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Oxidative stress increases pulmonary vascular permeability in diabetic rats through activation of transient receptor potential melastatin 2 (TRPM2) channels

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Abstract

Objective—*In vitro* superoxide activates pulmonary endothelial TRPM2 channels and increases the capillary filtration coefficient (Kf). We hypothesized that pulmonary capillary Kf is increased in a model of type I diabetes due to elevated vascular superoxide and resultant TRPM2 channel activation.

Methods—Type I diabetes was induced in Zucker rats using streptozotocin. Half of the streptozotocin animals were treated with apocynin, a NOX inhibitor. After 4 weeks, lung Kf was measured in the isolated lung in the presence or absence of TRPM2 inhibitors (2-APB and flufenamic acid). In an additional set of experiments, Kf was measured in non-diabetic Zucker rats after applying the superoxide donor (PMS).

Results—As compared to control rats, hyperglycemic rats exhibited increased vascular superoxide and Kf, along with decreased lung vascular TRPM2-L expression. Apocynin treatment reduced superoxide and Kf in hyperglycemic rats with no effect in control rats. TRPM2 channel inhibition decreased Kf in hyperglycemic rats with no effect in control rats. PMS increased the lung Kf in control rats, with TRPM2 inhibition attenuating this response.

Conclusion—Diabetic rats exhibit a TRPM2-mediated increase in lung Kf, which is associated with increased TRPM2 activation and increased vascular superoxide levels.

Keywords

Hyperglycemia; superoxide; lung permeability; TRPM2

INTRODUCTION

Type I diabetic humans and animals exhibit increased vascular oxidative stress and lung complications attributed to elevated capillary filtration coefficient (Kf) (16, 17, 23, 25, 26,

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CONTRIBUTIONS

S. L. designed the study, performed experiments, analyzed data, and wrote the manuscript. All authors contributed to data interpretation and critical revision of the manuscript.

DISCLOSURES

We have no conflicts to disclose.

29, 30, 34). However, the mechanisms for the elevated lung vascular permeability and the possible role of ROS on Kf have not been determined in a setting type I diabetes.

Superoxide has been shown to increase endothelial permeability in different vascular beds (7, 10, 15, 41) through an increase in intracellular calcium concentration and the resultant cell retraction (43). Three transient receptor potential (TRP) channels, including TRP vanilloid (TRPV), TRP canonical (TRPC), and TRP melastatin (TRPM), are known to increase calcium entry and thus pulmonary Kf (4, 37, 38). TRPM2 is a major target of superoxide and hydrogen peroxide (H_2O_2) (19, 20). An over-expression of TRPM2 enhances H_2O_2 -mediated calcium entry in human pulmonary endothelial cells (19, 20). However, whether an elevated vascular superoxide in type I diabetics increases pulmonary Kf via activation TRPM2 has not been determined.

The STZ-induced type I diabetic model is characterized by chronic hyperglycemia and increased NOX-mediated vascular oxidative stress (8). We determined if pulmonary capillary Kf is increased in STZ-treated hyperglycemic rats due to elevated TRPM2 channel activation. Since NOX is a major source of vascular superoxide (27, 28), we also determined pulmonary permeability in STZ-treated rats with or without treatment with apocynin, a NOX inhibitor. We hypothesized that pulmonary endothelial permeability is increased during chronic hyperglycemia due to superoxide-induced activation of TRPM2 channels.

METHODS

Male lean Zucker rats (LZ, 8-9 weeks old) were acquired from Harlan Laboratories (Indianapolis, IN). Experimental protocols for this study were approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center and were carried out according to both the National Institutes of Health Guide for the Care and Use of Laboratory Animals and guidelines of the Animal Welfare Act.

STZ (50mg/kg) was injected once i.p. to induce chronic hyperglycemia. Half of the STZ-treated rats were treated with apocynin, a NOX inhibitor, in the drinking water (2 mM for 4 wks, approximately 46 mg/kg/d) from the time the STZ was injected. All animals were 12-13 weeks old at the time of the experiments.

Pulmonary Kf

Animals were anesthetized with pentobarbital (65mg/kg, i.p.), and the lung and heart were isolated and surgically removed. Pulmonary Kf was measured as previously described (39). Briefly, we measured pulmonary arterial pressure (Pa) and pulmonary venous pressure (Pv) by using a PowerLab system (Model: ML 118, Colorado Springs, CO). The lung weights were measured at 3.5 mmHg (Pv) and then again after 10 min at 8.5 mmHg (Pv). Pressure was controlled by changing the height of the perfusate reservoir. The weight increases (ΔW) were calculated. Capillary pressure (Pc) was determined by the equilibrated pressure after the occlusion of both the arterial and venous sides at 3.5 mmHg (Pv) and 8.5 mmHg (Pv). Kf was calculated by using the following formula: $Kf = \Delta W / \Delta Pc / \text{lung dry weight}$. ΔPc is the delta change of Pc from the 3.5 mmHg (Pv) to 8.5 mmHg (Pv).

