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Adiponectin protects against hyperoxic lung injury and vascular leak

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Abstract

Adiponectin (Ad), an adipokine exclusively secreted by the adipose tissue, has emerged as a paracrine metabolic regulator as well as a protectant against oxidative stress. Pharmacological approaches of protecting against clinical hyperoxic lung injury during oxygen therapy/treatment are limited. Earlier, we have reported that Ad inhibits the NADPH oxidase-catalyzed formation of superoxide from molecular oxygen in human neutrophils. Having this as the premise, we conducted studies to determine whether (i) exogenous Ad would protect against the hyperoxia-induced barrier dysfunction in the lung endothelial cells (ECs) *in vitro* and (ii) endogenously synthesized Ad would protect against hyperoxic lung injury in wild type (WT) and Ad-overexpressing transgenic (AdTg) mice *in vivo*. The results demonstrated that exogenous Ad protected against the hyperoxia-induced oxidative stress, loss of glutathione (GSH), cytoskeletal reorganization, barrier dysfunction, and leak in the lung ECs *in vitro*. Furthermore, the hyperoxia-induced lung injury, vascular leak, and lipid peroxidation were significantly attenuated in AdTg mice *in vivo*. Also, AdTg mice exhibited elevated levels of total thiols and GSH in the lungs as compared to WT mice. For the first time, our studies demonstrated that Ad protected against the hyperoxia-induced lung damage apparently through attenuation of oxidative stress and modulation of thiol-redox status.

Keywords

Adiponectin; Hyperoxia; Oxidative Stress; Lung Vascular Endothelium; Reactive Oxygen Species; Barrier Dysfunction; Cytoskeletal Rearrangement; Hyperoxic Lung Damage

INTRODUCTION

Adipose tissue is not merely a passive fat depot but has emerged as an endocrine organ that hosts and operates critical and complex metabolic functions in normal physiology and

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pathological states (1, 2). Adipose tissue secretes a wide array of bioactive peptides and proteins which are collectively called “adipocytokines” or “adipokines” (3, 4). Adipokines include adiponectin (Ad), leptin, tumor necrosis factor- α (TNF- α), plasminogen activator inhibitor type 1, adipsin, and resistin (Schema-1) (3). They are involved in a variety of physiological functions including lipid metabolism, insulin sensitivity, alternate complement system, hemostasis, regulation of blood pressure, angiogenesis, and regulation of energy balance (3).

Ad has gained considerable attention due to its potent physiological effects and its therapeutic potential to treat insulin resistance, type-2 diabetes, obesity, and atherosclerosis (5, 6). Ad exhibits pleiotropic actions including antidiabetic, antiatherogenic, and anti-inflammatory effects (7). Ad has also been shown to decrease the expression of vascular cell adhesion molecules known to modulate endothelial inflammatory responses (8). The vascular and myocardial protective actions of Ad are increasingly becoming evident (9). Vascular endothelial protection by Ad including the suppression of reactive oxygen and nitrogen species (ROS and RNS) and the attenuation of oxidative stress have been reported (10). Earlier, we have shown that Ad inhibits NADPH oxidase-catalyzed generation of superoxide by human neutrophils (12). Overall, Ad appears to possess anti-inflammatory and oxidative stress protective actions.

