.5	128.2 ± 41.1	138.8 ± 32.1	144.5 ± 31.0
LAP (mm Hg)	6.7 ± 1.8	6.5 ± 1.0	6.8 ± 3.7	6.4 ± 3.5
RAP (mm Hg)	5.0 ± 1.3	5.7 ± 1.3	5.1 ± 1.4	4.9 ± 1.4
HR (beats/min)	124.1 ± 24	119.1 ± 11.1	125.7 ± 21.3	123.3 ± 24.2

Hemodynamic values before (baseline) and after the administration of ACh and iNO. Values are mean ± SD.

ACh, acetylcholine; HR, heart rate; iNO, inhaled nitric oxide; LAP, left atrial pressure; LPBF, left pulmonary artery blood flow; PAP, pulmonary arterial pressure; RAP, right atrial pressure.

* $P < 0.05$ as compared with baseline value.

Figure 1. Changes in pulmonary vascular resistance (PVR), expressed as percentage change from baseline, in response to acetylcholine (ACh, 1 µg/kg), an endothelium-dependent agent, and inhaled nitric oxide (NO, 40 ppm), an endothelium-independent agent, in vehicle- and rosiglitazone (RG)-treated shunt lambs. In vehicle-treated shunt lambs (white bars), PVR decreased in response to inhaled NO, but not to ACh. In RG-treated shunt lambs (black bars), PVR decreased in response to both ACh and inhaled NO. $n = 6$ for RG-treated group; $n = 5$ for vehicle-treated group. Values are mean ± SD. * $P < 0.05$ as compared with baseline.

lambs and RG-treated shunt lambs (Figure 5b, ANOVA, $P < 0.05$).

PPAR-γ Protein Expression

Lung tissue PPAR-γ protein levels in vehicle-treated shunt lambs were lower than in control lambs and RG-treated shunt lambs (Figure 6, ANOVA, $P < 0.05$).

DISCUSSION

A vast and expanding body of literature has established the importance of endothelial dysfunction in the pathogenesis

of pulmonary vascular disease. However, endothelial abnormalities are defined broadly. They include alterations in structure, perturbations in the normal production and/or balance of endothelial-derived factors, and functional abnormalities, including increased constriction and/or impaired relaxation. This study used a model of a congenital cardiac defect with increased PBF that mimics the early functional impairment in pulmonary vascular endothelium-dependent relaxation that occurs in infants and children with large left-to-right cardiac shunts, before the development of advanced structural changes and large elevations in PAP. Indeed, in the current era, in which surgical repair in neonates and infants is relatively safe and common, it is these early derangements that account for a substantial portion of the morbidity rather than the advanced structural disease that occurred in previous decades. The primary finding was that chronic treatment with RG prevented impairment in agonist-induced endothelium-dependent pulmonary vascular relaxation in shunt lambs. The association of this finding with an increase in lung tissue NO_x levels, increased eNOS activity, increased eNOS phosphorylation (Ser1177), decreased NADPH oxidase activity and subunit expression, and decreased lung tissue superoxide and 3-NT levels suggests two potential mechanisms for this effect: PPAR-γ activation may increase bioavailable NO through a decrease in NADPH oxidase activity that results in a decrease in NO scavenging by superoxide, and PPAR-γ activation may increase NO production from eNOS by increasing eNOS activity via increased phosphorylation at Ser1177.

In vitro and *in vivo* studies indicate that PPAR-γ signaling can alter oxidative stress and that RG treatment can decrease superoxide production in the systemic vasculature (9–11,14). In the pulmonary vasculature, RG treatment was shown to attenuate pulmonary arterial hypertension and vascular remodeling in chronic-hypoxia mouse models. Mechanisms include alterations in NADPH subunit expression and

