Activation of the GLP-1 Receptor by Liraglutide Increases ACE2 Expression, Reversing Right Ventricle Hypertrophy, and Improving the Production of SP-A and SP-B in the Lungs of Type 1 Diabetes Rats

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Diabetes alters microvascular function in the vascular beds of organs, including the lungs. Cardiovascular complications of pulmonary vascular affectation may be a consequence of the overactivation of the vasoconstrictive and proliferative components of the renin-angiotensin system. We previously reported that pulmonary physiology and surfactant production is improved by the glucagon-like peptide 1 receptor (GLP-1R) agonist liraglutide (LIR) in a rat model of lung hypoplasia. Because we hypothesized that streptozotocin-induced diabetes rats would show deficiencies in lung function, including surfactant proteins, and develop an imbalance of the renin-angiotensin system in the lungs. This effect would in turn be prevented by long-acting agonists of the GLP-1R, such as LIR. The induction of diabetes reduced the surfactant protein A and B in the lungs and caused the vasoconstrictor component of the renin-angiotensin system to predominate, which in turn increased angiotensin II levels, and ultimately being associated with right ventricle hypertrophy. LIR restored surfactant protein levels and reversed the imbalance in the renin-angiotensin system in this type 1 diabetes mellitus rat model. Moreover, LIR provoked a strong increase in angiotensin-converting enzyme 2 expression in the lungs of both diabetic and control rats, and in the circulating angiotensin(1–7) in diabetic animals. These effects prompted complete reversion of right ventricle hypertrophy. The consequences of LIR administration were independent of glycemic control and of glucocorticoids, and they involved NK2 homeobox 1 signaling. This study demonstrates by first time that GLP-1R agonists, such as LIR, might improve the cardiopulmonary complications associated with diabetes. **(*Endocrinology* 156: 3559–3569, 2015)**

Glucagon-like peptide 1 (GLP-1) is a hormone produced by the L-cells of the distal ileum in response to food intake. When interacting with its receptor, GLP-1 receptor (GLP-1R), GLP-1 increases insulin secretion (1) and reduces glucagon release (2) in a glucose-dependent manner, thereby contributing to glucose homeostasis. GLP-1R is a G protein-coupled membrane receptor detected not only in the cells of the pancreatic islets but also in several other tissues, such as the central nervous system, kidneys, heart, blood vessels and lungs (3). Long-acting GLP-1R agonists are used to treat type 2 diabetes mellitus (DM2), because their beneficial effects in glycemic control (4). GLP-1 not only improves glycemia, but it also elicits several extrapancreatic effects, such as inhibition of food

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Abbreviations: *A(1–7)*, angiotensin(1–7); ACE, angiotensin-converting enzyme; *AII*, angiotensin II; BNP-32, B-type natriuretic peptide-32; D, day; DM2, type 2 diabetes mellitus; GLP-1, glucagon-like peptide 1; GLP-1R, GLP-1 receptor; HPA, hypothalamic-pituitary-adrenal; LIR, liraglutide; LV, left ventricle; NKX2.1, NK2 homeobox 1; RAS, renin angiotensin system; RV, right ventricle; S, septum; SP, surfactant protein; STZ, streptozotocin; T2PN, type 2 pneumocyte; VEH, vehicle.

intake (5), reduction of meal-stimulated gastric acid secretion and emptying (6), and activation of lipolytic and lipogenic processes (7). Further effects include the activation of the hypothalamic-pituitary-adrenal (HPA) axis (8), an increase in cardioprotection (9, 10) and an up-regulation of pulmonary surfactant synthesis (11, 12).

Diabetes is linked to lung dysfunction (13), as characterized by thickening of the walls of the pulmonary microvasculature (14), up-regulation of extracellular matrix synthesis in the interstitial tissue, and a reduction of lung elasticity. Further consequences are a decrease in alveolar space (15), a reduction in gas diffusion rate (16) and the eventual collapse of local alveolar. Additionally, diabetes is linked to alterations of the pulmonary circulation, which they might include higher pulmonary arterial pressure, right ventricular (RV) hypertrophy by overload and a greater risk of sudden death (17, 18). Some of the cardiovascular complications of diabetes are linked to an imbalance in the activity of the components of the renin angiotensin system (RAS) (19, 20). The angiotensin-converting enzyme (ACE), synthesizes angiotensin II (*AII*) from angiotensin I, whereas ACE2 converts *AII* into angiotensin(1–7) (*A(1–7)*). *AII* induces vasoconstriction and proliferation, whereas *A(1–7)* stimulate vasodilatation and apoptosis (21). The increased vasoconstrictive activity of the RAS in lung tissue leads to cardiopulmonary anomalies, including an increase in the mean wall thickness of small arterioles and RV hypertrophy. These irregularities may be prevented by an increase in the activity of the vasoprotective branch of the RAS, which is mediated by *A(1–7)* (20, 22). Therefore, elevated pulmonary *AII* may contribute to the progression of vascular disorders in the lungs (23), endothelial dysfunction (24), and thickening, hypertrophy of vascular smooth muscle and enhanced local vascular tone (25).

Quantitative and qualitative aberrations due to surfactant production in the lungs may also trigger respiratory dysfunction (26). Surfactant production is reduced in models of diabetes mellitus, whereas expression of specific protein components of the pulmonary surfactant is altered. Increased blood levels of surfactant protein (SP)-A have recently been demonstrated to be associated with insulin resistance, whereas weaker production of SP-D is linked to a higher risk of developing diabetes in humans (27, 28). GLP-1R agonists can not only increase the production of surfactant phosphatidylcholine (12), but also, they can modulate SP-A and SP-B levels while ameliorating the changes in the functional ultrastructure of the lung in a model of pulmonary hypoplasia (11). Because GLP-1R is expressed more strongly in the lungs than in any other tissue, we assessed the effects of GLP-1 in the context of the lung alterations evident in animal models of diabetes. Accordingly, we evaluated the effects of the long-acting GLP-1R agonist liraglutide (LIR) on the pulmonary disorders that develop in an animal model of DM1 induced by streptozotocin (STZ) administration, in which insulin secretion is abolished.

In our study, we quantified the surfactant proteins SP-A and SP-B, and the balance between the components of the pulmonary RAS (ACE and ACE2 enzymes, and the peptides *AII* and *A(1–7)*). Pulmonary alterations in diabetes include changes in the local vasculature and therefore, we also studied the consequences of lung dysfunction in the RV and left ventricle (LV) of diabetic STZ rats. LIR was shown to be very effective in restoring lung function and in reversing RV hypertrophy, even after a short period of administration (1 wk). These effects were shown to be independent of changes in glycemia and of the levels of insulin.

