Type 1 Diabetes-Induced Hyper-Responsiveness to 5-Hydroxytryptamine in Rat Pulmonary Arteries via Oxidative Stress and Induction of Cyclooxygenase-2

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ABSTRACT
Recent epidemiological data suggest that diabetes is a risk factor for pulmonary arterial hypertension. The aim of the present study was to analyze the link between type 1 diabetes and pulmonary arterial dysfunction in rats. Male Sprague-Dawley rats were randomly divided into a control group (saline) and a diabetic group (70 mg/kg streptozotocin). After 6 weeks, diabetic animals showed a down-regulation of the lung bone morphogenetic protein receptor type 2, up-regulation of 5-hydroxytryptamine (5-HT) 2A receptors and cyclooxygenase-2 (COX-2) proteins as measured by Western blot analysis, and increased contractile responses to 5-HT in isolated intrapulmonary arteries. The hyper-responsiveness to 5-HT was endothelium-independent and unaffected by inhibition of nitric-oxide synthase but prevented by indomethacin, the selective COX-2 inhibitor N-[2-(cyclohexyl氧yl)-4-nitrophenyl]-methane sulfonamide (NS-398), superoxide dismutase, and the NADPH oxidase inhibitor apocynin or chronic treatment with insulin. However, diabetic rats at 6 weeks did not develop elevated right ventricular pressure or pulmonary artery muscularization, whereas a longer exposure (4 months) to diabetes induced a modest, but significant, increase in right ventricular systolic pressure. In conclusion, type 1 diabetes mellitus in rats induces a number of changes in lung protein expression and pulmonary vascular reactivity characteristic of clinical and experimental pulmonary arterial hypertension but insufficient to elevate pulmonary pressure. Our results further strengthen the link between diabetes and pulmonary arterial hypertension.

Introduction

Despite the fact that type 1 and type 2 diabetes are strongly associated with systemic cardiovascular diseases (Rutter et al., 2005) the relationship with pulmonary vascular disease has been almost disregarded (Fouty, 2008). However, epidemiological studies suggest a link between diabetes and pulmonary arterial hypertension (PAH) (Movahed et al., 2005; Makarevich et al., 2007; Zamanian et al., 2009). In addition, maternal diabetes is an independent risk factor for persistent pulmonary hypertension of the newborn (Hernandez-Diaz et al., 2007). There is also some experimental evidence linking type 2 diabetes with PAH. Thus, male ApoE(-/-) mice on a high-fat diet, an animal model associated to insulin resistance, developed PAH that was prevented by the antidiabetic drug rosiglitazone (Hansmann et al., 2008). PAH exhibits a complex pathophysiology, unlikely to be explained by a single factor (Chan and Loscalzo, 2008; Rabinovitch, 2008). Mutations in bone morphogenetic protein receptor type 2 (BMPR2) underlie many heritable and sporadic cases of PAH (International PPH Consortium, et al., 2008).
hypertrophy. We also further analyzed the mechanisms con-
type 1 diabetes, we have found pulmonary endothelial dys-
the endothelial dysfunction and vascular pathology found in
of nitric-oxide bioavailability is an additional component of
loss of 5-HT receptors or the 5-HT transporter attenu-
encoding 5-HT receptors or the 5-HT transporter are associ-
Wanstall, 2003; Rodat et al., 2007). Elevated plasma levels of
was visualized by its green autofluorescence. Small arteries were
vein and placed into the right ventricle (RV).
animals developed the expected increase in blood glucose (488 ± 39 mg/dl; P < 0.01 versus 134 ± 13 mg/dl in control animals) and decrease in body weight (BW) (196 ± 13 g; P < 0.01 versus 367 ± 10 g in control animals). We found no significant changes in RVSP or the ratios of the free wall of the RV to BW, free wall of the left ventricle (LV) plus septum (S) to BW or RV/(LV+S) compared with controls (Fig. 1A). Moreover, diabetes did not modify the percentage of muscular, partially muscular, or nonmuscular.