In vivo hyperoxic exposure causes severe lung inflammation, fibrin deposition, and pulmonary edema (13). Despite its potential to cause lung damage, oxygen therapy can be life-saving for critically ill patients with respiratory failure, but prolonged exposure to high concentrations of oxygen results in acute lung injury (14). Microvasculature of the lung is a critical target for hyperoxic insult. Lung vascular leak caused by vascular endothelial dysfunction contributes to several lung diseases including the hyperoxic lung damage. Earlier, we have shown that the lung vascular ECs are also capable of generating ROS under hyperoxia, through the activation of NAD[P]H oxidase (15). Membrane phospholipid peroxidation has been also reported in hyperoxic lung (16). ROS, generated by either neutrophils or lung cells, apparently contribute to hyperoxic lung vascular endothelial barrier dysfunction and vessel leak (17, 18). ROS and hyperoxia have been shown to cause cytoskeletal reorganization in the cultured vascular ECs (19–21). So far, many antioxidants have been tested to treat pulmonary oxidative stress with modest protection in animals and humans (17). However, the protective action of Ad, a naturally present adipokine in circulation and tissues, on the hyperoxic lung and vascular damage has not been reported to date. Hence, in the current study we investigated to determine whether (i) exogenous Ad would protect against the hyperoxia-induced barrier dysfunction in the lung endothelial cells (ECs) *in vitro* and (ii) endogenously synthesized Ad would protect against hyperoxic lung injury in wild type (WT) and Ad-overexpressing transgenic (AdTg) mice *in vivo*. The results demonstrated that exogenous Ad protected against the hyperoxia-induced oxidative stress and barrier dysfunction in the lung ECs *in vitro*. Furthermore, the hyperoxia-induced lung injury, vascular leak, and lipid peroxidation were significantly attenuated in AdTg mice *in vivo*.

MATERIALS AND METHODS

Materials

Bovine pulmonary artery endothelial cells (BPAECs) (passage 2) were obtained from Cell Applications Inc. (San Diego, CA). Fetal bovine serum (FBS), trypsin, minimum essential medium (MEM), phosphate-buffered saline (PBS), and non-essential amino acids were obtained from Gibco Invitrogen Corp. (Grand Island, NY). T-octylphenoxypolyethoxyethanol (Triton X-100), bovine serum albumin (BSA), 36.5% formaldehyde solution, 2', 7'-Dichlorofluorescein diacetate (DCFDA), fluorescein isothiocyanate (FITC)-dextran were obtained from Sigma Chemical Co. (St. Louis, MO). Mouse anti-ZO-1 and occludin antibody, AlexaFluor 488, AlexaFluor 568, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), and rhodamine-phalloidin were purchased from Invitrogen Co. (Carlsbad, CA). Polyoxyethylene sorbitan monolaurate (Tween-20) and 10× Tris-buffered saline (TBS) were obtained from Bio-Rad Laboratories (Hercules, CA). Glutathione (GSH) assay kit (GSH-Glo) was obtained from Promega Corporation (Madison, WI). Recombinant mouse Ad was obtained from BioVision Inc. (Mountain View, CA). Modular incubator chamber was obtained from Billups-Rothenburg Inc. (Del Mar, CA). Ad enzyme-linked immunoassay (ELISA) kit was obtained from R & D Systems Inc. (Minneapolis, MN). Rabbit polyclonal antibody raised against (E)-4-Hydroxynonenal [anti-HNE PAb] was obtained from Enzo Life Sciences International, Inc. (Farmingdale, NY). Evans blue dye and all other reagents of high purity were obtained from Sigma Chemical Company (St. Louis, MS). Anti-HNE Michael adducts antibody was obtained from CalBiochem (San Diego, CA). Lipid peroxidation assay kit was obtained from Oxford Biomedical Research Inc. (Oxford, MI).

Cell culture

BPAECs were cultured in MEM supplemented with 10% FBS, non-essential amino acids, antibiotics, and endothelial growth factor as described previously (22, 23). Cells in culture were maintained at 37° C in a humidified environment of 5% CO₂ – 95% air and grown to contact-inhibited monolayers with typical cobblestone morphology. BPAECs, from passages 5 to 20 (75–90% confluence) were used in the experiments.

Normoxia and hyperoxia exposure of ECs

Mouse recombinant Ad was prepared in sterile medium according to the manufacturer's instructions (BioVision) and then diluted to desired concentrations in MEM. BPAECs were then pre-treated with MEM alone or with MEM containing Ad (0.1–1 µg/ml) for the desired lengths of time. Following the Ad pre-treatment, cells were exposed to normoxia (5% CO₂ – 95% air) or hyperoxia (5% CO₂ – 95% O₂) in presence or absence of Ad for the desired lengths of time (3–24 h). Hyperoxia condition (90% oxygen) was achieved by using the modular incubator chamber (Billups-Rothenburg).

