Diabetes induces pulmonary artery endothelial dysfunction by NADPH oxidase induction

Jose G. Lopez-Lopez, Javier Moral-Sanz, Giovanna Frazziano, Maria J. Gomez-Villalobos, Jorge Flores-Hernandez, Eduardo Monjaraz, Cogolludo A, Perez-Vizcaino F. Diabetes induces pulmonary artery endothelial dysfunction by NADPH oxidase induction. Am J Physiol Lung Cell Mol Physiol 295: L727–L732, 2008. First published August 22, 2008; doi:10.1152/ajplung.90354.2008.—Recent data suggest that diabetes is a risk factor for pulmonary hypertension. The aim of the present study was to analyze whether diabetes induces endothelial dysfunction in pulmonary arteries and the mechanisms involved. Male Sprague-Dawley rats were randomly divided into a control (saline) and a diabetic group (70 mg/kg streptozotocin). After 6 wk, intrapulmonary arteries were mounted for isometric tension recording, and endothelial function was tested by the relaxant response to acetylcholine. Protein expression and localization were measured by Western blot and immunohistochemistry and superoxide production by dihydroethidium staining. Pulmonary arteries from diabetic rats showed impaired relaxant response to acetylcholine and reduced vasconstrictor response to the nitric oxide (NO) synthase inhibitor L-NAME, whereas the response to nitroprusside and the expression of endothelial NO synthase remained unchanged. Endothelial dysfunction was reversed by addition of superoxide dismutase or the NADPH oxidase inhibitor apocynin. An increase in superoxide production and increased expression of the NADPH oxidase regulatory subunit p47phox were also found in pulmonary arteries from diabetic rats. In conclusion, the pulmonary circulation is a target for diabetes-induced endothelial dysfunction via enhanced NADPH oxidase-derived superoxide production.

independent risk factor for persistent pulmonary hypertension of the newborn (13). There is also some experimental evidence linking type 1 and type 2 diabetes with pulmonary hypertension. Thus, male apoE−/− mice on a high-fat diet, an animal model of insulin resistance and metabolic syndrome, develop PAH (12). Right ventricular hypertrophy, suggestive of PAH, has been found in rats treated with streptozotocin, a model of type 1 diabetes (1).

Endothelial dysfunction, classically characterized by a reduced capacity of endothelial cells to induce vasodilation via the release of nitric oxide (NO), is an early and independent predictor of poor prognosis in most forms of cardiovascular disease (25, 29), including PAH (2, 7), and also in COPD (3). A considerable body of evidence in humans indicates that endothelial dysfunction is a key factor in the development of diabetic retinopathy, nephropathy, and atherosclerosis in both type 1 and type 2 diabetes (15, 26, 30). The signaling pathway of NO, cGMP, and cGMP-dependent protein kinase has been shown to be downregulated under diabetic conditions and contributes to the development of diabetic vascular complications (6, 31, 32).

In the present study, we have characterized the effects of type 1 diabetes on pulmonary arteries. We demonstrate for the first time that diabetes induces endothelial dysfunction in pulmonary arteries, an effect that might explain a higher incidence of PAH in diabetic patients. Moreover, we show that diabetes-induced endothelial dysfunction is due to an upregulation of NADPH oxidase and the subsequent increase in superoxide (O2•−), leading to a reduction in the bioavailability of NO.

MATERIALS AND METHODS

Animals and experimental groups. The investigation conforms with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1996), and the procedures were approved by our institutional review board. Male Sprague-Dawley rats (150–200 g) were randomly divided into a control and a diabetic group. Diabetes was induced by a single intraperitoneal injection of 70 mg/kg streptozotocin (controls were injected with saline) and followed for 6 wk. Hyperglycemia (>350 mg/dl) in the diabetic rats was confirmed using a OneTouch Ultra glucometer.

Vascular reactivity studies. Intrapulmonary artery rings (2–3 mm long, internal diameter ~0.5–0.8 mm) were dissected and mounted under 0.75 g of resting tension in organ chambers as previously described (8). After equilibration, rings were precontracted by
μmol/l phenylephrine, and concentration-response curves to acetylcholine (Ach; 1 nmol/l-100 μmol/l) or sodium nitroprusside (0.1 nmol/l-30 μmol/l in the dark) were performed by cumulative addition in the absence or presence of the NO synthase (NOS) inhibitor L-NAME (100 μmol/l), superoxide dismutase (SOD; 100 U/ml), or the NADPH oxidase inhibitor apocynin (300 μmol/l). In some rings, a concentration-response curve to phenylephrine (1 nmol/l–30 μmol/l) was carried out by cumulative addition of the drug.

Western blot analysis. Pulmonary artery or whole homogenates were run on a SDS-PAGE, and Western blot was performed as described ((18) using primary monoclonal mouse anti-eNOS (Transduction Laboratories, San Diego, CA), anti-SOD-1 antibodies (StressGen, Victoria, Canada), polyclonal rabbit anti-p47phox antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and mouse anti-α- or anti-β-actin (Sigma, Tres Cantos, Madrid).