Figure 2. Lung tissue nitric oxide synthase (NOS) activity, NO $_x$ levels, and Ser1177 endothelial NOS (eNOS) protein in normal (white bars) and vehicle- (black bars) and rosiglitazone (RG)-treated (gray bars) shunt lambs. **(a)** NOS activity was greater in vehicle-treated shunt lambs than in normal lambs, and greater in RG-treated shunt lambs than in both normal lambs and vehicle-treated shunt lambs. **(b)** Lung tissue NO $_x$ levels were higher in RG-treated shunt lambs than in normal lambs and vehicle-treated shunt lambs. **(c)** Lung tissue protein expression of Ser1177 eNOS was decreased in vehicle-treated shunt lambs as compared with normal lambs and increased in RG-treated shunt lambs as compared with vehicle-treated shunt lambs. Representative western blots are shown. Densitometric values for Ser1177 eNOS protein are shown relative to normal. $n = 5$ for normal group; $n = 5$ for vehicle-treated shunt group; $n = 6$ for RG-treated shunt group. Values are mean $\pm$ SD. * $P < 0.05$ as compared with normal group. † $P < 0.05$ as compared with vehicle-treated shunt group.

Figure 3. Lung tissue protein levels of nicotinamide adenine dinucleotide phosphate oxidase subunits **(a)** p47^{phox}, **(b)** p67^{phox}, and **(c)** rac1 in normal (white bars) and vehicle- (black bars) and rosiglitazone (RG)-treated (gray bars) shunt lambs. **(a,b)** Differences between lung tissue protein expression of p47^{phox} and p67^{phox} (ANOVA, $P = 0.08$) did not reach statistical significance. **(c)** Lung tissue protein expression of rac1 was greater in vehicle-treated shunt lambs than in normal lambs, and was lower in RG-treated as compared with vehicle-treated shunt lambs (ANOVA, $P < 0.05$). Representative western blots are shown. Densitometric values shown relative to normal. $n = 5$ for normal group; $n = 5$ for vehicle-treated shunt group; $n = 6$ for RG-treated shunt group. Values are mean $\pm$ SD. * $P < 0.05$ as compared with normal group. † $P < 0.05$ as compared with vehicle-treated shunt group.

superoxide levels, regulation of Nox4 expression through NF- κ B, and a downregulation of PPAR- γ through transforming growth factor- β (15–17). Thus, the findings of the current study are consistent with this emerging literature but are unique in demonstrating a role for PPAR- γ in early selective pulmonary vascular endothelial dysfunction secondary to increased PBF.

In our study, a role for NADPH oxidase is supported by changes in its activity and a decrease in superoxide signaling in the EPR assay with apocynin (an NADPH oxidase inhibitor) in vehicle-treated shunt lambs. Of note, rac1 protein levels normalized in RG-treated shunt lambs and apocynin had no impact on superoxide levels. Although these data strongly implicate PPAR- γ signaling in increased NADPH activity under conditions of increased PBF, the controlling mechanisms require further investigation.

Lung tissue eNOS phosphorylation at Ser1177 decreased in vehicle-treated lambs as compared with normal lambs and normalized in shunt lambs with RG treatment, in association with a significant increase in eNOS activity. Phosphorylation at Ser1177 is known to increase electron flux and enzyme activity of eNOS (18,19). Therefore, the increased lung tissue NO $_x$ levels in RG-treated shunt lambs, which were independent of changes in eNOS expression, may relate, in part, to an increase in NO release from endothelial cells apart from, or in addition to, diminished scavenging of NO by superoxide. PBF (with its associated shear forces) is a well-established and strong stimulus of NO release from eNOS (20). Therefore, the increases in NO $_x$ and eNOS activity in shunt lambs as compared with normal lambs were expected and consistent with previous studies (1). Alterations in bioavailable NO cannot be simply understood by analysis of lung tissue NO $_x$ levels. Rather, the demonstration

Figure 4. Lung tissue nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity in normal (white bar) and vehicle- (black bar) and rosiglitazone-treated (gray bar) shunt lambs. Lung tissue NADPH oxidase activity was measured by a low concentration-based lucigenin (5 μ mol/l) assay. Control activity was arbitrarily set at 100%. $n = 4$ for each group. Values are mean $\pm$ SD. * $P < 0.05$, as compared with normal group. † $P < 0.05$ as compared with vehicle-treated shunt group.