WT and AdTg animals

The FVB wild-type (WT) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The Ad transgenic (TG) mice (FVB background) were a kind gift from Dr. Philipp E.

Scherer of the Touchstone Diabetes Center, University of Texas Southwestern Medical Center, and were subsequently bred at the Ohio State University animal facility. The AdTg colony has been maintained with proper genotyping of the mice (24). Male AdTg and FVB WT mice of 8 wk of age and free of murine-specific pathogens were used.

Hyperoxia exposure of animals

WT and AdTg mice ($n = 3$ or 6), C57BL/6/J or FVB, male or female mice, 6–8 weeks of age were acclimated in a custom-made Plexiglass chamber (dimensions: $31 \times 18.5 \times 17$ cm) for 48 h. These mice were exposed to normoxia (room air, RA) or hyperoxia (90% O_2) for the desired lengths of time. A 12 h light/dark cycle was regulated during the experiment. For hyperoxia exposure of the animals, a gas cylinder, providing 100% O_2 was connected to the chamber through a flow meter (flow rate: 3 L/min). Likewise, for the normoxia exposure of animals, a compressed air (room air) cylinder was also connected to the chamber through a flow meter (flow rate: 14 L/min). Mice had free access to fresh chow and water at all times during the entire length of the experiment. The O_2 concentrations inside the chambers were monitored continuously using a gas analyzer (OxyStar-100 oxygen monitor) and recorded using a computerized data acquisition system during the entire length of the experiment.

Tissue collection

At the end of the experiment, mice were sacrificed by carbon dioxide euthanasia. The chest cavity was exposed to allow for lung expansion, after which blood was immediately drawn from the heart for determination of plasma Ad levels. The recovered bronchoalveolar lavage (BAL) fluid was centrifuged at $4^\circ C$ and the cellularity was determined. BAL was also analyzed for Ad levels by using the ELISA kit. For the determination of the pulmonary thiol-redox status and Ad levels, lungs of WT and AdTg mice were removed, washed thoroughly with PBS, snap frozen in liquid nitrogen, homogenized, and sonicated in ice-cold PBS.

Histology of lung

Histological analysis of the lungs was done according to the published procedures (25, 26). Immediately following the carbon dioxide euthanasia, the lungs were inflated to total lung capacity with PBS. Following inflation, the right lungs was removed and fixed with 4% formaldehyde and for histological examination in paraffin-embedded sections. Hematoxylin- and eosin-stained tissue sections were examined under the light microscope at $100\times$ magnification and images were captured digitally. Two veterinary pathologists scored the randomly selected lung sections from 1 (slight) to 5 (severe) with reference to alveolar and interstitial edema, infiltration with neutrophils, hemorrhage, and epithelial/endothelial necrosis (27). Four to six individual lung samples from each normoxia- and hyperoxia-exposed groups were subjected to the histological analysis.

Bronchoalveolar lavage (BAL) fluid collection

Mouse lungs were inflated with 1 ml PBS, and bronchoalveolar lavage (BAL) fluid was subsequently extracted. The BAL fluid was then centrifuged at 1300 ref for 5 min. Supernatant was aspirated and stored at $-80^\circ C$ for further analysis.

Adiponectin (Ad) determination

Ad levels in serum, BAL, cells, and lung were determined according to our previously established procedure (28) utilizing the Ad ELISA kit (R & D Systems Inc. Minneapolis, MN).

Cell morphology examination

Morphological alterations in BPAECs were studied according to our previously published procedure (29). BPAECs grown in 35-mm dishes (90% confluence) following exposures to normoxia and hyperoxia without and with Ad treatment were examined under the Nikon Eclipse TE2000-S at 20× magnification and digital images were captured.

ROS determination in ECs

The extent of intracellular ROS formation in BPAECs exposed to normoxia and hyperoxia without and with Ad treatment was determined using our previously published DCFDA fluorescence method (15).

