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Hyperglycemia-Mediated Oxidative Stress Increases Pulmonary Vascular Permeability

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Abstract

Objective—Hyperglycemia in diabetes mellitus is associated with endothelial dysfunction as evidenced by increased oxidative stress and vascular permeability. Whether impaired glucose control in metabolic syndrome impacts pulmonary vascular permeability is unknown. We hypothesized that in metabolic syndrome, hyperglycemia increases lung vascular permeability through superoxide.

Methods—Lung capillary filtration coefficient (Kf) and vascular superoxide were measured in the isolated lungs of lean Zucker (LZ) and obese Zucker rats (OZ). OZ were subjected to 4 weeks of metformin treatment (300 mg/kg/day orally) to improve insulin sensitivity. In a separate experiment, lung vascular permeability and vascular superoxide were measured in LZ exposed to acute hyperglycemia (30 mM).

Results—As compared to LZ, OZ had impaired glucose and insulin tolerance and elevated vascular superoxide which was associated with an elevated lung Kf. Chronic metformin treatment in OZ improved glucose control and insulin sensitivity which was associated with decreased vascular oxidative stress and lung Kf. Acute hyperglycemia in isolated lungs from LZ increased lung Kf, which was blocked with the NADPH oxidase inhibitor, apocynin (3mM). Apocynin also decreased baseline Kf in OZ.

Conclusions—These data suggest that hyperglycemia in metabolic syndrome exacerbates lung vascular permeability through increases in vascular superoxide, possibly through NADPH oxidase.

Keywords

hyperglycemia; lung permeability; metabolic syndrome

INTRODUCTION

Clinical evidence demonstrates obesity and insulin resistance is associated with an increased risk for acute respiratory distress syndrome (20), a pathological condition involving increased pulmonary vascular permeability and pulmonary edema. We have previously shown, in an animal model of metabolic syndrome, an increased baseline pulmonary

capillary filtration coefficient (Kf) and greater pulmonary edema after injury (54), however the mechanisms have not been addressed.

Several epidemiological studies demonstrate insulin resistance and elevated postprandial glucose values increase the risk of morbidity and mortality from cardiovascular disease (9, 32, 46). Also, postprandial hyperglycemia is thought to contribute to the development of vascular dysfunction in diabetes (12, 51) through oxidative stress (31). Indeed, in human vasculature, intermittent high glucose levels are damaging and cause higher oxidative stress than a constant hyperglycemia (37, 39). ROS have been shown to play an important role in regulating lung vascular permeability (5, 30). But whether hyperglycemia or insulin resistance alters permeability through ROS pathways is unclear in the lung vasculature, a vascular bed particularly vulnerable to ROS.

In chronic hyperglycemia and diabetes, NADPH oxidase is activated, leading to increases reactive oxygen species (ROS) in the lung (28, 55) and pulmonary vasculature (28). We have previously shown that obese Zucker rats (OZ), a widely used model of metabolic syndrome and insulin resistance, have an increased postprandial hyperglycemia along with increased NADPH oxidase activity in the vasculature (29). Improving insulin sensitivity in OZ reduces NADPH oxidase activity and vascular superoxide (53). Also, we have shown in lean Zucker rats (LZ) with chronic hyperglycemia, there is increased pulmonary permeability due to increases in superoxide levels from NADPH oxidase activation (30). Therefore, we hypothesized that the impaired glucose control in metabolic syndrome and the resultant increase in vascular superoxide levels results in an increase in lung vascular permeability at baseline. To test this hypothesis, this study determined the impact of hyperglycemia on lung vascular permeability and vascular superoxide production in LZ, OZ, and OZ treated with metformin to improve insulin sensitivity. To confirm glucose as a possible factor in regulating vascular superoxide and lung vascular permeability, isolated lungs and vasculature from LZ were subjected to hyperglycemia. We also determined the role of lung NADPH oxidase during hyperglycemia by perfusing isolated lungs with the NADPH oxidase inhibitor, apocynin.