Materials and Methods

Animal model

Adult Sprague-Dawley male rats weighing 250–300 g were provided by the Universidad de Santiago de Compostela. They were maintained in the animal housing facility at the Universidad de Vigo, where they were kept at 20°C–22°C on 12-hour light, 12-hour dark cycles, and with ad libitum access to water and food (A04-Panlab). Experimental procedures were performed in accordance with European Union guidelines for animal experimentation (council directive 2010/63/UE) and approved by the Ethics Committee at the University of Vigo.

Diabetes was induced by ip injection of STZ (70 mg/kg; Sigma-Aldrich) as described previously (8). Glycemia was measured 24 hours after induction on day 2 (D2), and only animals with severe hyperglycemia (25 mmol/L) were included in the study (STZ, n = 26). The control group (n = 26) received only 0.1M citrate buffer (pH 4.5).

STZ-induced diabetes rats received sc injections of insulin (1 IU/kg; Insulatard FlexPen, Novo-Nordisk) every 12 hours for the after 6 days. From D7 to D13, half the STZ-treated rats and the control rats received LIR (100 μg/kg [Bachem]; STZ+LIR, n = 7 and control+LIR, n = 7) or its vehicle (VEH) alone (0.01% acetic acid, control+VEH, n = 7 and STZ+VEH, n = 7), both applied sc every 12 hours. From D7 to D13, the insulin dose was reduced to 0.5 IU/kg in all the STZ animals. Blood samples were collected in BD vacutainer tubes (250 kIU of aprotinin and 15% K3EDTA) by jugular venipuncture under pentobarbital anesthesia (50 mg/kg) on D2, D6, D8, and D10. Plasma was stored at −20°C to later analyze hormone levels. The animals were weighed daily, and 7 animals from each group were killed by decapitation on D14. The trunk blood from each group was collected, and the right lower lung lobules, and right and left heart ventricles, were removed and weighed. The duodenum, pancreas, and fundus were isolated from the control+VEH and control+LIR groups. The tissues were stored at −80°C until they were processed for RNA and protein extraction. The heart tissues of members of each group were analyzed morphometrically.

Six rats from each group were used for lung angiographic analysis.

Reverse transcription PCR

The relative expression of mRNA was analyzed as described previously (11) using 150 ng of cDNA to assess the SP-A, SP-B, GLP-1R, and NK2 homeobox 1 (NKX2.1) transcripts, with 18S employed as a housekeeping gene. PCR conditions and primer sequences are detailed in Supplemental Table 1. Real-time PCR to analyze ACE and ACE2 gene expression was carried out with the Real Time Ready Single Assay (ACE, ID503125 and ACE2, ID505454) and Fast Start Universal Probe Master Rox (Roche Diagnostics) on 20-ng samples of cDNA, amplifying 18S (ID502300) in parallel for each sample as an internal control.

Western blotting

Total protein samples from the lungs and RVs were isolated and quantified as described previously (11). Protein samples (24 μg for SP-A and 48 μg for SP-B) were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories) that were then probed overnight at 4°C with antibodies against SP-A (1:200, sc-7700; Santa Cruz Biotechnology, Inc), SP-B (1:250, 251406; Abbiotec, LLC), and β-Actin (1:500, sc-130657; Santa Cruz Biotechnology, Inc). Antibody binding was detected by incubating the membranes for 1 hour at room temperature with antigoat IgG horseradish peroxidase conjugated (1:10 000, sc2020; Santa Cruz Biotechnology, Inc) in the case of SP-A and antirabbit IgG horseradish peroxidase conjugated (1:2500, sc-2004; Santa Cruz Biotechnology, Inc) in the case of SP-B and β-Actin (Table 1). The luminescence signal was visualized as previously described (11).

Hormone and glucose determination

Glucose plasma levels were measured using the Glucose-RTU kit (Biomérieux). Plasma corticosterone and c-peptide were measured using specific RIA kits (DRG-Instruments). An ELISA kit was used to measure the levels of *A(1–7)* (Uscn Life Science). C18 columns (Strata C18-E; Phenomenex) were used to enrich *AII* from the plasma according to the manufacturer's instructions. The final concentration was determined by a specific RIA kit (Phenix Pharmaceuticals). The interassay coefficient of variation at ED_{50} was calculated to be 2.18%, 6.59%, 6.23%, and 6.00% for corticosterone, c-peptide, *AII*, and *A(1–7)*, respectively.

Type 2 pneumocyte (T2PN) isolation

Lungs were isolated from 2 rats from the control+VEH group. After harvesting, tissues were immediately placed in a cold saline solution (4°C). T2PNs were isolated as previously described (29), and RNA from these cells was isolated and RT-PCR analysis was performed (11).

RV hypertrophy analysis

The Fulton index, the ratio between the wet weight of the RV and the LV plus septum (S), was used as an indicator of RV hypertrophy. For morphometric analysis, hearts were fixed in a relaxed state in 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). After 24 hours, the hearts were placed in 15% or 30% sucrose solutions and stored at −80°C. Images of hematoxylin-eosin-stained midventricular cross-sections of hearts (60 μm, CM1510S; Leica) were analyzed with the ImageJ 1.47 software to quantify the area of the free walls of the RV and LV but not the S, calculating the ratio thereof. Images of the sections were captured on a Nikon Coolpix 990 superhigh performance camera using a Nikon SMMZ645 magnifying glass.

The concentration of B-type natriuretic peptide-32 (BNP-32), a molecular marker of mass cavity hypertrophy and ventricular dysfunction (30), was measured in the RV and LV using the AssayMax BNP-32 ELISA kit (Assaypro). The intra and interassay coefficients of variation at ED_{50} were 4.34% and 8.5%, respectively.

Lung angiography

The animals were anesthetized, and the jugular vein was cannulated with polyethylene tubing 20 for pulmonary angiogram. All rats were heparinized (10 U/mL) and a single bolus of contrast agent (0.6 mL, Iomeron 350; Bracco Imaging) was administered. Images of the dissected animals were taken with the In-Vivo MS FX PRO Imaging System (Bruker, Inc). Three-minute x-ray images were taken at 35 Kvp. Aperture settings included an F-stop = 4. For vessel enhancement, a morphological Tophat filter was applied to the images followed by a hysteresis threshold to segment the vessels (31). Vascular density was determined using Image-Pro Plus software (MediaCybernetics, Inc) (32).