***In vitro* EC paracellular transport/leak assay**

The extent of EC paracellular transport/leak of FITC-dextran across BPAEC monolayers exposed to normoxia and hyperoxia without and with Ad treatment was determined according to the previously published procedure (30). BPAECs grown to 100% confluence on sterile inserts (0.4 µm) in 12-well culture plates were exposed to normoxia or hyperoxia for designated lengths of time. Following the exposures, FITC-dextran paracellular transport/leak across the BPAEC monolayer was determined at 1 h by measuring the fluorescence of FITC-dextran in the medium below the EC monolayer at 480 nm excitation and 530 nm emission.

Confocal fluorescence microscopy of actin cytoskeletal rearrangement in ECs

Actin cytoskeletal rearrangement (stress fibers) in ECs exposed to normoxia and hyperoxia without and with Ad treatment was studied by using the confocal fluorescence microscopy according to our previously published procedure (29). Actin stress fibers were visualized by staining the cells with rhodamine-phalloidin (1:50 dilution). Nuclei were visualized by DAPI staining. Fluorescence images were captured digitally under the Zeiss LSM 510 confocal/multiphoton microscope at 543 nm excitation and 565 nm emission under 63× magnification.

Confocal immunofluorescence microscopy of cytoskeletal and tight junction proteins and lipid peroxidation in ECs

Cytoskeletal reorganization (cortactin redistribution), tight junction proteins (ZO-1 and occludins), and lipid peroxidation [4-Hydroxynonenal (4-HNE) formation] in BPAECs exposed to normoxia and hyperoxia, without and with Ad treatment, were studied according our previously published procedure by using the confocal immunofluorescence microscopy (19, 29). ZO-1, occludin, cortactin, and 4-HNE in cells were visualized by staining with the anti-ZO-1, anti-occludin, anti-cortactin, and anti-4-HNE antibodies (1:200 dilution), respectively. Following the primary antibody treatment, cells were treated with AlexaFluor

568 (1:200 dilution) for visualization of ZO-1, occludin, and cortactin, and with AlexaFluor 488 (1:200 dilution) for visualization of 4-HNE. Nuclei were visualized by DAPI staining. Fluorescence images were captured digitally under the Zeiss LSM 510 confocal/multiphoton microscope at 568 nm excitation and 603 nm emission for the visualization of ZO-1, occludin, and cortactin and at 495 nm excitation and 519 nm emission for the visualization of 4-HNE, under 63× magnification.

GSH and thiols determination in ECs and lungs

BPAECs grown up to 90% confluence in 96 well plates were exposed to normoxia and hyperoxia without and with Ad treatment. At the end of the experiment, intracellular GSH levels were determined by using the GSH-Glo glutathione assay kit according to the manufacturer's recommendations (Promega Corp. Madison, WI). GSH levels in the lung homogenates from the WT and AdTg, in PBS, were determined using the GSH-Glo glutathione assay kit as described earlier. Total thiol levels in the lung homogenates were determined according our previously published method (23). The GSH and thiol levels of the ECs and lung tissue were normalized to their protein values.

***In vivo* lung vascular leak determination**

Following the exposure of mice to normoxia and hyperoxia, the animals were euthanized with CO₂ lungs were explanted, and the lung vascular leak was determined by the extravasation of Evans blue (2 g/100 ml stock) in the isolated perfused lung according to the published procedure (31, 32). Following the Evans blue perfusion, lungs were photographed and then homogenized. The homogenates were treated with two volumes of formamide for 18 h at 60° C, centrifuged at 12,000 × g for 30 min, and the absorbance of the supernatant was determined spectrophotometrically at 620 nm and 740 nm.

Lipid peroxidation assay in lungs

Following the hyperoxia exposure of the mice, lungs were removed and snap frozen in liquid nitrogen. Frozen lung samples were homogenized in 20 mM phosphate buffer (pH 7.4) containing 0.5 mM butylated hydroxytoluene. The homogenate was then analyzed for malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) as the index of lipid peroxidation, by using the lipid peroxidation assay kit (Oxford biomedical research group). The concentrations of MDA and HAE were expressed as μmoles/μg total protein.