of impaired agonist-induced selective endothelium-dependent pulmonary relaxation is of primary relevance. However, we have previously demonstrated that impaired bioavailable NO can be understood by analyzing NO_x levels relative to eNOS activity. We have shown previously that shunt lambs develop a progressive decrease in NO_x levels relative to eNOS activity as compared with age-matched normal lambs from 2 to 8 wk of age (1). In the current study, NOS activity was increased in shunt lambs compared with normal lambs, as expected, and NO_x levels relative to eNOS activity were greater in normal lambs as compared with shunt lambs. Of note, RG treatment resulted in a marked increase in NO_x and eNOS activity in shunt lambs.

Superoxide reacts avidly with NO to produce peroxynitrite. The determination of peroxynitrite levels *in vivo* is challenging. Peroxynitrite readily nitrates protein tyrosine residues, and thus quantification of nitrotyrosine (3-NT) provides an indirect measure of peroxynitrite levels. Consistent with previous studies, we found an increase in 3-NT levels in vehicle-treated shunt lambs as compared with normal lambs. Moreover, consistent with our hypothesis that RG treatment would decrease oxidative stress in shunt lambs, we found the RG-treated shunt lambs had 3-NT levels that were lower than those of vehicle-treated shunt lambs and similar to those of normal lambs.

Of note, we found that PPAR- γ expression was decreased in vehicle-treated shunt lambs as compared with normal lambs and was increased in RG-treated shunt lambs. Little is known about the regulation of PPAR- γ expression in lung tissue in either health or disease. A recent study demonstrated that NO can activate PPAR- γ via a p38 mitogen-activated protein kinase pathway, which is intriguing given the increased NO_x levels in RG-treated shunt lambs (21). However, whether cross talk between NO and PPAR- γ results in increased PPAR- γ expression in lung tissue is unclear. In addition,

Figure 5. Superoxide and 3-nitrotyrosine (3-NT) protein levels in lung tissue from normal (white bars) and vehicle- (black bars) and rosiglitazone (RG)-treated (gray bars) shunt lambs. (a) Superoxide levels estimated by electron paramagnetic resonance (EPR). Superoxide levels were higher in vehicle-treated shunt lambs than in normal lambs, and lower in RG-treated than in vehicle-treated lambs. Specificity of the EPR assay for superoxide was confirmed by a reduction in the waveform amplitude with the addition of SOD to the samples in vehicle-treated shunt lambs. The addition of apocynin (an NADPH oxidase inhibitor) to the samples resulted in no change in EPR amplitude of normal and RG-treated lambs but a ~37% signal reduction in vehicle-treated lambs. (b) Lung tissue 3-NT levels were increased in vehicle-treated shunt lambs as compared with normal lambs, and decreased in RG-treated as compared with vehicle-treated shunt lambs. Representative western blots are shown. $n = 5$ for normal group; $n = 5$ for vehicle-treated shunt group; $n = 6$ for RG-treated shunt group. Values are mean $\pm$ SD. * $P < 0.05$ as compared with normal group. † $P < 0.05$ as compared with vehicle-treated shunt group. NADPH, nicotinamide adenine dinucleotide phosphate; SOD, superoxide dismutase.

whether PPAR- γ activation, from RG-treatment, alone accounted for the preservation in endothelium-dependent pulmonary vascular relaxation or whether the increase in PPAR- γ expression was necessary remains an interesting but unanswered question.

We conclude that PPAR- γ activation with chronic RG treatment attenuates the impairment in agonist-induced endothelium-dependent pulmonary vascular relaxation in 4-wk-old lambs with a model of increased PBF. We propose that an increase in bioavailable NO underlies this effect, and that it may be mediated by two potential mechanisms: a decrease in NADPH oxidase activity that results in lower superoxide levels with less NO scavenging, and an increase in NO production secondary to increased eNOS activity due to increased phosphorylation at Ser1177. The potential ability to use PPAR- γ agonists to diminish endothelial injury and/or dysfunction in infants and children with common congenital cardiac defects that result in increased PBF is exciting given that this class of medications is currently commercially available for clinical