Determination of lung Kf following TRPM2 channel inhibition

We determined the role of TRPM2 channel activation in regulating Kf in chronic hyperglycemia using the isolated lung preparation. In isolated lungs from control and hyperglycemic animals, we added 2-APB to the perfusate 15 minutes before the increase in Pv. 2-APB is shown to selectively inhibit TRPM2 channels at a concentration lower than 10 μ M (36).

Role of superoxide on TRPM2 channel activation and lung Kf

In a group of control (non-hyperglycemic) animals we performed isolated lung experiments in which we added phenazine methosulfate (PMS, 1 μ M), a superoxide donor, to the perfusate 15 minutes before the increase in Pv. Kf was determined as described above. To determine if TRPM2 channels were responsible for the superoxide-mediated increase in Kf, we added two TRPM2 channel inhibitors, 2-APB (1 μ M) and flufenamic acid (FA, 100 μ M), to the perfusate using the protocol described above. Additionally, it has been shown that the TRPC/store-operated calcium channel (SOC) also regulates pulmonary endothelial permeability (1, 19). ROS are reported to activate TRPC/SOC channels (19, 20). To exclude the effect of TRPC/SOC activation on Kf in our study, a TRPC/SOC inhibitor, SKF96365 (SKF, 1 μ M), was applied to the perfusate using the protocol as described above.

Superoxide level and NOX activity

Superoxide levels in aortic tissue were measured using dihydroethidium (DHE) fluorescence (28). Aortic segments were rinsed in a physiological salt solution [PSS; containing (in mM) 119.0 NaCl, 4.7 KCl, 1.6 CaCl₂, 1.18 NaH₂PO₄, 1.17 MgSO₄, and 24.0 NaHCO₃]. These segments were incubated in light-protected PSS (37°C) containing 5 μ M DHE for 30 min. Segments were rinsed in DHE-free PSS and split longitudinally and placed endothelium side up on a coverslip. A drop of Fluotrogel with tris buffer (EMS, PA) was applied to keep the tissue moist. The endothelium was visualized, and images were obtained using a laser scanning confocal microscope (Leica Microsystems, Buffalo Grove, IL).

To validate that the PMS treatment increased vascular superoxide, aortas were collected from control animals, and superoxide levels were measured using DHE (37°C, 5 μ M) fluorescence, as we previously described (38), in untreated tissue and in tissue incubated for 15 min with PMS (1 μ M).

NOX activity was measured in pulmonary arteries using lucigenin chemiluminescence as we have published previously (25). Homogenates were prepared from pulmonary arteries. The homogenates were incubated with lucigenin (5 μ M) for chemiluminescence detection using a Berthold luminometer. For measurement of NOX activity, NADPH was added (100 μ M), and chemiluminescence was measured. A luminescence reading was obtained for an overall measuring time of 5 min for each sample. Enzyme activity is expressed in relative light units (RLU) per second and normalized by protein concentration from each sample.

TRPM2 channel protein expression

Pulmonary arteries from LZ control and hyperglycemic LZ were collected, homogenized and centrifuged at 13,000 rpm. The supernatant was separated by electrophoresis on a

Criterion TGX AnykD Gel (Bio-Rad, Hercules, CA), and transferred to a nitrocellulose membrane for Western blotting. TRPM2-L, the only functional TRPM2 channel isoform (NB110-81601, Novus Biologicals, Littleton, CO) and β -actin antibodies were applied after incubation with an Odyssey Blocking Buffer (LI-COR, Lincoln, NE). The TRPM2-L antibody recognizes a C-terminal portion of the rat TRPM2 protein (within residues 1430-1508), specific for TRPM2-L. The membrane was read by a LI-COR Odyssey scanner (LI-COR, Lincoln, NE).

Reagents and chemicals

Apocynin, STZ, PMS, 2-APB, FA, and SKF were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Apocynin was given in the tap water. STZ was given i.p. in saline. 2-APB, FA, and SKF were dissolved in 100% ethanol and diluted as 1:1000-10000 in the isolated lung perfusate.