SDS-PAGE and Western blotting analysis of 4-HNE Michael adducts

Lung tissue lysates preparation and SDS-PAGE and Western blotting analysis of proteins for 4-HNE Michael adducts (as an index of lipid peroxidation) were carried out according to the previously published procedure (19). Proteins were probed with the primary anti-HNE Michael adducts primary antibody (1:2000 dilution) and horseradish peroxidase-conjugated goat anti-rabbit (1:2000 dilution) secondary antibody and immunodetected with the enhanced chemiluminescence (ECL) reagents according to the manufacturer's recommendation (PerkinElmer, Waltham MA). The blots were digitally quantified.

Protein Determination

Protein was determined by the BCA protein assay (Thermo Scientific, Rockford, IL).

Statistical analysis of data

Standard deviation (SD) for each data point was calculated from triplicate samples. Data were subjected to one-way analysis of variance, and pair wise multiple comparisons was done by Dunnett's method with $P < 0.05$ indicating significance.

RESULTS

Adiponectin protects against hyperoxia-induced alterations in lung EC morphology

Alterations in cell morphology are widely used as an index of cytotoxicity, and therefore here we investigated whether a hyperoxia environment would alter the cell morphology and if Ad would protect against such alterations in ECs. Light microscopic images revealed that BPAECs treated with hyperoxia (24 h) exhibited loss of cell morphology, random enlargement of some cells, extensive formation of filipodia and lamellipodia, and loss of tight junctions in hyperoxia-exposed ECs. Treatment of BPAECs with Ad (0.1 $\mu\text{g}/\text{ml}$) protected against the hyperoxia-induced loss of cell morphology (Figs. 1A–1B). These results revealed that hyperoxia caused complete loss of cell morphology and Ad offered protection against such adverse effect in BPAECs.

Adiponectin attenuates hyperoxia-induced ROS production in lung ECs

Hyperoxia has been established to cause ROS production in ECs (15). Therefore, here we investigated whether Ad treatment would modulate the hyperoxia-induced ROS formation in lung ECs. Ad (0.05 $\mu\text{g}/\text{mL}$) treatment significantly attenuated the hyperoxia-induced ROS production in BPAECs at 3 h of exposure as compared to the same in the cells exposed to hyperoxia alone as revealed by the DCFDA fluorescence method (Fig. 2). These results demonstrated that Ad attenuated the hyperoxia-induced ROS production in lung ECs.

Adiponectin attenuates hyperoxia-induced lipid peroxidation in lung ECs

Membrane lipid peroxidation has been shown to be a key player in oxidative stress including the hyperoxic lung damage (16, 33). In the current study, one of the highly reactive products of lipid peroxidation, 4-hydroxynonenal (4-HNE), was measured using confocal immunofluorescence microscopy in order to determine if Ad would modulate its formation in BPAECs. Hyperoxia exposure for 24 h significantly induced the formation of 4-HNE in BPAECs. Treatment of cells with Ad (0.1 $\mu\text{g}/\text{ml}$) completely attenuated the hyperoxia-induced 4-HNE formation in BPAECs at 24 h of exposure as compared to the same in the cells exposed to hyperoxia alone (Figs. 3A–3B). These results established that Ad attenuated the hyperoxia-induced lipid peroxidation in lung ECs.