Vascular Reactivity. Intrapulmonary artery rings (2-3 mm long, internal diameter ~0.5–0.8 mm) were dissected and mounted in Krebs’ solution under 0.75 g of resting tension in organ chambers as described previously (Cogolludo et al., 2006b). In some experiments, the endothelium was removed by gently rubbing the intimal surface of the rings with a metal rod. The endothelium removal procedure was verified by the inability of acetylcholine (10⁻⁶ M) to relax arteries precontracted with 10⁻⁶ M phenylephrine. After equil-
other contractions were reached they were washed with Krebs’ solution for 30 min, and concentration-response curves to 5-HT (10⁻⁸ to 10⁻⁴ M) were performed by cumulative addition in the absence or presence of drugs.

**Kᵥ Current Recordings.** PA smooth muscle cells were enzymat-
ally isolated, and membrane currents were recorded using the whole-cell configuration of the patch-clamp technique as described previously (Cogolludo et al., 2006b).

**Western Blot Analysis.** Pulmonary artery or whole lung ho-
logenates were run by SDS-polyacrylamide gel electrophoresis, and Western blot was performed as described previously (Moreno et al., 2004) using primary monoclonal mouse anti-α- or anti-β-
actin (Sigma-Aldrich, St. Louis, MO), anti-BMPR2 (BD Biosciences Transduction Laboratories, Lexington, KY), anti-SHTA₂ (BD Biosciences Pharmingen, San Diego, CA), anti-cyclooxygenase-2 (COX-2) (Cayman Chemical, Ann Arbor, MI), or anti-Kᵥ₁.5 (Alomone Labs, Jerusalem, Israel) antibodies.

**Material and Methods**

**Animals and Treatments.** The investigation conforms with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication 85-23, revised 1996), and the procedures were approved by the institutional review board (Comité de Experimentación Animal, Universidad Complutense de Madrid, Madrid, Spain). Male Sprague-Dawley rats were randomly divided into control and diabetic groups. Diabetes was induced by a single intraperitoneal injection of 70 mg/kg streptozotocin (controls were injected with saline) and followed for 6 weeks (one group was followed for 4 months). In a further experimental series, streptozotocin-treated rats were randomly cotreated with insulin glargine (5 units/kg, once daily) or saline. Blood glucose was analyzed using a clinical glucometer.

**Pressure Measurements.** Systolic and diastolic systemic arterial pressures (SAPs) were analyzed with a pressure transducer via a catheter located in the right carotid artery in anesthetized (pentobarbitone, 30–50 mg/kg i.p.) rats ventilated with room air. Right ventricular systolic pressure (RVSP) was then measured in open-chest rats with a pressure transducer via a catheter advanced through the right jugular vein and placed into the right ventricle (RV).

**Lung Histology.** The right lung was inflated in situ with formol (via a column of 25-cm height through the trachea) and embedded in paraffin. Lung sections were stained by hematoxylin/eosin and Masson trichrome techniques and examined by light microscopy. Elastic was visualized by its green autofluorescence. Small arteries were analyzed in a blinded fashion and categorized as muscular, partially muscular, or nonmuscular.

**Results**

**Right Ventricular Systolic Pressure and Right Ven-
tricular Weight.** After 6 weeks of streptozotocin treatment, animals developed the expected increase in blood glucose (488 ± 39 mg/dl; P < 0.01 versus 134 ± 13 mg/dl in control animals) and decrease in body weight (BW) (196 ± 13 g; P < 0.01 versus 367 ± 10 g in control animals). We found no significant changes in RVSP or the ratios of the free wall of the RV to BW, free wall of the left ventricle (LV) plus septum (S) to BW or RV/(LV+S) compared with controls (Fig. 1A). Moreover, diabetes did not modify the percentage of muscular, partially muscular, or nonmuscular arteries (Fig. 1B).