MATERIALS AND METHODS

Animals

Male LZ (12–13 wk) and OZ (8–9 wk) were obtained from Harlan Laboratories (Indianapolis, IN, USA). All animal procedures and experimental protocols in this study were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center and were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the guidelines of the Animal Welfare Act. Animals were housed 2–3 per cage at a 12 hour dark/light cycle with food and water ad libitum. At the time of experiments, all animals were 12–13 weeks old.

Metformin treatment

In a group of animals, OZ (8–9 wk) were given metformin (Major Pharmaceuticals, Livonia, MI) via oral gavage (300 mg/kg/day) for 4 weeks. Metformin administration was withheld

from animals 24 hours before all experiments to exclude any effects from acute metformin treatment.

Body Composition

To examine changes in body composition induced by metformin administration, LZ, OZ, and OZ treated with metformin were weighed and subjected to magnetic resonance imaging (EchoMRI-900TM, Echo Medical System, Houston, TX). Fat mass and lean mass were measured in duplicate for each animal.

Glucose and insulin tolerance

To perform an oral glucose tolerance test (OGTT), animals were fasted 6 hours and given an oral gavage of 0.7 g/ml glucose solution (3g/kg) to assess glucose tolerance. Plasma glucose levels were measured via tail vein using a glucometer (ACON Laboratories, San Diego, CA) at 0, 15, 30, 45, 60, 120, and 180 minutes after the glucose administration. Glucose tolerance was assessed by both the plasma glucose curve and area under the curve (AUC). AUC was calculated for the 180-minute glucose curves using the trapezoidal rule.

To assess insulin sensitivity, animals were fasted 6 hours and subjected to an insulin tolerance test (ITT). Animals were given an intraperitoneal injection of insulin (human recombinant insulin, Sanofi, Paris, France) at a dosage of 1 U/kg. Plasma glucose levels were assessed via the tail vein at 0 and 60 minutes after the injection. After a minimum of 7 days following the OGTT and ITT procedures, isolated lung experiments were performed.

Before isolating the lung, venous blood samples (~2 mL) from each animal were collected by cutting the pulmonary artery. Plasma concentrations of insulin were measured via ELISA (R&D Systems, Minneapolis, MN). Animals were fasted 4 – 6 hours at the time of blood removal.

Isolated lung preparation

Animals were anesthetized using pentobarbital (65 mg/kg, i.p.). The lung and heart were excised and used to measure lung Kf, as we have previously described (54, 55). Briefly, a tracheotomy was preformed, and the lungs were ventilated via a trachea tube using a positive-pressure rodent ventilator (Model 680, Harvard Apparatus, South Natick, MA) at 55 breaths/min and a 2.5 ml tidal volume using humidified room air. Peak inspiratory pressure was fixed at 9 cmH₂O and positive end-expiratory pressure was fixed at 3 cmH₂O. After a median sternotomy, heparin (100 U) was injected into the right ventricle. Using a peristaltic pump, the physiological salt solution (PSS) perfused the isolated lung through the pulmonary artery. The PSS perfusate contained (in mM): 129.8 NaCl, 19 NaHCO₃, 5.4 KCl, 1.8 CaCl₂, 0.83 MgSO₄, and 5.5 glucose with 4% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) and was warmed to 37°C using a water-heated bubble trap (Radnoti, Monrovia, CA). For hyperglycemic or osmotic equivalent conditions, the PSS was prepared with 30 mM glucose or mannitol. To see the effects of a NADPH oxidase inhibitor on lung Kf in control OZ, control LZ, and LZ treated with hyperglycemia, apocynin (3mM; Sigma-Aldrich, St. Louis, MO) was dissolved in the PSS. The left ventricle was cannulated, and the isolated lung and heart were surgically removed and suspended on a force-displacement