Quantitative and statistical analyses

Superoxide levels before and after PMS treatments were compared using t-test. The superoxide levels and NOX activity in non-hyperglycemic, hyperglycemic, and apocynin-treated hyperglycemic animals were compared using one-way ANOVA. The Kf under baseline, PMS-treated, PMS+SKF-treated conditions were compared by one-way ANOVA. TRPM2 channel expression in LZ and hyperglycemic LZ were compared using a t-test. All of the other data were compared by using two-way ANOVA. Where significant effects occurred, individual groups were compared using the Holm-Sidak method. All of the data are presented as means $\pm$ SEM. A probability of $p < 0.05$ was accepted as statistically significant for all comparisons.

RESULTS

STZ induces chronic hyperglycemia in lean Zucker rats

As compared with control LZ, the STZ-treated LZ exhibited significantly higher fasting glucose levels from the third day after STZ injection (Table 1). Four weeks after STZ injection the LZ (12-13 wks old) had significantly lower body weights than their age-matched controls (Table 1). Apocynin treatment had no effect on glucose levels or body weights (Table 1).

TRPM2 channel expression decreased in STZ-treated hyperglycemic LZ

There was a significantly lower pulmonary artery TRPM2-L channel expression in hyperglycemic LZ as compared to control LZ (Figure 1).

Superoxide levels and NOX activity are elevated in type I diabetic rats

STZ-treated LZ, as compared with control LZ, exhibited significantly higher aortic superoxide levels indicated by DHE fluorescence (Figure 2A) and higher NOX activity in pulmonary arteries measured by RLU of chemiluminescence (Figure 2B). Apocynin treatment in hyperglycemic LZ significantly decreased both vascular superoxide levels and NOX activity (Figures 2A and 2B, respectively).

Chronic hyperglycemia increases pulmonary Kf via superoxide-mediated TRPM2 activation

Figure 3 presents the isolated lung Kf from control, hyperglycemic, and apocynin-treated hyperglycemic LZ with or without TRPM2 channel inhibition. Kf was significantly elevated in the LZ with chronic hyperglycemia. This increase in Kf was attenuated by apocynin treatment (Figure 3). 2-APB had no effect on the Kf in non-hyperglycemic LZ but significantly inhibited pulmonary Kf in both STZ-treated and apocynin-treated hyperglycemic LZ (Figure 3).

TRPM2 inhibition prevents superoxide-induced increases in pulmonary Kf

Figure 4 presents the pulmonary Kf changes in normoglycemic LZ (control) after application of the superoxide donor, PMS, with and without treatment with two TRPM2 channel inhibitors. PMS application significantly increased pulmonary Kf (Figure 4). 2-APB (1 μ M) significantly inhibited the PMS-induced Kf increase but had no effect on basal Kf (Figure 4). The effects of FA (100 μ M), another TRPM2 channel inhibitor, were similar to 2-APB (Figure 4). Inhibition of TRPC/SOC by SKF had no effect on pulmonary Kf after PMS application (Figure 5). PMS significantly increased superoxide levels in the aorta (data not shown).

In Figures 3 and 4, the control group with or without treatment of 2-APB are from the same animals. In Figures 4 and 5, the PMS-treated LZ group represents data from the same animals.

DISCUSSION

The major findings of this work are: 1) type I diabetic LZ exhibited increased vascular oxidative stress, pulmonary Kf, and decreased vascular TRPM2-L channel expression; 2) inhibition of NOX with apocynin treatment decreased vascular oxidative stress and pulmonary Kf in the diabetic rats; 3) inhibition of TRPM2 channel decreased lung Kf in diabetic rats, with this inhibitory effect attenuated in the apocynin-treated diabetic LZ; and 4) a superoxide donor increased the pulmonary Kf in non-hyperglycemic LZ, with this increase blunted following TRPM2 channel inhibition.

Pulmonary Kf in diabetic rats is increased due to oxidative stress

Chronic hyperglycemia has been shown to increase inter-endothelial gaps in the lung capillary endothelium (2, 22, 30, 32). We found that rats with chronic hyperglycemic exhibited an increased pulmonary Kf (Figure 3) in the isolated lungs perfused with a physiological saline solution (PSS) with normal glucose concentration (5.5 mM). Chronic hyperglycemia is known to increase vascular superoxide levels (7, 10, 15, 41), and NOX is one of the major sources of endothelial superoxide generation during chronic hyperglycemia (27, 28). In the current study, we found significant increases in vascular NOX activity and superoxide levels in diabetic rats (Figure 1). The lung vasculature is vulnerable to oxidative stress (11), and the pathological changes are correlated with an increased pulmonary Kf (3, 40). We found that, in diabetic rats, inhibition of superoxide after apocynin treatment

significantly reduced lung Kf. These results suggest that NOX-mediated oxidative stress increases pulmonary endothelial permeability in the context of chronic hyperglycemia.