Statistical analysis

Statistical analysis of the data was performed using the STATISTICA 6 software package. Data were represented as the mean ± SE of 5–7 independent samples, each obtained from a different animal. The normal distribution of data was analyzed by Shapiro-Wilk test. Comparisons between sets of 2 independent groups were performed by the parametric Student's *t* test and when more than 2 by the two-way ANOVA in which the effect of diabetes and treatment were analyzed by post hoc Fisher's test. By convention, a value of $P < .05$ was accepted as statistically significant.

Results

The effects of LIR on glycemic control and body weight

The administration of STZ to rats destroys β-pancreatic cells causing acute diabetes characterized by severe

Table 1. Antibody Table

Peptide/protein target	Name of antibody	Manufacturer, catalog number, and/or name of individual providing the antibody	Species raised in; monoclonal or polyclonal	Dilution used
SP-A		sc-7700; Santa Cruz Biotechnology; Inc	Goat; policlonal	1:200
SP-B		251406; Abbiotec	Rabbit; policlonal	1:250
B-Actin		130657; Santa Cruz Biotechnology, Inc	Rabbit; policlonal	1:500

hyperglycemia and very low c-peptide concentrations. In this model, exogenous insulin is required for the animals to survive. From D8 to D14, STZ treatment led to a 10-fold reduction in c-peptide plasma levels with respect to the control, whether or not LIR was administered (Figure 1A). By D10, LIR eventually modify the concentration of c-peptide in nondiabetic but not in STZ diabetic animals which lacking β-cells. From D2 to D14, STZ-treated rats displayed marked elevations in glycemia (Figure 1B), and no changes in glucose levels were observed in either diabetic or nondiabetic rats when treated with LIR.

Despite being administered insulin, STZ-induced diabetes rats experienced a weight loss of 13 ± 4.73 g between D1 and D7 (Figure 1C) that persisted during treatment with LIR. By contrast, the mean weight of the nondiabetic rats increased significantly by 26 ± 2.23 g from D1 to D7 (Figure 1C), and it remained unchanged from D8 to D14 during the application of LIR.

LIR reverses the surfactant protein deficiency due to STZ-induced diabetes

STZ treatment led to a 1.5- and 3.4-fold reduction in the mRNA levels of SP-A and SP-B, respectively (Figure 2, A and B). LIR treatment had an opposite effect in STZ-treated animals and caused 3.0- and 9.2-fold increases in SP-A and SP-B, respectively, whereas just a 1.5-fold increase was observed for both proteins in the controls. In Western blottings 2 SP-A isoforms were observed, a 32-kDa native form and a glycosylated form migrating at 36 kDa (Figure 2C). The expression of both SP-A isoforms was comparable in the lungs of STZ-treated and control rats and LIR administration did not modify SP-A isoform levels in either cohort. Likewise, STZ treatment did not affect the amount of SP-B protein (Figure 2D) respect to controls, but LIR administration to STZ-treated rats markedly increased the SP-B protein levels (Figure 2D).

The mRNA levels for NKX2.1, a key transcription factor regulating surfactant protein expression (33, 34), were found to be reduced by a 1.6-fold in STZ-treated rats (Figure 2E). Subsequent administration of LIR reversed this decrease producing a net increase in expression 40% above that observed in control animals (control+VEH).

Because LIR treatment increased the SPs mRNA levels in lungs from diabetic animals, to ensure a potential direct effect, the GLP-1R expression in T2PN was examined. The analysis of these cells isolated from lungs of control animals, revealed the presence of GLP-1R (Figure 3A). The STZ treatment itself did not affect the levels of GLP-1R mRNA (Figure 3B) in lungs, but LIR administration promoted a 6.9- and 3.2-fold increase in the levels of GLP-1R mRNA of both STZ-treated and untreated animals, respectively. The effect of LIR on the GLP-1R expression in lungs from nondiabetic rats appears to be specific but not exclusive of the lungs. Thus, LIR did not modify the expression levels of the receptor in the pancreas (Figure 3C) neither in the gastric fundus (Figure 3D), whereas the administration of LIR increases (5.8-fold) the expression of GLP-1R in the duodenum (Figure 3E), suggesting differential organ targeting and functional roles for GLP-1.

We previously reported that GLP-1R agonists activated the HPA axis, thereby increasing glucocorticoid levels in circulation (8). In addition, glucocorticoids are known to potently induce the production of lipid and protein components of surfactants (35). Hence, it might be pos-

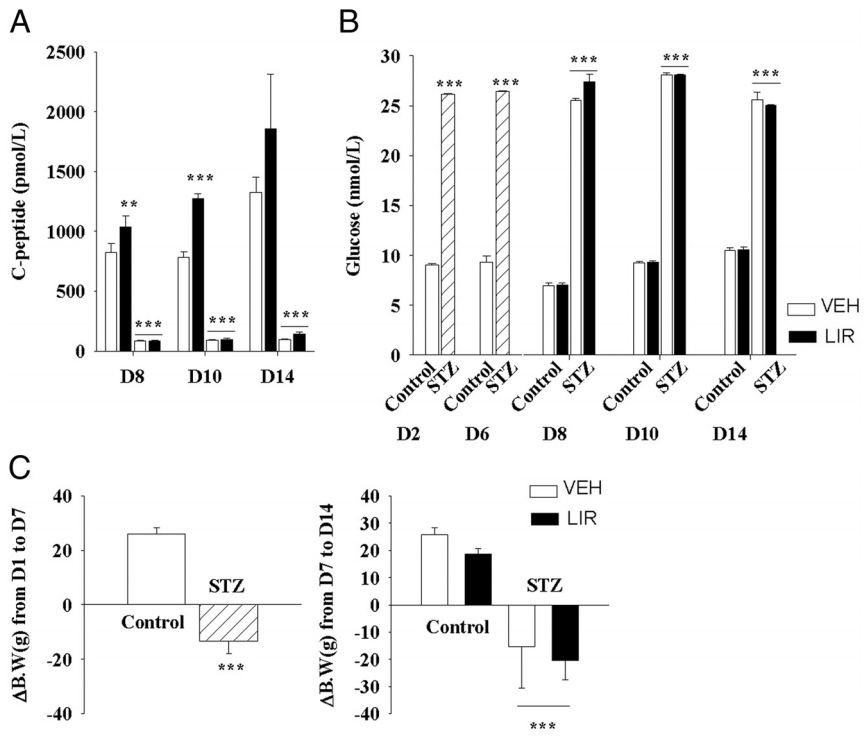

Figure 1. Initial parameters of STZ-induced diabetes rats. A, Plasma concentrations of c-peptide in nondiabetic (control) and STZ diabetic rats (STZ) treated with LIR or the VEH 8, 10, and 14 days after inducing diabetes (D8, D10, and D14). B, Glucose plasma levels on D2, D6, D8, D10, and D14 after inducing diabetes. C, Rat body weight gain before treatment (D1–D7) and during LIR or VEH administration from D7 to D14. The data are expressed as the mean ± SEM (n = 7 per group): **, $P < .01$; ***, $P < .001$ vs the control+VEH group (in B, D2–D6, and in C, D1–D7, Student's *t* test was used; two-way ANOVA followed by post hoc Fisher's test was applied for data in B, D8 onwards, and C to compare all the groups from D8 to D14).