Figure 6. Lung tissue protein expression of peroxisome proliferator-activated receptor (PPAR- γ) in normal (white bar) and vehicle- (black bar) and rosiglitazone (RG)-treated (gray bar) shunt lambs. PPAR- γ was decreased in vehicle-treated shunt lambs as compared with normal lambs and increased in RG-treated shunt lambs as compared with vehicle-treated shunt lambs. Representative western blots are shown. Densitometric values for PPAR- γ protein are shown relative to normal. $n = 5$ for normal group; $n = 5$ for vehicle-treated shunt group; $n = 6$ for RG-treated shunt group. Values are mean $\pm$ SD. * $P < 0.05$ as compared with normal group. † $P < 0.05$ as compared with vehicle-treated shunt group.

use. Therefore, we believe that further investigation of these pathways is warranted.

METHODS

Surgical Preparation

A total of 11 mixed-breed Western pregnant ewes (137–141 d gestation, term = 145 d) were anesthetized. Fetal exposure was obtained through the pregnant horn of the uterus and a left lateral thoracotomy was performed on the fetal lamb. With the use of side biting vascular clamps, an 8.0-mm vascular graft was anastomosed between the ascending aorta and main pulmonary artery of the fetal lambs. This procedure was previously described in detail (1).

Four weeks after spontaneous delivery, shunt lambs were anesthetized and catheters were placed into the right and left atrium and main pulmonary artery. An ultrasonic flow probe (Transonics Systems, Ithaca, NY) was placed around the left pulmonary artery to measure PBF. Arterial blood and lung biopsies were obtained. In addition, using identical anesthesia, arterial blood and lung tissue were harvested from five normal, nonshunted, 4-wk-old lambs for comparisons of biochemical data with those of vehicle- and RG-treated shunt lambs. Arterial blood and lung pieces were processed as previously described in detail (1).

At the end of the 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. All protocols and procedures were approved by the Committee on Animal Research of the University of California, San Francisco.

Drug Protocol

Beginning immediately after birth, lambs were treated with either RG (GlaxoSmithKline, Brentford, UK, $n = 6$, 3 mg/kg/d) or empty gelatin capsules ($n = 5$, vehicle control). The drug or vehicle capsules were administered orally once daily for a 4-wk period. The number of vehicle capsules administered was increased to match the upwardly adjusted weight-based dosing of RG.

Measurements

Pulmonary and systemic arterial, and right and left atrial pressures, heart rate, and PBF were measured. All hemodynamic variables were measured continuously using the Gould Ponemah Physiology Platform (Version 4.2) and Acquisition Interface (Model ACG-16, Gould, Cleveland, OH). Blood gases and pH were measured, and PVR was calculated using standard formulae. Shunt fraction (pulmonary flow/systemic flow) was determined using the Fick principle. These procedures were previously described in detail (1).

Pulmonary Vascular Reactivity

Pulmonary vascular responses were assessed after the administration of ACh and inhaled NO. Lambs were instrumented as above and allowed to rest for a minimum of 30 min. Mechanical ventilation was adjusted to maintain $p\text{CO}_2$ between 35 and 40 mm Hg and pH between 7.35 and 7.40. Sodium bicarbonate was administered to correct metabolic acidosis if it occurred. The inspired oxygen concentration was maintained at 21%. Baseline hemodynamics and PBF were recorded. In random order, ACh (1 $\mu\text{g/kg}$) or 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 10 min. The inspired concentrations of NO and nitrogen dioxide were continuously quantified by electrochemical methodology (Inovent, Ohmeda, Liberty, NJ). 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 hemodynamics returned to baseline.