**Lung BMPR2 But Not Kᵥ₁.5 Expression Is Down-
Regulated in Diabetes.** Because BMPR2 and Kᵥ₁.5 are key proteins involved in PAH we examined its expression in diabetic lungs. Diabetes induced a clear down-regulation of lung BMPR2 expression (Fig. 2A). In contrast, the expression of Kᵥ₁.5 channel protein was not significantly modified in these diabetic animals (Fig. 2B). In isolated PA smooth mus-
Fig. 1. Effects of diabetes on RVSP, right ventricular hypertrophy, and PA muscularization. A, systolic and diastolic SAP, RVSP, right ventricular weight relative to body weight (RV/BW), and right ventricular weight relative to left ventricular plus septum weight (RV/LV+S) in control (open columns) and streptozotocin-treated (solid columns) rats (n = 5 and 8, respectively). B, percentage of muscular, partially muscular, and nonmuscular arteries in control and diabetic rats (n = 4). Each column represents the mean ± S.E.M.

PA from Diabetic Rats Show Endothelium-Independent Hyper-Responsiveness to 5-HT. 5-HT induced a concentration-dependent contractile response that was higher in diabetic rats compared with control rats (Fig. 3A). The analysis of the concentration-response curve indicated that diabetes induced an increase in the maximal contractile response (E_max; Fig. 3C) without significant changes in the concentration of 5-HT required for half-maximal contraction (pD2; Fig. 3C). Because this increased response could be attributed to a reduced nitric-oxide bioavailability (Lopez-Lopez et al., 2008) similar experiments were carried out in the presence of the competitive antagonist of 5-HT2A receptors, ketanserin. This drug produced a rightward shift of the concentration response to 5-HT (Fig. 4A). The calculated pK_B values of ketanserin from this shift, an indicator of the potency of the antagonist, were 8.2 in both intact and denuded arteries (Fig. 4B). These pK_B values are in agreement with the expected potency of the antagonist for 5-HT2A receptors (8.1–9.7) (Alexander et al., 2008), indicating that these receptors seem to play a major role in 5-HT contraction as reported previously (MacLean et al., 1996). The potency of ketanserin was similar in control and diabetic animals in both intact and denuded vessels. To analyze the possible role of changes in 5-HT2A receptors, its expression was analyzed in lung homogenates by Western blot. The expression of the 5-HT2A receptors was increased ~2-fold in diabetic rats (Fig. 4C).

Role of 5-HT2A Receptors. We analyzed the contractile response of 5-HT in endothelium-intact and -denuded PA in the presence of the competitive antagonist of 5-HT2A receptors, ketanserin. This drug produced a rightward shift of the concentration response to 5-HT (Fig. 4A). The calculated pK_B values of ketanserin from this shift, an indicator of the potency of the antagonist, were 8.2 in both intact and denuded arteries (Fig. 4B). These pK_B values are in agreement with the expected potency of the antagonist for 5-HT2A receptors (8.1–9.7) (Alexander et al., 2008), indicating that these receptors seem to play a major role in 5-HT contraction as reported previously (MacLean et al., 1996). The potency of ketanserin was similar in control and diabetic animals in both intact and denuded vessels. To analyze the possible role of changes in 5-HT2A receptors, its expression was analyzed in lung homogenates by Western blot. The expression of the 5-HT2A receptors was increased ~2-fold in diabetic rats (Fig. 4C).
Role of Superoxide. Because we have reported previously an increase in NADPH oxidase-derived superoxide in PA from diabetic rats (Lopez-Lopez et al., 2008), we analyzed the contractile response to 5-HT in endothelium-intact PA treated with superoxide dismutase (SOD) or the NADPH oxidase inhibitor apocynin. The contraction induced by 5-HT in control rats (Fig. 6) was similar to that observed in the absence of the drugs (Fig. 3A). However, both treatments prevented enhanced response to 5-HT in diabetic rats.