In situ detection of vascular superoxide production. Unfixed pulmonary arteries were cryopreserved, included in OCT, frozen, and cut into 20-μm cross sections as described (8). Sections were incubated for 30 min in HEPES-buffered solution containing dihydroethidium (10 μmol/l), counterstained with the nuclear stain DAPI, and photographed. Fluorescence was quantified using ImageJ (version 1.32j, National Institutes of Health). Superoxide production was estimated from the ratio of ethidium:DAPI fluorescence (18).

Immunohistochemistry. Sections (20 μm) of pulmonary arteries were prepared as described above for dihydroethidium fluorescence and fixed with 4% paraformaldehyde for 1 h, and immunohistochemistry was performed as described (18).

Statistical analysis. Results are expressed as means ± SE of measurements. Statistical analysis was performed by comparing the control and diabetic groups with an unpaired Student’s t-test. P < 0.05 was considered statistically significant. Individual cumulative concentration-response curves were fitted to a logistic equation. The maximal drug effect (E_{max}) and the negative log molar drug concentration producing 50% of the E_{max} (pD_{2}) were calculated from the fitted curves for each ring.

RESULTS

Endothelial dysfunction in diabetic pulmonary arteries. Stimulation of pulmonary artery rings with 80 mmol/l KCl results in a contractile response that is usually regarded as indicative of the smooth muscle contractile capacity. This response was similar in pulmonary artery rings from control and diabetic rats (374 ± 14 and 373 ± 94 mg, respectively). After washing KCl, a cumulative concentration-response curve to the α-adrenoceptor agonist phenylephrine showed an increased maximal response in the diabetic group compared with the control one (Fig. 1A, E_{max} 106 ± 6 vs. 83 ± 7% of the response to KCl) without changes in the concentration of the vasoconstrictor required for half-maximal activation (pD_{2} values 7.40 ± 0.25 vs. 7.37 ± 0.10, respectively). In rings precontracted with phenylephrine (100 nmol/l, i.e., ~60–75% of its maximal response), increasing concentrations of ACh induced a relaxant response that is considered as an index of endothelial function. This relaxant response was dramatically reduced in pulmonary arteries from diabetic rats compared with controls (Fig. 1B). The analysis of the concentration-response curve indicated that diabetes induced a change in the maximal relaxant response (E_{max} values were 71 ± 5% and 34 ± 6% in the control and diabetic group, respectively, P < 0.01) without significant changes in the concentration of ACh required for half-maximal relaxation (pD_{2} 6.28 ± 0.10 vs. 6.38 ± 0.11, respectively).

NO dependence. The relaxant response induced by ACh was fully inhibited by the NOS inhibitor L-NAME in both groups (Fig. 2A). These results suggest that the NO pathway is impaired in the diabetic animals. Moreover, the NOS inhibitor L-NAME induced a significant contraction in pulmonary arteries from control rats that was negligible in the diabetic animals (Fig. 2B). These results indicate that, in control animals, there is a basal NO synthesis regulating pulmonary artery tone that is absent in the diabetic animals. To analyze whether the impaired response to endothelial-derived NO is due to a reduced bioavailable NO or due to a defect in the signaling of NO in vascular smooth muscle, we analyzed the effects of nitroprusside, which directly activates soluble guanylyl cyclase in vascular smooth muscle, mimicking the effects of endogenous NO. The relaxation induced by the soluble guanylyl cyclase activator nitroprusside was not different between groups (Fig. 2C). To analyze the possible role of changes in endothelial NO (eNOS), its expression was analyzed in lung homogenates by Western blot. The expression of this enzyme was similar in diabetic compared with control rat lungs (Fig. 2D).

Role of superoxide. We analyzed the relaxant response of ACh in pulmonary arteries treated with SOD. SOD produced an increase in the relaxant response of ACh, with this effect much more marked in the diabetic compared with the control arteries (Fig. 3A). Therefore, in the presence of SOD, the
maximal relaxant responses to ACh were not significantly different in control and diabetic rats (Emax = 91 ± 5% and 83 ± 4%, respectively). To characterize and localize superoxide production within the vascular wall, ethidium red fluorescence was analyzed in sections of pulmonary arteries incubated with dihydroethidium. Dihydroethidium is oxidized by superoxide to yield ethidium, which stains DNA. Positive red staining, indicative of superoxide production, was quantified and normalized to the blue fluorescence of the nuclear stain DAPI, allowing comparisons between different sections. Rings from diabetic rats showed a marked increase in adventitial, medial, and endothelial cells compared with control (Fig. 3B). We also determined whether there were changes in the expression of SOD-1, the main lung antioxidant enzyme responsible for superoxide clearance. Paradoxically, SOD-1 expression was increased in pulmonary arteries from diabetic rats (Fig. 3C).

