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 except by indomethacin, the selective COX-2 inhibitor N-[2-(cyclohexylxoyl)-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., 2007).

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., 2005).

ABBREVIATIONS: PAH, pulmonary arterial hypertension; 5-HT, 5-hydroxytryptamine (serotonin); BMPR2, bone morphogenetic protein receptor type 2; BW, body weight; COX, cyclooxygenase; $E_{\text{max}}$, maximal drug effect; $K_v$, voltage-gated potassium channel; LV, left ventricle; PA, pulmonary artery; S, septum; SAP, systemic arterial pressure; RV, right ventricle; RVS, right ventricular systolic pressure; SOD, superoxide dismutase; TXA$_2$, thromboxane A$_2$; NS-398, N-[2-(cyclohexylxoyl)-4-nitrophenyl]-methane sulfonamide; U46619, (2E)-7-[(1S,3S,4S)-3-[(E,3S)-3-hydroxyoct-1-enyl]-5-oxabicyclo[2.2.1]heptan-2-yl]hept-5-enic acid; LNAME, N’-nitro-L-arginine methyl ester; SC236, 4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide; TP, thromboxane prostanoid; ROS, reactive oxygen species.
and down-regulation of its expression is a common feature of several forms of PAH (Atkinson et al., 2002). Inactivation, down-regulation, or gene polymorphisms of voltage-gated potassium channels (\(K_C\)) have also been implicated in PAH (Yuan et al., 1998). Several lines of evidence also indicate that serotonin \([5\text{-hydroxytryptamine (5-HT)}]\) plays a central role in the pathogenesis of this entity. Thus, 5-HT stimulates pulmonary artery (PA) contraction and smooth muscle cell proliferation and blocks \(K_C\) (MacLean et al., 2000; Cogolludo et al., 2006b). Hyper-responsiveness to 5-HT in large and small PAs is a common feature of PAH (Le Cras et al., 2000; Sato et al., 2000; Keegan et al., 2001; Thomas and Wanstall, 2003; Rodat et al., 2007). Elevated plasma levels of 5-HT and overexpression or polymorphisms in the genes encoding 5-HT receptors or the 5-HT transporter are associated with the disease (MacLean et al., 2000). It has also been suggested that local endothelium-derived 5-HT acting in a paracrine manner may be involved in PAH (Eddahibi et al., 2006). Furthermore, pharmacological inhibition or genetic deletion of 5-HT receptors or the 5-HT transporter attenuates PAH and prolongs survival (MacLean et al., 2000). Loss of nitric-oxide bioavailability is an additional component of the endothelial dysfunction and vascular pathology found in PAH (Coggins and Bloch, 2007).

In rats treated with streptozotocin, a widely used model of type 1 diabetes, we have found pulmonary endothelial dysfunction associated with increased superoxide production and up-regulation of the NADPH oxidase subunit \(p47\text{phox}\) (Lopez-Lopez et al., 2008). Right ventricular hypertrophy has also been found in this model (Al-Shafei et al., 2002). We hypothesized that type 1 diabetes could lead to the development of PAH. Therefore, the present study was designed to analyze the effects of streptozotocin on pulmonary markers of PAH, including the pulmonary expression of key proteins of the disease, \(K_C\) currents, PA pressure, and right ventricular hypertrophy. We also further analyzed the mechanisms contributing to the vascular hyper-reactivity of PA to 5-HT.

**Materials 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 1986), 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 cotedrated 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 formaldehyde (via a column of 25-cm height through the trachea) and embedded in paraffin. Lung sections were stained by hematoxylin/eosin and Mason trichrome techniques and examined by light microscopy. Elastin was visualized by its green autofluorescence. Small arteries were analyzed in a blinded fashion and categorized as 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 and 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 \((10^{-6} \text{ M})\) to relax arteries precontracted with \(10^{-6} \text{ M}\) phenylephrine. After equilibration, rings were precontracted by 80 mM KCl, and once a stable contraction was reached they were washed with Krebs’ solution for 30 min, and concentration-response curves to 5-HT \((10^{-8} \text{ to } 10^{-4} \text{ M})\) were performed by cumulative addition in the absence or presence of drugs.

**K\(_C\) Current Recordings.** PA smooth muscle cells were enzymatically 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 homogenates were run by SDS-polyacrylamide gel electrophoresis, and Western blot was performed as described previously (Moreno et al., 2004) using primary monoclonal mouse anti-\(\alpha\)- or anti-\(\beta\)-actin (Sigma-Aldrich, St. Louis, MO), anti-BMPR2 (BD Biosciences Transduction Laboratories, Lexington, KY), anti-SHT\(_{\alpha2}\) (BD Biosciences Pharmingen, San Diego, CA), anti-cyclooxygenase-2 (COX-2) (Cayman Chemical, Ann Arbor, MI), or anti-\(K_C\),1.5 (Alomone Labs, Jerusalem, Israel) antibodies.

**Statistical Analysis.** Results are expressed as means \pm S.E.M. of measurements. Statistical analysis was performed by an unpaired Student’s \(t\)-test and for multiple comparisons by one-way analysis of variance followed by a Newman-Keuls test. \(P < 0.05\) was considered statistically significant. Individual cumulative concentration-response curves were fitted to a logistic equation. The maximal drug effect \((E_{\text{max}})\) and the drug concentration producing 50% of the \(E_{\text{max}}\) \((EC_{\text{50}})\) expressed as negative log molar value \((pD_2)\) were calculated from the fitted curves for each ring. Apparent \(pK_B\) values were calculated according to the equation \(pK_B = \log(DR - 1) - \log[B]\), where DR is the ratio of the mean \(EC_{\text{50}}\) values of the agonist in the presence and absence of a given concentration \((IB)\) of the antagonist.