Effects of Exogenous Addition of Superoxide and a Thromboxane A₂ Analog. The responses to 5-HT in PA from control rats were analyzed in the presence of the superoxide-generating drug pyrogallol or the thromboxane A₂ (TXA₂) analog (Z)-7-[(1S,3S,4S)-3-[(E,3S)-3-hydroxyoct-1-eny]-5-oxabicyclo[2.2.1]heptan-2-yl]hept-5-enolic acid (U46619). After washing the initial response to KCl, pyrogallol or U46619 was added at concentrations titrated to produce 5 to 15% of the response to KCl (3 × 10⁻⁶ and 10⁻⁸ M, respectively). After 15 min the concentration-response to 5-HT was performed. Pyrogallol increased the Eₘₐₓ to 5-HT without changing the pD₂ value, and this effect was prevented by indomethacin (Fig. 7A), mimicking the results in diabetic rats. On the other hand, U46619 produced a leftward shift of the curve to 5-HT (increase in pD₂) with a nonsignificant increase in the Eₘₐₓ, and this effect was not prevented by apocynin (Fig. 7B).

Role of Cyclooxygenase-2. To analyze a possible role of COX, we tested the effects of the nonselective COX inhibitor indomethacin in endothelium-intact vessels. This drug produced a weak rightward shift of the curve to 5-HT in PA from control rats (Fig. 5A), leading to a significant decrease in the pD₂ value (Fig. 5C). It is noteworthy that indomethacin abolished the hyper-responsiveness to 5-HT in diabetic rats. In the presence of the selective COX-2 inhibitor N-[2-(cyclohexylamino)-6-nitrophenyl]-methylene sulfonamide (NS-398) the responses to 5-HT in control rats were similar to those in its absence. However, in diabetic rats, this drug prevented the enhanced response to 5-HT and also induced a weak rightward shift of the curve (Fig. 5B), leading to a significant decrease in the pD₂ value (Fig. 5C). Consistent with a role for COX-2 in the vascular responses, the amount of this protein was strongly increased in PA from diabetic rats (Fig. 5D).
Despite the well known link between diabetes and systemic cardiovascular disease, the relationship with pulmonary vascular disease has been largely overlooked. In a preclinical cardiovascular disease, the relationship with pulmonary vascular oxidative stress. RVSP and RV/(LV + S) were significantly different in diabetic rats compared with nondiabetic controls at 6 weeks, whereas at 4 months we found a significant increase in diabetic rats. Because PAs from BMPR2(-/-) mice also exhibit increased contractile responses to 5-HT (Long et al., 2006) it seems likely that in the diabetic rats hyper-responsiveness to 5-HT is a consequence of the BMPR2 down-regulation. It is noteworthy that PAH is not evident in BMPR2(-/-) mice but it does develop after chronic 5-HT infusion, an effect that is exaggerated under hypoxic conditions (Long et al., 2006).

The increased response to 5-HT was maintained in endothelium-denuded vessels or in the presence of l-NAME, in-
down-regulated and it has been also reported in diabetic rat kidneys (Wang et al., 2001). Our data are consistent with the concept that BMPR2 mutation or down-regulation is a predisposing factor but may not be sufficient for PAH 5-HT (Long et al., 2006). In contrast, in diabetic rats there was no significant change in KV currents and KV1.5 expression. This is consistent with the small, if any, change in both parameters found in the fawn hooded rats, an animal model of genetic predisposition to PAH, at 20 weeks of age (prehypertensive) (Bonnet et al., 2006).

Hyper-responsiveness to 5-HT in large and small PA rings is a common feature of animal models of PAH, including cardiopulmonary bypass-, chronic hypoxia-, intermittent hypoxia-, or monocrotaline-induced PAH (Brown et al., 1998; Sato et al., 2000; Keegan et al., 2001; Thomas and Wanstall, 2003; Rodat et al., 2007). We also found a marked increase in the response to 5-HT in intrapulmonary arteries from diabetic rats. Because PAs from BMPR2(-/-) mice also exhibit increased contractile responses to 5-HT (Long et al., 2006) it seems likely that in the diabetic rats hyper-responsiveness to 5-HT is a consequence of the BMPR2 down-regulation. It is noteworthy that PAH is not evident in BMPR2(-/-) mice but it does develop after chronic 5-HT infusion, an effect that is exaggerated under hypoxic conditions (Long et al., 2006).