transducer (Grass Technologies model FT03, Warwick, RI) above a humidified chamber maintained at 37°C. The perfusion rate was gradually increased to 30 ml/min/kg for the LZ. The OZ flow rate was based on age-matched LZ body weight. Pulmonary arterial pressure (Pa) and venous pressure (Pv) were monitored using a PowerLab system (ADInstruments, Colorado Spring, CO). After a 15-minute equilibration period with the Pv adjusted to 3.5 mmHg, capillary pressure (Pc) was determined by double occlusion on both the arterial and venous sides, and this was considered baseline Pc. After equilibration, Pv was then increased to 8.5 mmHg for a period of 15 minutes by increasing the height of the reservoir. The increase in lung weight in the first 5 minutes was considered to be pulmonary vascular recruitment and distension. The change in weight over the subsequent 10 minutes (ΔW) was used to represent transcapillary filtration (1). After the 15-minute period, Pc was measured again. After the end of the experiment, lungs were stored at 60°C for a week until a stable weight was reached. Kf was calculated using the formula: $Kf = \Delta W / \Delta Pc / \text{lung dry weight}$.

Pc is the Pc change from the baseline Pv pressure of 3.5 mmHg to 15 minutes after the increase of Pv to 8.5 mmHg. Vascular resistance was calculated by $(Pa - Pv) / \text{flow rate}$.

Vascular superoxide

Aortic superoxide levels were measured using dihydroethidium (DHE) fluorescence as we have previously shown (29). Aortic segments were then rinsed with PSS (in mM: 119 NaCl, 4.7 KCl, 1.6 CaCl₂, 1.18 NaH₂PO₄, 1.17 MgSO₄, and 24 NaHCO₃). Aortas were protected from light and incubated for 30 minutes in PSS containing 5 μ M DHE at 37°C. After incubation, segments were rinsed in PSS, split longitudinally, and placed endothelial side up on the slide. Fluotrogel with tris buffer (Electron Microscope Sciences, PA) was applied to keep the tissues hydrated. The endothelium was visualized at 20 $\times$ magnification using a laser scanning confocal microscope (Leica Microsystems, Buffalo Grove, IL, USA). Six random locations were selected for each aorta to quantitate DHE fluorescence. The same visualization parameters and laser settings were used for all aorta segments. Time was minimized for each microscope session to minimize variability and DHE instability. To compare the effects of obesity and metformin treatment, aorta segments from LZ, OZ and metformin-treated OZ were incubated in control PSS. Each slide contained one aorta segment from each animal group. DHE fluorescence is presented as the % of the LZ control aorta segment from each slide (LZ control = 100%). In a separate experiment, to compare the effect of acute hyperglycemia in LZ vasculature, each slide contained 2 aorta segments from each animal (4 total segments) with one segment treated with PSS containing 5.5 mM glucose and the other with PSS containing 30 mM glucose. Additionally, apocynin (3 mM) was added to the hyperglycemic PSS in separate LZ aorta segments and compared to aortas incubated in the control PSS. Data is presented as the % of DHE fluorescence relative to the control segment from the same animal.

Data and Statistical Analyses

Data are presented as mean $\pm$ standard error. Statistical analyses were performed using SigmaStat software (Systat, Richmond, CA). The plasma glucose levels during the OGTT were compared using a two-way repeated measures analysis of variance (ANOVA) test to compare all animal groups. Two-way ANOVA was used to compare ITT and Kf among LZ and OZ treated with and without apocynin treatment. All other groups were compared with

one-way ANOVA, as appropriate. Where significant effects occurred, the Holm-Sidak method was used to compare individual groups. A probability of $p < 0.05$ was accepted as statistically significant for all comparisons.

RESULTS

OZ fat mass was significantly greater than LZ, whereas lean mass was lower in OZ as compared to LZ (Table 1). During the 4 week metformin treatment, OZ had significantly lower body weight and fat mass as compared to control OZ (Figure 1A). As compared to LZ, metformin-treated OZ had significantly greater body weight and fat mass but lower lean mass. There were no differences found in fasting plasma glucose levels among all animal groups, however, fasting plasma insulin was significantly greater in OZ as compared to LZ (Table 1). Metformin treatment significantly reduced fasting insulin levels as compared to control OZ. Insulin levels in OZ treated with metformin were greater than LZ (Table 1).