**Results**

**Right Ventricular Systolic Pressure and Right Ventricular Weight.** After 6 weeks of streptozotocin treatment, animals developed the expected increase in blood glucose \((488 \pm 39 \text{ mg/dl}; P < 0.01\) versus 134 \pm 13 \text{ mg/dl in control animals}) and decrease in body weight (BW) \((196 \pm 13 \text{ g}; P < 0.01\) versus 367 \pm 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). However, in the group treated for 4 months with streptozotocin (blood glucose 473 \pm 31 \text{ mg/dl}; \(P < 0.01\) versus 125 \pm 5 \text{ mg/dl in control animals}) there was a significant increase in RVSP compared with parallel controls \((22.4 \pm 1.9, n = 6\) versus 16.7 \pm 0.7 mm Hg, \(n = 6; P < 0.05\)).

**Lung BMPR2 But Not \(K_C\),1.5 Expression Is Down-Regulated in Diabetes.** Because BMPR2 and \(K_C\),1.5 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 \(K_C\),1.5 channel protein was not significantly modified in these diabetic animals (Fig. 2B). In isolated PA smooth mus-
Lopez et al., (2008) similar experiments were carried out in arterioles, was similar in both groups (14 ± 2 and 14 ± 1 pF in control and diabetic, respectively; not significant). We also observed a nonsignificant decrease in the amplitude of the $K_v$ currents in diabetic animals (Fig. 2C).

**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 ($E_{\text{max}}$; Fig. 3C) without significant changes in the concentration of 5-HT required for half-maximal contraction ($pD_2$; 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 nitric-oxide synthase inhibitor L-NAME. This drug increased the maximal response to 5-HT in both diabetic and control animals ($P < 0.01$), but the difference between both groups remained highly significant ($P < 0.01$; Fig. 3, B and C). Likewise, in endothelium-denuded arteries the response to 5-HT was higher ($P < 0.01$) than in intact ones, but again diabetic-denuded vessels were strongly hyper-responsive ($P < 0.01$; Figs. 3C and 4A). In endothelium-denuded arteries from rats cotreated with streptozotocin plus insulin (blood glucose = 178 ± 5 mg/dl) the $E_{\text{max}}$ of 5-HT was reduced (115 ± 17%; $P < 0.05$) compared with the parallel group of streptozotocin-treated rats (glucose = 440 ± 26; $E_{\text{max}}$ = 201 ± 17%).

**Role of 5-HT$_{2A}$ Receptors.** We analyzed the contractile response of 5-HT in endothelium-intact and -denuded PA in the presence of the competitive antagonist of 5-HT$_{2A}$ 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-HT$_{2A}$ 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-HT$_{2A}$ receptors, its expression was analyzed in lung homogenates by Western blot. The expression of the 5-HT$_{2A}$ 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-etyl]-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 E₉₅ max 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 E₉₅ max, 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-(cyclohexyl- loxyl)-4-nitrophenyl]-methane 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).
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-

**Fig. 5.** Role of COX-2. A and B, effects of the nonselective COX inhibitor indomethacin (INDO; 10−6 M) (A) and the COX-2-selective inhibitor NS-398 (10−5 M) (B) on the vasoconstrictor responses to 5-HT in endothelium-intact PA (n = 6). C, Emax and pD2 values calculated from A and B and in the absence of drug (untreated, calculated from Fig. 3). D, expression of COX-2 in PA from control (n = 5) and diabetic (n = 6) rats. *, P < 0.05; **, P < 0.01, diabetic versus control rats. †, P < 0.05, indomethacin versus untreated.

**Discussion**

Despite the well known link between diabetes and systemic cardiovascular disease, the relationship with pulmonary vascular disease has been largely overlooked. In a previous study, insulin resistance in ApoE(−/−) mice was associated with PAH (Hansmann et al., 2007). We found previously PA endothelial dysfunction, a characteristic feature of PAH, in rats with type 1 diabetes, and right ventricular hypertrophy was also reported in streptozotocin-treated rats (Al-Shafei et al., 2002; Lopez-Lopez et al., 2008). Herein, we show that rats treated with streptozotocin share a number of pulmonary vascular abnormalities with animal models and patients with PAH such as down-regulation of 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...
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 x 10^-6 M) in the absence or presence of indomethacin (indo; 10^-6 M) (A) and the TXA2 analog U46619 (10^-6 M) (B) in the absence or presence of apocynin (apoc; 3 x 10^-4 M) on the vasoconstrictor responses to 5-HT in PA (n = 4-8). **P < 0.01 versus control.

indicating 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 contraction indicates that the responses to 5-HT are mediated mainly by 5HT2A receptors in both control and diabetic animals. However, a possible additional role for other receptors such as 5HT1B cannot be ruled out (MacLean et al., 2000). The overexpression of 5HT2A receptors in diabetic animals could be responsible for the higher contractile response of 5-HT. Nevertheless, this possibility does not explain the acute reversal by COX inhibitors or reactive oxygen species (ROS) scavengers as discussed below.

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 D2 from exogenous arachidonic acid (Watts et al., 1982). However, whether COX-2 is benefici-
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-HT_{2A} 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-HT_{2A} 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.

Conducted experiments: Lopez-Lopez, Moral-Sanz, Frazziano, Gomez-Villalobos, Moreno, Menendez, and Flores-Hernandez.


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