Preparation of Protein Extracts and Western Blot Analysis

Lung protein extracts were prepared and western blot analysis was performed as previously described (1,22). Briefly, western blot analysis was performed with a 1:2,500 dilution of the NOS antiserum; 1:100 dilution of PPAR- γ ; 1:500 dilution of Rac1, p47^{phox}, and p67^{phox}; 1:1,000 dilution of 3-NT antibody, and antiphospho Ser1177 eNOS; or a 1:10,000 dilution of β -actin, washed with tris-buffered saline containing 0.1% Tween, and then incubated with a goat antimouse immunoglobulin G horseradish peroxidase. After washing, the protein bands were visualized with chemiluminescence using a Kodak Digital Science Image Station (NEN Life Science Products, Boston, MA) and analyzed using the KED-1 software (KDE, Brighton, MA). All captured and analyzed images were determined to be in the dynamic range of the system. The eNOS, Rac1, p47^{phox}, and PPAR- γ antisera were obtained from Transduction Laboratories (Lexington, KY); 3-NT antibody was obtained from Calbiochem (San Diego, CA); and antiphospho Ser1177 eNOS was obtained from Cell Signaling Technology (Danvers, MA). To normalize for protein loading, blots were reprobed with β -actin.

Measurement of NO_x

To quantify bioavailable NO, NO and its metabolites were determined in lung tissue as we have previously described (1,23,24). The sensitivity is 1×10^{-12} moles, with a concentration range of 1×10^{-9} to 1×10^{-3} molar of nitrate.

Assay for NOS Activity

NOS activity was determined using the conversion of 3H-L-arginine to 3H-L-citrulline as described by Bush (25) and as we have previously described in detail (1). All activities were normalized to the amount of protein in each lysate. To determine the potential contribution of inducible NOS to total NOS activity, assays were repeated without calcium supplementation.

Lung Tissue NADPH Oxidase Assay

Lung tissue from normal lambs and vehicle- and RG-treated shunt lambs was collected and homogenated with Tris-sucrose buffer (10 mmol/l Tris base (Fischer, Fairlawn NJ)), 340 mmol/l sucrose (Mallinkrodt Baker, Phillipsburg, NJ), 1 mmol/l EDTA (Mallinkrodt

Baker), and 10 µg/ml protease inhibitor mixture (Sigma, St. Louis, MO). The homogenate protein concentration was measured. NADPH oxidase activity was measured by a luminescence assay in the reaction buffer with 5 µmol/l lucigenin, 1 mmol/l EGTA, and 50 mmol/l phosphate buffer, pH 7.0. A total of 100 µg of homogenate protein + 100 µmol/l NADPH as substrate (Sigma) was added to an 8 mm test tube with 500 µl of reaction buffer, then incubated at 37°C for 5 min. Photon emission was measured at 15 s in a luminometer (model TD-20/20; Turner Designs, Sunnyvale, CA) (26,27).

Superoxide Quantitation

Superoxide levels in lung tissue were estimated by EPR assay using the spin-trap compound CM in the presence and absence of polyethylene glycol-superoxide dismutase, as we have previously described (1,13). To determine the specificity of the detected EPR signal, additional sample groups were immersed in EPR buffer supplemented with 100 U/ml of the membrane-permeable form of superoxide dismutase (polyethylene glycol-conjugated superoxide dismutase). In addition, to determine the relative contribution of NADPH oxidase activity to superoxide production, equivalent samples were preincubated in 100 µmol/l apocynin (Sigma, St. Louis, MO), an NADPH oxidase inhibitor, as we have previously described (1,28). Samples were analyzed for protein content using Bradford analysis (Bio-Rad, Hercules, CA). Sample volumes were then adjusted with EPR buffer and 25 mg/ml CM hydrochloride to achieve equal protein content and a final CM concentration of 5 mg/ml. Samples were analyzed with a MiniScope MS200 ESR (Magnetech, Berlin, Germany). Resulting EPR spectra were analyzed using ANALYSIS v.2.02 software (Magnetech), whereby the EPR maximum and minimum spectral amplitudes for the CM superoxide spin-trap product waveform were quantified.

Statistical Analysis

Statistical calculations were performed using the GraphPad Prism V. 4.01 software (GraphPad Software, La Jolla, CA). The mean ± SD or SE was calculated for all samples, and significance was determined either by the unpaired *t*-test or ANOVA. For ANOVA, Newman-Keuls *post hoc* testing was also used.

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