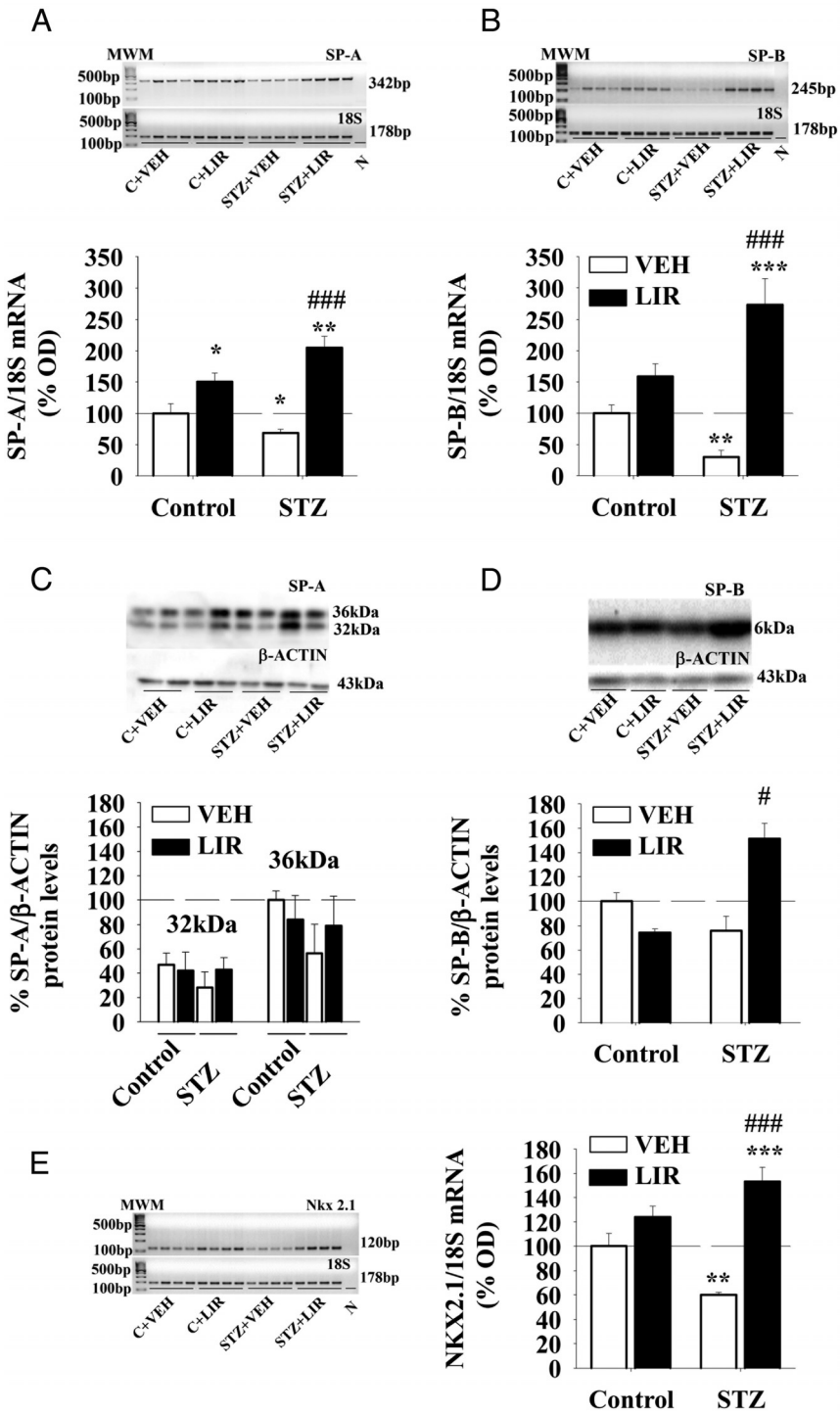

Figure 2. Effect of LIR on the pulmonary expression of surfactant proteins and NKX2.1 in a model of DM1. Effect of LIR on SP-A and SP-B mRNA (A and B) and protein expression (C and D) in the lungs of diabetic (STZ) or nondiabetic animals (control) on D14 after inducing diabetes. NKX2.1 (E) mRNA in lungs from experimental groups. Representative RT-PCR and Western blot assays (upper panels) expressed data as mean ± SEM (n = 5 per group, lower panels): *, $P < .05$; **, $P < .01$; ***, $P < .001$ vs control+VEH; #, $P < .05$; ###, $P < .001$ vs STZ+VEH (two-way ANOVA followed by post hoc Fisher's test). N, PCR negative control; MWM, molecular weight marker.

tulated that LIR would promote changes in surfactant protein production indirectly via an increase in circulating glucocorticoids. The activation of the HPA axis by GLP-1 analogues is dose dependent. Because for present experiments we selected LIR doses that in our previous experience did not affect glucocorticoid levels, we measured the plasma corticosterone variations throughout the experimental period (Supplemental Figure 1). As expected in diabetes, corticosterone levels were elevated from D1 to D8 in STZ-treated animals (36), with a 3.7-fold increase with respect to the untreated controls observed the day after the onset of LIR administration. Afterward, no significant differences in corticosterone levels were found between the experimental groups, except for an eventual reduction in STZ rats treated with LIR at D8, and no increases could therefore be attributed to the use of LIR (Supplemental Figure 1)

LIR prevents RV hypertrophy in STZ-induced diabetic rats

STZ diabetic rats were found to have a significantly heavier RV than control rats (0.84 ± 0.05 vs 0.6 ± 0.06 mg/g of body weight, $P = .009$) (Figure 4A), whereas no differences were observed in the LV mass (Figure 4B). The RV to LV+S ratio was also markedly increased in STZ-treated rats with respect to the controls (0.37 ± 0.03 vs 0.26 ± 0.04, $P = .008$) (Figure 4C). However, application of LIR prevented the increase in RV weight in STZ-treated rats, which after 7 days of treatment had RV masses and RV to LV+S ratios similar to those of the control animals (Figure 4, A and C). Similarly, no changes in the LV weights were observed after LIR administration in either STZ or control rats (Figure 4B). The changes in RV mass were also reflected in the cross-sectional area of the RV free wall, because the ratio between the area of the free wall of both ventricles (RV/LV) was increased significantly in STZ rats compared with controls (0.383 ± 0.033 vs 0.284 ± 0.010, $P = .036$) (Figure 4D).