As compared to LZ and OZ treated with metformin, OZ had significantly greater plasma glucose responses to an oral gavage of glucose (3g/kg) (Figure 1B) and greater glucose AUC (Table 1). However, as compared to LZ, metformin-treated OZ had similar OGTT responses as shown by plasma glucose and glucose AUC (Figure 1B and Table 1, respectively). After insulin injection (1 U/kg, i.p.), plasma glucose fell significantly in all animal groups (Figure 1C). However, the plasma glucose response to insulin was blunted in OZ as compared to LZ. As compared to control OZ, metformin-treated OZ had significantly lower plasma glucose after 60 minutes post-insulin injection. There were no differences in insulin-induced glucose responses between LZ and metformin-treated OZ.

At baseline, OZ had significantly larger lung Kf as compared to LZ (Figure 2A). Acute apocynin treatment in OZ significantly reduced lung Kf as compared to control OZ. OZ treated with metformin had significantly lower Kf as compared to OZ (Figure 2A). Both acute apocynin and chronic metformin treatment in OZ normalized Kf to levels comparable to LZ. There were no differences in pulmonary vascular resistance between any groups (Figure 2B). The data for lung Kf in control LZ are replicated in Figure 4 for comparative purposes.

As compared to each control LZ sample, OZ vasculature had significantly elevated superoxide (Figure 3). Metformin-treated OZ had significantly lower superoxide as compared to control OZ vasculature. There were no significant differences found between metformin-treated OZ and control LZ aortic segments. As compared to control LZ aortas under normoglycemic conditions, LZ aortas incubated with hyperglycemic PSS produced significantly greater superoxide levels. In LZ, the addition of apocynin to the hyperglycemic PSS significantly reduced superoxide as compared to the aorta segments incubated in hyperglycemic PSS alone (Figure 3).

Figure 4 demonstrates a significant increase in Kf with the addition of glucose (30 mM) in LZ as compared to control LZ. However, with the addition of mannitol to the perfusate (30 mM), there were no differences seen in Kf in either LZ or OZ as compared to baseline (Figure 4A). In LZ lungs, apocynin added to the control perfusion PSS had no effect on

baseline Kf (Figure 4A). However, apocynin added to the hyperglycemic perfusion PSS reduced the glucose-stimulated increase in lung Kf. There were no differences in lung Kf between baseline or acute hyperglycemia with apocynin treatment in LZ. There were no significant differences in vascular resistance in any group (Figure 4B).

DISCUSSION

The major new findings of this study are: 1) baseline lung Kf was increased in OZ as compared to LZ, with either acute inhibition of NADPH oxidase or improvement in chronic glycemic control in OZ decreasing lung Kf to similar levels seen in LZ; 2) in LZ, hyperglycemia increased lung vascular permeability and vascular superoxide; with this hyperglycemic-induced increase in lung Kf blocked with apocynin. These results suggest that impaired glucose control seen in metabolic syndrome may increase lung vascular permeability through increases in superoxide, possibly from NADPH oxidase activation.

The increased baseline pulmonary permeability in OZ may be clinically significant. Critically ill obesity patients have an increased risk for acute respiratory distress syndrome (20), of which pulmonary permeability is a primary factor that increases the risk of mortality (25). Also, clinical studies reveal obese trauma patients are associated with increased incidences of lung injury and pulmonary complications (10, 33). In agreement, we have shown that, as compared to LZ, OZ have greater lung injury after trauma characterized by increased lung permeability, pulmonary edema, and mortality (55). Whether an elevated baseline lung permeability in obese patients is a contributing factor to the increased risk of lung injury and pulmonary edema in these patients remains to be addressed.