Superoxide increases pulmonary endothelial Kf via activation of the TRPM2 channel

Calcium entry through TRP channels, including TRPC, TRPV, and TRPM channels, increases endothelial permeability (4, 9, 19, 20, 18, 24). 2-APB has been shown to selectively inhibit TRPM2 channels at a concentration lower than 10 μ M (36). In current study, we found that 2-APB decreased Kf in STZ-treated rats, suggesting that TRPM2 plays an important role in lung Kf during chronic hyperglycemia. We have previously shown that treatment with PMS, a superoxide donor, can increase the capillary Kf in the isolated lung (39). Since the TRPM2 channel is an oxidant-activated calcium channel, we applied PMS to verify if the superoxide-mediated increase in lung Kf is TRPM2 channel-dependent. Because there is a lack of commercial extracellular agonists, we used two different TRPM2 inhibitors, 2-APB and FA. We found that the PMS-induced increase in lung Kf was blunted by treatment with either 2-APB or FA (Figure 4), suggesting that TRPM2 channel activation by superoxide may be responsible for the increase in lung Kf during chronic hyperglycemia. Furthermore, the inhibitory effect of 2-APB on Kf was attenuated in the diabetic rats following apocynin treatment, suggesting that the increased basal lung Kf is due, at least in part, to NOX-mediated superoxide production. 2-APB still decreased the Kf in the diabetic rats treated with apocynin, suggesting that other factors may also contribute to the TRPM2-mediated increase in Kf.

TRPC channels, acting through SOC and receptor-operated calcium entry, are involved in the regulation of vascular permeability (1, 4). Oxidants are also known to induce calcium entry through TRPC/SOC channels (14, 18). SKF is used to inhibit SOC and TRPC channels (13, 33). Although SKF is not a selective inhibitor for SOCs, in our isolated lung experiment, SKF has no effect on the PMS-induced Kf increase (Figure 5). This indicates that the channels which are inhibited by SKF, including SOC, play a minor role in mediating the increase in Kf. In addition, inhibition of TRPM2 channel by 2-APB significantly decreased but failed to normalized the Kf in STZ rats compared to the control group. This could be due to the activation of other calcium channels in STZ rats which are not inhibited by 2-APB, such as TRPV4 channels. Townsley's laboratory reported that activation of TRPV4, a mechanically activated receptor, also increases pulmonary endothelial permeability (4, 38). However, TRPM2 channel inhibitors significantly decreased the lung Kf in hyperglycemic rats without affecting the Kf in control animals, suggesting that TRPM2 channels play a major role in the increased lung Kf in diabetic rats. In addition to the increased superoxide-mediated activation of TPRM2, we found a down-regulation of TRPM2-L channel expression in the lung vasculature from diabetic rats (Figure 1). The mechanisms responsible for this are unclear. Besides TRPM2-L, 3 isoforms of TRPM2 channels have been determined: TRPM2-S (short), TRPM2- N (a deletion of 20 amino acids in the N-terminus), and TRPM2- C (a deletion of 34 amino acids in the CAP domain) (20, 42). TRPM2-L is the only fully functional isoform. Thus, we determined the TRPM2-L expression level in our model. The decreased TRPM2-L expression during chronic hyperglycemia might be due to the overexposure of superoxide and a resultant negative feedback-mediated down regulation (31). Nevertheless, these results collectively suggest

that the function of TRPM2 channels in regulating lung Kf is minor under control conditions, but is increased in response to the elevated superoxide in diabetics. Further studies are clearly needed to determine the pulmonary TRPM2 channel sensitivity in control and diabetic animal models by using pharmacological and electrophysiological approaches.

In conclusion, these results suggest that, in chronic hyperglycemic rats, oxidative stress activates TRPM2 channels, leading to an increase in pulmonary capillary Kf. This TRPM2 channel-mediated elevation in Kf may contribute to the increased susceptibility to lung complications seen in patients with type I diabetes. This study provides novel findings that point to possible TRPM2 channel blockade-based therapeutics in type I diabetic patients with lung injury.