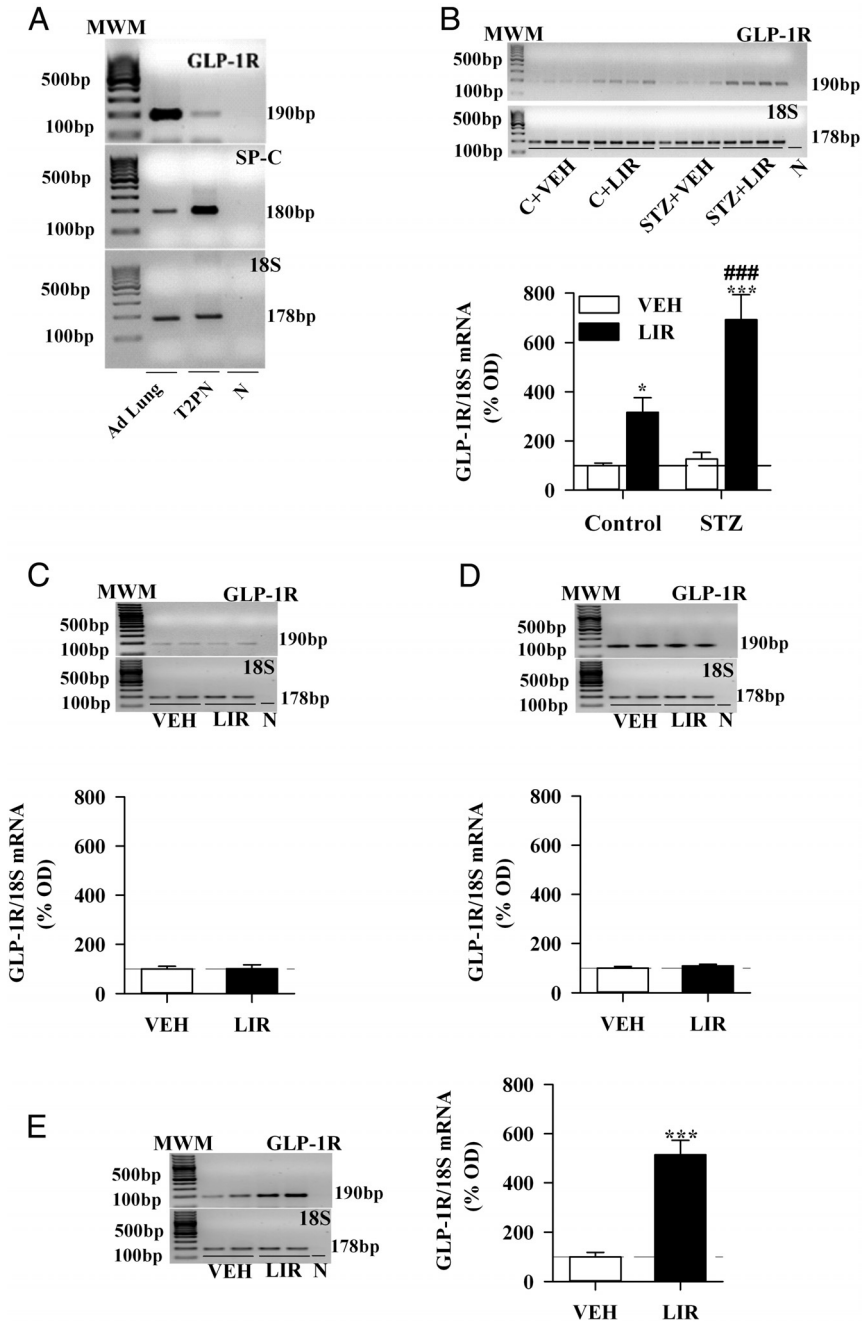

Figure 3. GLP-1R expression in T2PN and the effect of LIR on GLP-1R expression in different tissues by semiquantitative RT-PCR. A, GLP-1R and SP-C mRNA expression in T2PN from adult lungs and total adult lung (Ad Lung). B, Effect of LIR on GLP-1R mRNA levels in lungs from diabetic (STZ) or nondiabetic animals (control) on D14 after inducing diabetes. GLP-1R mRNA expression in pancreas (C), gastric fundus (D), and intestine (duodenum) (E) from nondiabetic animals treated with LIR. The data are expressed as mean ± SEM (n = 5 per group): *, $P < .05$; ***, $P < .001$ vs control+VEH; ###, $P < .001$ vs STZ+VEH (in B, two-way ANOVA followed by post hoc Fisher's test; in C–E, Student's *t* test was performed). MWM, molecular weight marker; N, PCR negative control.

LIR administration also blocked this increase and restored the mean area of the RV free wall to that observed in the control group.

In addition, we measured the BNP-32 protein in the RV and LV muscle. STZ-induced diabetes rats had an astounding 8.2-fold increase in the BNP-32 levels of the RV compared with the control group (1.15 ± 0.32 vs 0.14 ± 0.01 ng/mg, respectively; $P = .003$) (Figure 4E). Conversely, the BNP-32 concentration in the LV was similar in both STZ and control rats. Consistent with the changes in mass, LIR markedly reduced the levels of BNP-32 in the RV of diabetic rats, restoring them to the levels in control rats. Moreover, no variation in the levels of BNP-32 was observed in response to LIR administration in the LV in either the control or STZ diabetes rats.