Increased vascular permeability in diabetes mellitus has been well recognized, but the mechanisms are still unclear, and much less understood in a model of pre-diabetes like metabolic syndrome. Vascular permeability has been shown to be increased in patients with type 1 or 2 diabetes (7) and metabolic syndrome (16). The current study indicates that the high glucose levels in metabolic syndrome may play a major role in increasing lung vascular permeability through superoxide production. The metabolism of high glucose levels activates the DAG/PKC pathway (21), which can activate NADPH oxidase (40), producing superoxide. Superoxide and other ROS can directly increase vascular permeability (2) or alter calcium regulation which changes endothelial permeability (57). Although, the superoxide measured in the current study was from the aorta; the same ROS physiology and components are present in lung vasculature (28) and both locations are exposed to the same metabolic disorder. NADPH oxidase inhibition reverses complications such as vascular dysfunction (41) and vascular hyperpermeability (30) during poor glucose control. We show here that hyperglycemia alters lung vascular permeability through an ROS pathway, as shown by the increased vascular superoxide and lung vascular permeability after incubation with high glucose levels in LZ and the normalization of these factors with NADPH oxidase inhibition. These data suggest that superoxide is a significant mediator of hyperglycemia's ability to increase lung vascular permeability. Further experimental evidence is needed to confirm the sources and effects of this oxidative stress in the setting of metabolic syndrome.

Metabolic syndrome is an established risk factor for increased oxidative stress (3, 36). As demonstrated in a large clinical study, the number of metabolic syndrome components in patients is correlated with increasing oxidative stress (18). Similar with animal studies, the current study reveals the importance of superoxide in metabolic syndrome. Animal studies have shown an increased vascular superoxide (29, 43) in metabolic syndrome. Similarly, in diabetes, the expression of the NADPH oxidase protein has been shown to be upregulated in pulmonary (28) and non-pulmonary vasculature (52). In OZ, coronary (26) and cerebral (17) vasculature as well as the aorta (53) have increased superoxide production as compared to LZ; and vascular ROS in OZ is reduced with improvement of the insulin resistance (53). The use of antioxidants in metabolic syndrome remains relatively unexplored. Some studies demonstrate protection against cardiovascular disease in patients taking antioxidants (11, 42). We are unaware of any studies investigating the relationship between oxidative stress and lung vascular permeability in metabolic syndrome.

Metformin is a widely used insulin-sensitizing treatment for type 2 diabetes and metabolic syndrome. It reduces insulin resistance, plasma lipids, glucagon signaling, and hepatic gluconeogenesis (4, 23) by activating AMP activated protein kinase (AMPK) (8, 35). In the present study, metformin treatment in OZ improved insulin sensitivity and reduced fat mass, similar to other findings in OZ (6, 45) and in obese humans (19, 50, 56). We have previously shown that, although having similar fasting levels of plasma glucose, OZ have elevated plasma glucose levels throughout the majority of the night (peaking at 250 and 175 mg/dL for OZ and LZ, respectively) (53). We believe that this hyperglycemia was effectively reduced by metformin treatment and that this decrease in hyperglycemia played a significant role in improving lung vascular permeability.

There are limitations in this study. Apocynin reversed the hyperglycemia-induced permeability in LZ as well decreased baseline lung permeability in OZ, which we assume involved vascular NADPH oxidase. Indeed, apocynin inhibits NADPH oxidase by oxidizing thiols on p47phox (14, 48), the cytosolic subunit, subsequently preventing its translocation to the membrane-bound subunits, which induces NADPH oxidase activation (24). However, there has been criticism of apocynin's selectivity. Apocynin may have an additional effect to cause vasodilation by inhibiting the Rho kinase pathway (47). Additionally, apocynin may act as a general antioxidant, as demonstrated in cell culture subjected to high H₂O₂ concentrations (22). However, in the present study, apocynin had no effect on the control LZ Kf or pulmonary vascular resistance, suggesting that non-specific effects of apocynin were not present in the isolated lung preparation. Also, hydrogen peroxide may directly activate NADPH oxidase in vascular cells (15), which further complicates the interpretation of these previous findings.

In the current study, we believe that the poor glucose homeostasis was improved with metformin and contributed to the reduced vascular superoxide and lung vascular permeability in OZ. However, another limitation of the current study is that, independent of glucose control and insulin sensitivity, metformin and other compounds that block mitochondrial respiration have been shown to affect ROS, to either increase ROS production (27, 49) or inhibit ROS production (34, 38). Also, metformin improves insulin sensitivity which could affect other metabolic parameters and risk factors for cardiovascular disease.