The increased response to 5-HT was maintained in endothelium-denuded vessels or in the presence of l-NAME, in-

Discussion

Described previously as BMPR2, COX-2 induction, up-regulation of 5-HT2A receptors, and vascular hyper-responsiveness to 5-HT in addition to those described previously such as endothelial dysfunction and pulmonary vascular oxidative stress. RVSP and RV/(LV + S) and PA muscularization were not significantly different in diabetic rats compared with nondiabetic controls at 6 weeks, whereas at 4 months we found a significant increase in RVSP. This increase was modest, much lower than classic models of PAH.

Both clinical and experimental forms of PAH are associated with a decrease in KV currents and diminished pulmonary expression of BMPR2 (Atkinson et al., 2002; Takahashi et al., 2006; Morty et al., 2007) and KV1.5, KV3.1, and KV2.1 channels (Yuan et al., 1998; Bonnet et al., 2006; Guignabert et al., 2006). In diabetic animals lung BMPR2 protein was
dicitating that a major component of this phenomenon is not related to acute release of endothelial vasoactive factors. However, differences tended to be smaller in endothelium-denuded compared with intact vessels, suggesting that endothelial dysfunction (Al-Shafei et al., 2002; Lopez-Lopez et al., 2008) might also play a role. Moreover, it seems to be secondary to the high blood glucose rather than a direct effect of streptozotocin because it was reduced by cotreatment with insulin. On the other hand, the similar potency of ketanserin, a specific 5HT2A receptor competitive antagonist, on 5-HT vasoconstrictor responses to 5-HT in PA (n = 4–8). ** *, P < 0.01 versus control.

![Fig. 7.](#)

**Fig. 7.** Superoxide and a TXA2 analog enhance 5-HT contractile responses in PA from control rats. Effects of the nonenzymatic generator of superoxide pyrogallol (pyrog; 3 × 10⁻⁶ M) in the absence or presence of indomethacin (indo; 10⁻⁵ M) (A) and the TXA2 analog U46619 (10⁻⁵ M) (B) in the absence or presence of apocynin (apoc; 3 × 10⁻⁴ M) on the vasoconstrictor responses to 5-HT in PA (n = 4–8). ** *, P < 0.01 versus control.

COX-2 protein levels are increased in lungs from rats with PAH induced by hypoxia (Chida and Voelkel, 1996) or high pulmonary blood flow (Sato et al., 2000; Lam et al., 2005) and in hypoxic human PA smooth muscle cells (Yang et al., 2002). In addition, elevated TXA2 levels have been demonstrated in several forms of PAH (Christman et al., 1992). Accordingly, we found increased COX-2 expression in the PA from diabetic rats. Moreover, streptozotocin-induced diabetes led to an exaggerated lung production of prostaglandin E₂, prostaglandin F₂α, and prostaglandin D₃ from exogenous arachidonic acid (Watts et al., 1982). However, whether COX-2 is beneficial or detrimental in PAH is controversial. Thus, inhibition of COX-2 by celecoxib exhibited beneficial effects against the development of monocrotaline-induced PAH (Rakotoniaina et al., 2008). In contrast, hypoxia-induced PAH was exacerbated by the coxibilicate derivative 4-[5-(4-chlorophenyl)-3-((tri-fluoromethyl)-1H-pyrazol-1-yl] benzenesulfonamide (SC236) (Pidgeon et al., 2004) and in COX-2 knockout animals (Cathcart et al., 2008; Fredenburgh et al., 2008). Herein, we found that acute inhibition of COX-2 can prevent the hypercontractile response to 5-HT in diabetic rats as previously found in PAH induced by intermittent hypoxia (Thomas and Wanstall, 2003) or high pulmonary blood flow (Sato et al., 2000). These results suggest that a COX-2-derived metabolite is responsible for the enhanced maximal response to 5-HT. The fact that the TP receptor agonist U46619 reproduces at least partially the effect of diabetes suggests that the COX-2 metabolite might be acting on these receptors. Likewise, COX-2-dependent and endothelium-independent vascular hyper-responsiveness have also been reported in several systemic arteries from animal models of type 1 and type 2 diabetes (Jarajapu et al., 2008; Shi and Vanhoutte, 2008). Moreover, COX-2 inhibitors and ROS scavengers prevent the vascular hyper-responsiveness in endothelium-denuded femoral arteries from streptozotocin-induced diabetic rats (Shi and Vanhoutte, 2008), suggesting that COX metabolites and ROS are generated in the smooth muscle. In addition, the TP receptor antagonist terutroban prevented the femoral vascular hyper-responsiveness to ROS in diabetic animals, suggesting that the COX metabolite is acting via TP receptors (Shi and Vanhoutte, 2008).