LIR administration restores the renin-angiotensin system imbalance in the lungs of STZ-induced diabetic rats

The expression of ACE mRNA in STZ rats was similar to that in control rats (Figure 5A), yet LIR administration promoted about 2.8-fold increase in ACE mRNA transcription in control animals (control+LIR, 3.23 ± 2.11 vs control+VEH, 1.08 ± 0.17; $P < .001$) and a 3.1-fold increase in diabetic rats (STZ+LIR, 2.11 ± 0.33 vs STZ+VEH, 0.68 ± 0.09; $P < .001$). By contrast, the mRNA levels of ACE2 were significantly reduced in STZ rats compared with the control rats (0.31 ± 0.08 vs 1.0 ± 0.35, respectively; $P = .048$, 2.5-fold reduction) (Figure 5B). LIR administration enhanced ACE2 mRNA expression in control rats 2.5-fold (control+LIR, 2.49 ± 0.74 vs control, 1.04 ± 0.35, $P < .001$) and restored the ACE2 transcript levels in STZ rats to those found in control rats receiving the VEH (Figure 5B). In addition, the ACE2 to ACE mRNA ratio in the lungs resulted in a 2.5-fold reduction in STZ rats (STZ+VEH, 0.37 ± 0.06 vs control+VEH, 0.92 ± 0.2, $P = .008$), which ratio was improved after LIR administration. LIR did not modify the ACE2 to ACE ratio in the lungs of nondiabetic rats (Figure 5C).

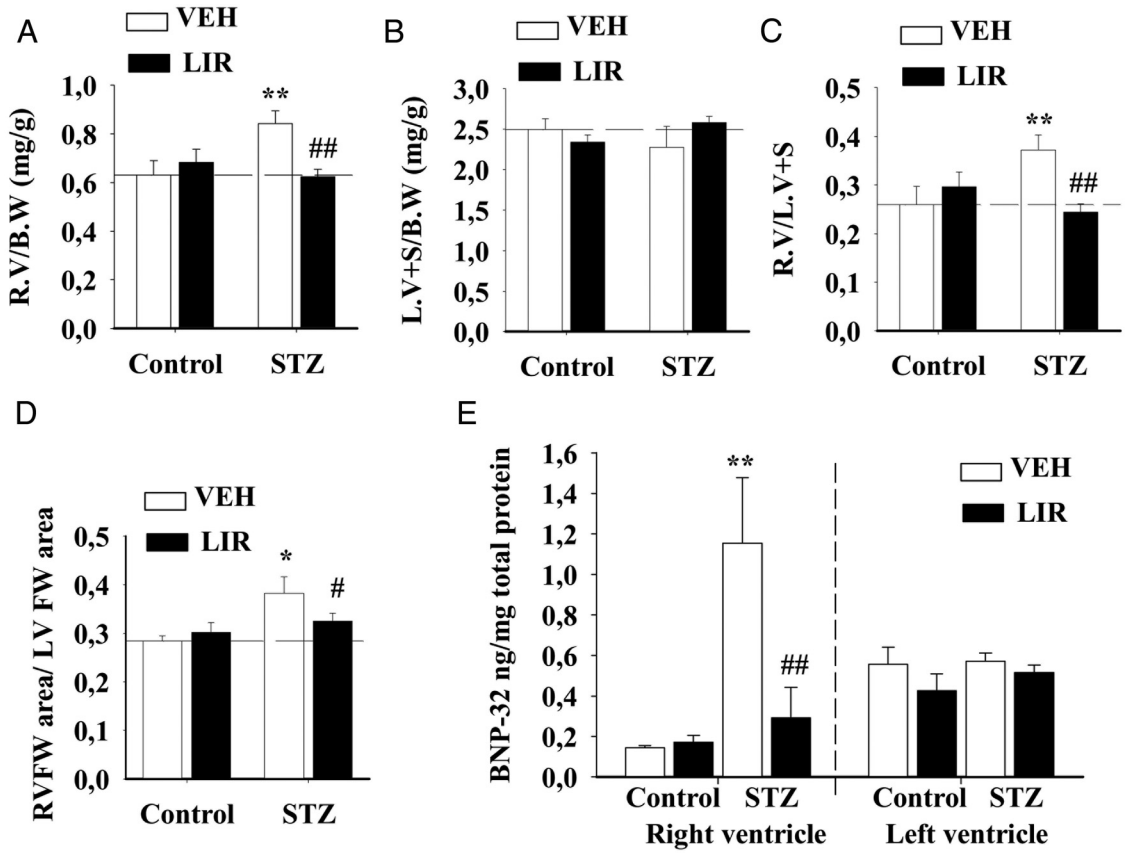

Figure 4. Effect of LIR treatment on diabetic (STZ) and nondiabetic rats (control) with respect to RV hypertrophy. RV (A) and LV wet weight (including the S, LV+S) (B) corrected for body weight (BW). RV hypertrophy was analyzed by the weight of the RV relative to the LV+S (RV/LV+S) (C). Cardiac morphometry was evaluated as the ratio between the area of the RV free wall of hematoxylin-eosin-stained cross-sections of hearts and the area of the LV free wall of each experimental group (D). BNP-32 concentration was determined in the RV and LV of STZ-treated and control rats (E): control+VEH (BW, 330.28 ± 5.00 g), STZ+VEH (BW, 242.81 ± 6.29 g), and STZ+LIR (235.81 ± 7.15 g). The data were expressed as mean ± SEM (n = 7 per group): *, $P < .05$; **, $P < .01$ vs control+VEH; #, $P < .05$; ##, $P < .01$ vs STZ+VEH (two-way ANOVA followed by post hoc Fisher's test). FW, free wall.

Circulating *AII* and *A(1–7)* levels were measured with the expectation that they would reflect the imbalance in ACE and ACE2 expression observed in the lungs. On D8, STZ-induced diabetes rats experienced a marked 3.1-fold increase in *AII* plasma levels with respect to the controls (48.70 ± 14.76 vs 15.47 ± 1.85 ng/mL, $P = .01$). On D14, there was a 2.0-fold increase with respect to nondiabetic rats (34.34 ± 5.84 vs 17.19 ± 3.59 ng/mL, $P = .038$) (Figure 6A), although this increase was reversed by LIR administration. Despite the reduction in ACE2 expression in the lungs of diabetic animals, we did not find significant variations in circulating *A(1–7)* levels in STZ+VEH animals at the end of the experiment (D14) with respect to controls (Figure 6B). By contrast, LIR administration led to an increase in *A(1–7)* plasma levels on D14 in STZ rats compared with diabetic controls (16.0 ± 2.2 vs 9.5 ± 0.79 ng/mL, $P < .017$) (Figure 6B), consistent with the increase in ACE2 activity.