However, we and others have provided evidence that emphasizes the importance of hyperglycemia as the important factor that increases vascular oxidative stress in metabolic syndrome and insulin resistance (44). For example, in type 2 diabetic patients, plasma glucose was significantly correlated with plasma ROS (13). Regardless, we believe the beneficial effects of metformin in OZ was due, at least in part, to the reduction of chronic glucose levels through improvements in insulin resistance.

CONCLUSIONS

Metabolic syndrome was associated with impaired glucose control, increased vascular superoxide, and an increased baseline pulmonary vascular permeability. The increased baseline vascular oxidative stress and lung vascular permeability in OZ was reduced by chronic metformin treatment. Hyperglycemia in LZ increased lung Kf, independent from any changes in pulmonary vascular resistance or osmolarity, suggesting glucose as a significant mediator of metabolic syndrome's effect on lung permeability. In addition, both the baseline lung Kf in OZ and hyperglycemic-induced increase in lung Kf in LZ were blocked with apocynin, suggesting that the source of superoxide may have been from NADPH oxidase. Further investigation is needed into the direct mechanisms by which ROS increases lung vascular permeability in metabolic syndrome.

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Figure 1.

Effects of metformin treatment in OZ. A) Body weights at 9 to 13 weeks of age for OZ and OZ treated with metformin. (# $P < 0.05$ vs. OZ; $n = 8$ for each group). B) Plasma glucose after oral glucose tolerance test (3g/kg). (+ $P < 0.05$ OZ vs. LZ; # $P < 0.05$ OZ vs. OZ + Metformin; $n = 6-8$ for each group). C) Plasma glucose after insulin tolerance test (1U insulin/kg body weight) (+ $P < 0.05$ LZ vs. OZ; # $P < 0.05$ OZ vs. OZ + Metformin; $n = 7-8$ for each group).

Figure 2.

A) Pulmonary Kf and B) vascular resistance for LZ, OZ, OZ acutely treated with apocynin (Apo), and OZ chronically treated with metformin (+P < 0.05 OZ vs. LZ; #P < 0.05 vs. OZ; n = 6–9 for each group except OZ + Apo, n=5).

A)

B)

Figure 3.

A) DHE fluorescent images in aortic segments from LZ, OZ, OZ treated with metformin, and LZ treated with glucose (30 mM), and LZ treated with glucose and apocynin (3 mM).

B) Percent DHE fluorescence from aortic segments from OZ, OZ treated with metformin, and LZ treated with glucose relative to LZ control (LZ control = 100%). (* $P < 0.05$ vs LZ control; # $P < 0.05$ vs. OZ; + $P < 0.05$ vs. LZ + Glucose; $n = 6-8$ for each group except LZ + Glucose + Apo, $n=4$).

Figure 4.

The effect of acute hyperglycemia on A) pulmonary Kf and B) vascular resistance for LZ and OZ. Isolated lungs were perfused with either control solution, mannitol solution (30 mM), or glucose solution (30 mM). Additionally, LZ lungs perfused with control and glucose solutions were also treated with apocynin (*P < 0.05 vs LZ; #P < 0.05 LZ + Glucose vs LZ + Mannitol; n = 8–10 for all groups except LZ + Apo, n = 4).

Table 1

Baseline characteristics of animals (fasted 4–6 hours).

	LZ	OZ	OZ + Metformin
Fat Mass (g)	29 ± 2	212 ± 7 *	156 ± 5 *#
Lean Mass (g)	293 ± 3	253 ± 4 *	258 ± 6 *
Glucose (mg/dL)	116 ± 2	118 ± 4	111 ± 3
Glucose AUC	449 ± 10	697 ± 18 *	483 ± 24 #
Insulin (mg/dL)	2.3 ± 0.2	22.2 ± 2.6 *	11.8 ± 3.1 *#

*
p < 0.05 vs LZ;#
p < 0.05 vs OZ