Type 1 and type 2 diabetes are associated with systemic oxidative stress (Keaney and Loscalzo, 1999; Meigs et al., 2007). In PA, diabetes induces an increase in superoxide and up-regulation of p47phox, the regulatory subunit of the superoxide-generating enzyme NADPH oxidase, which is involved in endothelial dysfunction (Lopez-Lopez et al., 2008). The down-regulation of BMPR2 in diabetic rat kidney was prevented by the antioxidant tiron (Yeh et al., 2009), suggesting that it was secondary to oxidative stress. In addition, oxidative stress is involved in the exaggerated response to vasoconstrictors in systemic arteries from diabetic animals (Shi and Vanhoutte, 2008). Thus, we hypothesized that scavenging superoxide using SOD or inhibiting its main source using apocynin, a widely used yet nonselective NADPH oxidase inhibitor, might also prevent 5-HT hyper-responsiveness. In fact, both approaches prevented the exaggerated response to 5-HT in diabetic PA without affecting the controls. On the contrary, exogenous addition of a superoxide-donating drug such as pyrogallol increased the maximal response to 5-HT in control PA, mimicking the effects of diabetes.

The relationship of COX-2 and reactive oxygen species is complex because COX-2 can generate superoxide directly or indirectly via the release of TXA2 (Cogollo et al., 2006a; Shi and Vanhoutte, 2008) and conversely, COX-2 activity can be stimulated by reactive oxygen species (Garcia-Redondo et al., 2009). Thus, we questioned what was first in the signaling pathway in diabetic PA COX-2 activity or increased oxidative stress. Our data fit better with the second possibility because indomethacin prevented the pyrogallol-induced hyper-responsiveness to 5-HT in control rats, whereas apocynin had no effect on U46619-induced sensitization. Taken together, the present results suggest that NADPH oxidase-
derived reactive oxygen species activate its downstream effector COX-2, leading to the enhanced response to 5-HT. This is further supported by the up-regulation of the main proteins involved in this pathway, p47phox, COX-2, and 5-HT2A receptors observed in the diabetic lungs.

In conclusion, consistent with data in humans and animal models of PAH, in diabetic rats BMP2R expression was down-regulated, 5-HT2A receptors, p47phox and COX-2 were up-regulated, and PAs were hyper-responsive to 5-HT. This latter effect was independent of the endothelium and seemed to be related to NADPH oxidase-induced superoxide production and COX-2-derived metabolites. All of these changes were not sufficient to induce a consistent increase in PA pressure or PA muscularization. However, a prolonged period of diabetes induced an increase in RVSP. Our results further strengthen the link between diabetes and PAH.

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Authorship Contributions
Participated in research design: Moreno, Lorente, Cogolludo, and Perez-Vizcaino.
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