Because diabetic rats showed an increase in *AII* levels and the administration of LIR to diabetic animals induced an increase in *A(1–7)* levels together with a reduction in *AII* to control levels, the vascular surface area in lungs from the different groups was determined by angiography. In agreement to the changes in the RAS components, the lungs from STZ diabetic animals showed a significant decrease in the total vascular density (Figure 6C), compatible with an increased pulmonary vascular resistance. The administration of LIR to diabetic animals led to the restitution of the vascular density to values observed in the lungs from the control group. This effect was not observed when LIR was administrated to nondiabetic animals (Figure 6C).

Discussion

Many pulmonary diabetic alterations are related to common comorbidities associated with DM2, such as obesity and sleep disturbances. However, hyperglycemia and diabetes mellitus are independent factors affecting the lung function (37). The reduction in lung compliance fre-

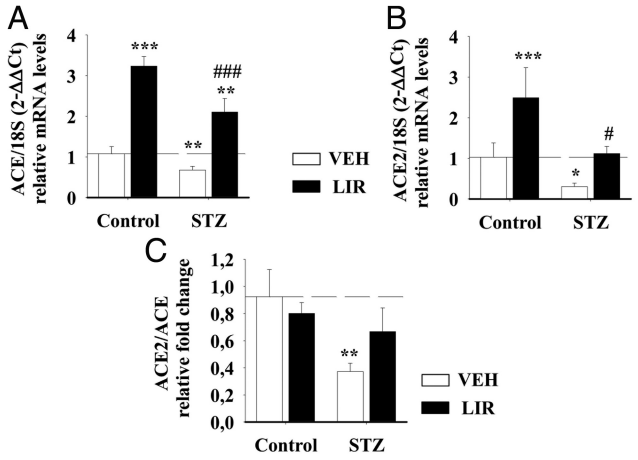

Figure 5. Balance between ACE2 and ACE. Effect of LIR on ACE (A) and ACE2 (B) mRNA expression in the lungs of STZ-treated and control rats. The relative expression of both enzymes with respect to the corresponding control group were used to calculate the ACE2 to ACE ratio (C). The data were expressed as the mean ± SEM (n = 5 per group): *, $P < .05$; **, $P < .01$; ***, $P < .001$ vs control+VEH; #, $P < .05$; ###, $P < .001$ vs STZ+VEH (two-way ANOVA followed by post hoc Fisher's test).

quently found in DM2 patients correlates with observed levels of hyperglycemia and insulin resistance. Indeed, diabetic patients were recently attributed reduction of 3%–10% in the forced expiratory volume in first second test with respect to normal subjects, which may be related to the increased mortality of these subjects (13).

Studies on STZ-induced diabetic rats have demonstrated that T2PN have an abnormal morphology (38) and

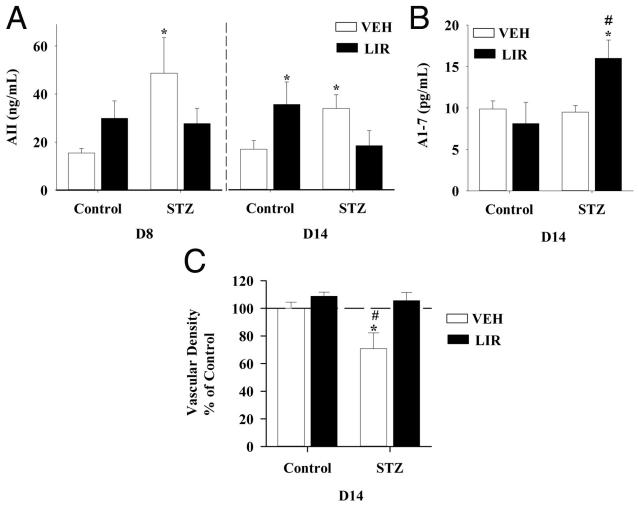

Figure 6. Circulating levels of *AII*, *A(1–7)*, and lung vascular density. Circulating *AII* concentration at D8 and D14 (A), and *A(1–7)* at D14 (B), in the plasma of STZ-treated and control rats. C, Lung vascular density was determined by analyzing the images by depicting the pixels in arbitrary units. The value from the control group was considered 100%, and the values for the different treatments were compared with the control group. The values were represented as mean ± SEM (n = 7 per group): *, $P < .05$ vs control+VEH; #, $P < .05$ vs STZ+VEH. Statistical differences were estimated using the two-way ANOVA followed by post hoc Fisher's test.

there are deficiencies in the synthesis of the lipid component of the pulmonary surfactant (39–42). In diabetes rat models induced by aloxan or STZ administration SP-B and SP-C mRNA expression appears to be reduced (43), whereas high-glucose levels have been shown to cause a reduction in SP-B and SP-C mRNA in lung explants (44). Moreover, up-regulation of SP-A was demonstrated in diabetic animals in vivo and in vitro (43, 45), yet subsequent studies indicated that SP-A mRNA expression was reduced after glucose treatment of lung explants (44). These discrepancies might be explained because SP-A production is involved in the early response to lung injury and thus, so depending on when it was measured, it may be up-regulated as consequence of the process whereby damaged alveoli are restored (27, 45). We found that there are fewer SP-A and SP-B mRNA transcripts in lungs of diabetic animals and that LIR was able to completely restore the levels of SP-A and SP-B mRNA. Other authors were unable to find any variation in the SP-A levels in response to incretins (LIR and Exendin 4), using a specific model of obstructive lungs pathology in female mice (46). We have previously shown that GLP-1R expresses differentially in the lungs of fetuses, newborns, and adult female and male rats, what in fact nicely correlates with the variations in SP-A and SP-B levels in each gender (11).

On the other hand, we have shown that LIR increases GLP-1R expression in the lungs of control and diabetic rats, a clear-cut case of a self-priming mechanism, commonly occurring in some hormonal responses (47, 48), and here may be contributing to the reinforcement of SP-A and SP-B levels increment. Moreover, NKX2.1 is a nuclear transcription factor with a key role in the regulation of SP-A and SP-B expression (33, 34). The surfactant protein deficiency associated with diabetes might be at least in part due to the reduction of NKX2.1 expression that we observed in the lungs of the diabetic rats. LIR was able to restore intracellular levels of NKX2.1, because being a possible mediator of the potent effect of GLP-1R activation on both surfactant proteins.