**Role of NADPH oxidase.** To analyze a possible role of NADPH oxidase, we tested the effects of apocynin, an inhibitor of this superoxide-generating complex, on the relaxant response of ACh in pulmonary arteries. As shown with SOD, apocynin also produced a marked increase in the relaxant response of ACh, again with this effect more marked in the diabetic compared with the control arteries (Fig. 4A; 108 ± 2% and Emax = 94 ± 4%, respectively). Therefore, we analyzed the expression of p47phox, the regulatory subunit of NADPH oxidase in pulmonary arteries from control and diabetic rats. Figure 4B shows that this NADPH oxidase subunit was markedly increased in the vessels of diabetic compared with control rats. Likewise, a strong increase in p47phox immunostaining was observed in the adventitia and in the media in sections of pulmonary arteries (Fig. 4C).

**DISCUSSION**

Herein we show that treatment with streptozotocin, an established model for type 1 diabetes, induced endothelial dysfunction in rat pulmonary arteries. This effect was associated with a reduced vasodilator response to the NOS inhibitor l-NAME, whereas the response to nitroprusside and the expression of eNOS remained unchanged. Endothelial function was restored by addition of SOD or the NADPH oxidase inhibitor apocynin. An increase in superoxide production and increased expression of the NADPH oxidase regulatory subunit p47phox was also found in pulmonary arteries from diabetic rats.

In pulmonary arteries from animals and humans, NO is the major vasoactive factor accounting for endothelium-dependent relaxation (16, 28). Likewise, in the present study, ACh-induced pulmonary artery relaxation in both control and diabetic rats was entirely dependent on endothelium-derived NO because it was abolished by l-NAME. Thus, the diminished ACh-induced relaxation indicates an impaired agonist-induced NO bioactivity. The impairment of the NO pathway for pulmonary artery relaxation is further supported by the lack of a contractile response induced by l-NAME in diabetic rats. Thus, not only agonist-induced but also basal NO bioactivity was impaired in diabetic pulmonary arteries.

Endothelium-dependent relaxation requires 1) Ca2+-dependent activation of eNOS in endothelial cells leading to NO synthesis, 2) NO diffusion to the adjacent smooth muscle cells, 3) NO-induced activation of soluble guanylyl cyclase leading to cyclic GMP synthesis, and 4) activation of protein kinase G. All these steps in the signaling pathway of NO in systemic vessels have been reported to be impaired by high glucose in
in vitro studies and/or in type 1 and type 2 diabetes (15, 26, 30, 31, 32). In the present study, the relaxant response to the activator of soluble guanylyl cyclase nitroprusside was similar in control and diabetic rats, indicating that alterations in the latter two steps mentioned above do not seem to contribute to diabetes-induced endothelial dysfunction in pulmonary arteries. On the other hand, changes in the expression of eNOS in pulmonary arteries may play a role in endothelial dysfunction. This is supported by the findings of elevated superoxide production and increased activity of NADPH oxidase in diabetic rats compared to control rats. The role of NADPH oxidase in pulmonary artery dysfunction is further supported by the observation that preincubation with the NADPH oxidase inhibitor apocynin significantly improves endothelial function in diabetic rats. Additionally, the expression of p47phox, a subunit of NADPH oxidase, was increased in diabetic rats, as shown by Western blot analysis. These findings suggest that modulation of NADPH oxidase activity could be a potential target for the treatment of diabetes-induced pulmonary artery dysfunction.
experimental models of diabetes are controversial. Thus both increased and decreased expression of eNOS in different systemic vessels has been reported in the same model used herein (22, 32). Our data show that in the pulmonary arteries, there is no significant change in eNOS expression, ruling out that deficient eNOS protein might account for endothelial dysfunction.

One of the key mechanisms of endothelial dysfunction involves the vascular production of reactive oxygen species, particularly superoxide, which reacts rapidly with and inactivates NO (27). Endothelial dysfunction associated with both diabetes and PAH has been reported to involve the vascular production of superoxide, which reacts rapidly with NO, limiting its diffusion (5, 7, 27). Dihydroethidium staining indicated a marked increase in superoxide production in pulmonary arteries from diabetic rats. The fact that in the diabetic pulmonary arteries the impaired relaxant response to ACh was significantly rescued by SOD, the main physiological scavenger of superoxide, indicates a functional role of the increased superoxide production in pulmonary arteries from diabetic rats. The impairment of the pulmonary vascular function described in the present paper further supports that type 1 diabetes is also associated with pulmonary vascular dysfunction.

In conclusion, our present results demonstrate that the pulmonary circulation is also a target for diabetes-induced damage via enhanced NADPH oxidase-derived superoxide and add experimental support for the recently reported association between diabetes and PAH.

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GRANTS

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REFERENCES


17. Liu S, Ma X, Gong M, Shi L, Lincoln T, Wang S. Glucose down-regulation of cGMP-dependent protein kinase I expression in vascular...