Acknowledgments

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List of Abbreviations

2-APB	2-aminoethoxydiphenyl borate
ALI	acute lung injury
DHE	dihydroethidium
FA	flufenamic acid
H₂O₂	hydrogen peroxide
Kf	lung capillary filtration coefficient
LZ	lean Zucker rats
NOX	nicotinamide adenine dinucleotide phosphate-oxidase
PMS	phenazine methosulfate
ROS	reactive oxygen species
SOC	store-operated calcium channel
STZ	streptozotocin
TRPM2	transient receptor potential melastatin 2
TRPM2-L	full length TRPM2 channel isoform

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PERSPECTIVES

Compared with normal individuals, diabetic and obese patients are at a higher risk of developing acute lung injury (ALI) after acute illness such as trauma and sepsis. An increased pulmonary capillary permeability is an important factor contributing to lung edema during ALI. The mechanisms responsible for the increased susceptibility are unclear. In this study, we determined the role of the TRMP2 channel activation in pulmonary permeability regulation in a type I diabetic rat model. Our data suggest that, in hyperglycemic rats, increased oxidative stress activates the TRPM2 channel and subsequently elevates lung Kf. This increased basal lung permeability in type I diabetes may be the potential mechanism responsible for the high susceptibility to ALI after acute illness in the diabetic and obese population.

Figure 1. Pulmonary arterial TRPM2-L channel expression in control LZ and STZ-treated LZ TRPM2-L channel is down-expressed in STZ-treated type I diabetic LZ (*, $p < 0.05$ vs. Control). $n = 6$ in Control LZ; $n = 6$ in STZ-treated LZ.

Figure 2. Aortic superoxide levels and pulmonary arterial NADPH oxidase activity in LZ with and without STZ/apocynin treatment

A) The top is the example of confocal images obtained using a confocal microscopy from DHE-treated aorta. STZ treatment significantly increases superoxide level in aorta compared with control group (*, $p < 0.05$ vs. control). Apocynin treatment partially normalizes superoxide in STZ-treated group (*, $p < 0.05$ vs. control; #, $p < 0.05$ vs. STZ group). $n = 6$ for control group, $n = 5$ for STZ group, $n = 6$ for STZ+apocynin group. **B)** STZ treatment significantly increases NOX activity in pulmonary arteries compared with control group (*, $p < 0.05$ vs. control). Apocynin treatment partially normalizes NOX activity in STZ-treated group (*, $p < 0.05$ vs. control; #, $p < 0.05$ vs. STZ group). $n = 6$ for control group, $n = 6$ for STZ group, $n = 6$ for STZ+apocynin group.

Figure 3. Pulmonary capillary Kf in control, STZ-treated, and STZ+apocynin-treated LZ with and without 2-APB application

STZ treatment significantly increase Kf compared with control group (*, $p < 0.05$ vs. control). Apocynin treatment partially normalizes Kf in STZ-treated group (*, $p < 0.05$ vs. control; #, $p < 0.05$ vs. STZ group). 2-APB (1 μ M) has no effect on control group, but partially normalizes Kf in STZ group (*, $p < 0.05$ vs. 2-APB-treated control; ^, $p < 0.05$ vs. STZ group), and in STZ+apocynin group (*, $p < 0.05$ vs. 2-APB-treated control; ^, $p < 0.05$ vs. STZ+apocynin group). n=5 for control, n=5 for 2-APB-treated control, n=5 for STZ, n=5 for 2-APB-treated STZ, n=6 for STZ+apocynin, n=6 for 2-APB-treated STZ+apocynin.

Figure 4. Pulmonary capillary Kf in control and PMS-treated LZ with and without 2-APB or FA application

PMS significantly increases Kf in LZ (*, $p < 0.05$ vs. control LZ). 2-APB (1 μ M) has no effect on control LZ but normalized Kf in PMS-treated group (#, $p < 0.05$ vs. PMS-treated LZ). Similarly, FA (100 μ M) has no effect on control LZ but partially normalized Kf in PMS-treated group (#, $p < 0.05$ vs. PMS-treated group; *, $p < 0.05$ vs. control LZ treated with FA). $n=5$ in all groups.

Figure 5. Pulmonary capillary Kf in PMS-treated group with and without SKF application
SKF (1 μ M) has no effect on Kf in PMS-treated lungs. n=5 in all groups.

Table 1

Body weight and blood glucose levels in control, STZ-treated, and STZ+apocynin-treated LZ.

	Control	STZ	STZ+apocynin
Body weight (g)	354±21	274±18 *	265±27 *
Glucose (mg/dL)	100±12	444±44 *	457±27 *

Values are means±SEM

* $p < 0.05$ vs. control; there is no significant difference in body weight and glucose levels between STZ and STZ+apocynin group. n=12 for control group, n=16 for STZ group, and n=18 for STZ+apocynin group.