We also quantified the ventricular masses in our model of STZ-induced diabetes, as previously reported that heart increased affection in diabetic patients and incidence of pulmonary hypertension (17). There was marked increase in the RV weight, RV to LV+S ratio, RV free wall area and RV BNP-32 protein levels in STZ rats. Thus, STZ-treated rats appear to develop RV hypertrophy which is likely compensatory to a postulated elevation in the vascular bed pulmonary resistance (14). LIR not only reduced the RV to LV+S ratio, but also, it restored the area of the RV free wall and prevented up-regulation of BNP-32. It is important to consider that LIR had no effect in the RV mass or in the levels of BNP-32 of control rats, and that it had no

effect on the mass of the LV of either STZ-treated or control rats. Together, the most likely explanation for the preventative effects of LIR on RV hypertrophy is that it not only acts directly on the heart but also at pulmonary vascular level. In STZ diabetic rats there is a decrease in total vascular density of the pulmonary bed, what justifies an increase in vascular resistance, RV overload and compensatory RV mass hypertrophy (Figure 6C). Interestingly, LIR completely restores vascular density, what is in agreement with the effective restitution of the RV mass in STZ diabetic rats (Figures 6C, 4A, and 4C).

The renin-angiotensin system is a major regulator of blood pressure and salt/water homeostasis. The components of the RAS are produced locally in a wide range of organs where they fulfill paracrine or autocrine actions (21). Some of the vascular complications associated with diabetes may be a consequence of an imbalance in the local activity of the RAS (20), including at the pulmonary vascular bed levels (22). ACE and ACE2 have been detected in pulmonary tissue (49) and the expression of ACE determines the rate of *AII* synthesis, because ACE cleaves angiotensin I to *AII*. Production of *AII* promotes vasoconstriction and proliferation, yet when ACE2 further cleaves *AII* to produce *A(1–7)*, vasodilatation and apoptosis are promoted. The ratio of ACE2 to ACE ultimately determines the predominant physiological effects of the system: vasoconstriction and proliferation when there is more ACE than ACE2; and vasodilatation and apoptosis when there is less. We show that diabetes could produce a local imbalance in ACE and ACE2 production in the lungs, leading to augmented vasoconstriction and vascular resistance in the pulmonary bed. In fact, we demonstrated that STZ-induced diabetes is associated with a decrease in ACE2 expression in the lungs and a reduced ACE2 to ACE ratio, which lowers the *A(1–7)* concentration and enhances *AII* accumulation. These findings suggest a strong tendency towards vasoconstriction in the pulmonary vascular bed and of proliferation in the lung endothelium capillaries in diabetic animals. Such tendencies will contribute to increased pulmonary vascular resistance and to tissue remodeling mediated by *AII* (50, 51). We found that the reduced ACE2 mRNA expression in lungs of STZ diabetic rats is linked to an increase in circulating *AII* but not to significant changes in *A(1–7)* production. Therefore, pulmonary ACE2 appears not to be the only enzyme responsible for *A(1–7)* production and other enzymes may also contribute to the synthesis of *A(1–7)*, such as prolylendopeptidase or the neutral endopeptidase-24.11 (52, 53).

The cardioprotective effects of LIR have been studied extensively, yet until now, it was unclear if LIR modified the activity of the RAS as part of the underlying mechanisms for heart protection. Here, we have demonstrated that LIR is very effective in restoring the local RAS balance in lungs of diabetic rats, as it increases the expression of both the ACE and ACE2 enzymes, and it restores the ACE2 to ACE ratio. The increase in ACE mRNA suggests stronger *AII* production, which does in fact occur in diabetic rats. This enhances the availability of ACE2 substrate, which is in turn stimulated by LIR, and leads to an increase the *A(1–7)* production as a final step in this cascade. Thus, our results show that LIR can fully restore the balance of the enzymes in the lungs, improving the conditions for a physiological vasomotor response in the pulmonary vascular bed of diabetic rats. The effects of LIR on the ACE and ACE2 balance is reflected in the circulatory angiotensin levels, and LIR restored the levels of *AII* in circulation to the normal range while increasing the plasmatic levels of *A(1–7)* in STZ diabetic rats.

LIR has been attributed with vasoprotective properties, because it increases nitric oxide production via activation of endothelial nitric oxide synthase (9), and it also reduces vascular cell adhesion molecule 1 and plasminogen activator inhibitor 1 production by endothelial cells under hyperglycemic conditions. These factors contribute to the improvement of endothelial function and the dampening of proinflammatory conditions in the vascular bed (10). According to our data, LIR may also trigger vasoprotective actions by increasing ACE2 expression, at least in the lungs. This would have general cardiovascular benefits and lead to improved pulmonary vascular and cardiac function.

The effects described here are independent of the known insulinotropic activities of LIR, as the STZ diabetes model used lacks the capacity to secrete insulin, and these animals maintained high hyperglycemia values throughout the experiment despite LIR administration. The pulmonary complications of diabetes developed precociously in the STZ-induced diabetes rats with strong repercussions for the functioning of the RV, which rapidly experienced hypertrophy. Importantly, this RV hypertrophy is also completely prevented by LIR. All these responses were obtained with a LIR dose sufficiently low that it did not affect the body weight of the rats nor did it elicit changes in glucocorticoids.

In conclusion, using the STZ-induced DM1 rat as a model, for the first time, we demonstrate that the changes in the expression of ACE and ACE2 in the lungs, the onset of right heart hypertrophy, and alterations in the levels of SP-A and SP-B, can be reversed by LIR administration. This reversal occurs independently of glycemic control, body weight change or glucocorticoid levels. More evidence is appearing that supports the proposal that reducing ACE and increasing ACE2 activity in the lungs should

play a key role in the treatment of pulmonary hypertension (49). To date, methods to address this possibility have employed ACE inhibitors (54), but heretofore, no drugs able to potentiate the inherent activity of pulmonary ACE2 (55, 56) have been described. We present a novel strategy to balance the activities of ACE and ACE2, using the GLP-1R agonist LIR to augment the expression levels of ACE2. Here, this strategy appears to work well in the lungs of both STZ rats and control animals. These results are of major interest and open promising new avenues for the treatment of severe pulmonary arterial hypertension. Especially encouraging is the fact that the effects of LIR are independent of glycemic control and insulin regulation. In this context, LIR shows very remarkable biological effects in the lungs of diabetic animals, which is consistent with the very high levels of GLP-1R expression in this organ. In light of the fact that GLP-1R agonists are currently available for the treatment of DM2 in humans, and that they considered are safe and effective, we postulate here the novel therapeutic benefits that this class of drugs can offer for the still relatively unexplored consequences of diabetes, such as lung complications. Our results suggest the need for the immediate study in humans of the newly reported advantages of using GLP-1R agonists.

Acknowledgments

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