Adiponectin attenuates hyperoxia-induced paracellular permeability in lung ECs

Earlier experiments of this study revealed that hyperoxia induced ROS formation and caused the alterations of cell morphology in BPAECs, which were attenuated by Ad treatment. Also, it has been established that oxidative stress induces vascular EC paracellular

permeability and leak (19, 31). This led us to hypothesize that the hyperoxia would induce the paracellular permeability/leak (barrier dysfunction) in lung EC monolayer, which could be modulated by Ad treatment. Here, the results demonstrated that hyperoxic exposure for 24 h significantly induced the increase in the paracellular permeability of FITC-Dextran across the BPAEC monolayer, which was significantly attenuated by Ad (0.1 mg/ml) treatment (Fig. 4A). Under identical conditions, the corresponding protective effects of Ad on the cell morphology in the BPAEC monolayers exposed to hyperoxia were evident (Fig. 4B). These results showed that Ad protected against the hyperoxia-induced lung EC barrier dysfunction.

Adiponectin protects against hyperoxia-induced cytoskeletal reorganization in lung ECs

Cytoskeleton plays a central role in the regulation of cell size and shape and motility (34). Actin cytoskeletal reorganization in ECs under oxidative stress is known to contribute to barrier dysfunction in the lung vascular EC monolayers *in vitro* and in lung vasculature *in vivo* (32). Also, in the earlier experiments of the current study, Ad was shown to attenuate the hyperoxia-induced ROS production, lipid peroxidation, and paracellular transport in BPAECs. Hence, we investigated here whether hyperoxia would cause the actin cytoskeletal reorganization (stress fiber formation) in BPAECs and if Ad would protect against such EC response. Hyperoxic exposure for 24 h caused the formation of actin stress fibers in BPAECs, which was completely attenuated by Ad (0.1 µg/ml) treatment (Fig. 5A). Cortactin, an adaptor protein responsible for actin regulation (35), has been shown to be modulated by hyperoxia in lung ECs (20). Therefore, here, we investigated the cortactin redistribution in BPAECs exposed to hyperoxia for 24 h and furthermore examined whether Ad would modulate the cortactin response. Hyperoxia caused a marked and dense redistribution of cortactin around the cell periphery and nucleus in BPAECs, which was completely reversed by Ad (0.1 µg/ml) treatment (Fig. 5B). Overall, these results demonstrated that Ad protected against the hyperoxia-induced actin cytoskeletal reorganization in lung ECs.

Adiponectin protects against hyperoxia-induced tight junction alterations in lung ECs

Tight junctions are highly crucial for the cell-to-cell contact and maintenance of tight barrier in EC monolayer (36). Occludins and ZO-1, important tight junction proteins, are key players in the maintenance of cellular tight junctions (37). Also, in the earlier experiments of this study, it was revealed that hyperoxia induced the cytoskeletal rearrangement in BPAECs, which was attenuated by Ad. Therefore, here, we investigated whether hyperoxia would alter the distribution of ZO-1 and occludins and if Ad would attenuate such alteration. As shown in Fig. 6A, hyperoxia (24 h) caused marked disappearance of ZO-1 localized around the cell periphery which was restored by Ad (0.1 µg/ml) treatment. Hyperoxia (24 h) also caused the redistribution of occludins in BPAECs as compared to the same in the control untreated cells under identical conditions, which was attenuated by Ad (0.1 µg/ml) treatment (Fig. 6B). These results demonstrated that hyperoxia induced the alterations in tight junction proteins in lung ECs, which were protected by Ad.

Adiponectin protects against hyperoxia-induced GSH depletion in lung ECs

Earlier in this study, we showed that hyperoxia caused ROS formation and lipid peroxidation (oxidative stress) in BPAECs which were attenuated by Ad treatment. Oxidative stress and thiol-redox imbalance are known to go hand in hand (38). Therefore, here, we investigated whether hyperoxia would cause the depletion of intracellular GSH in ECs which would be modulated by Ad treatment. Hyperoxic exposure for 24 h caused a significant depletion of intracellular GSH in BPAECs as compared to the same in the cells exposed to normoxia under identical conditions (Fig. 7). Ad (0.1–1 µg/ml), significantly but not completely, attenuated the hyperoxia-induced loss of intracellular loss of GSH in BPAECs, even at the lowest tested dose. Upon increasing the concentration of Ad up to 1 µg/ml, no apparent enhancement of restoration of the hyperoxia-induced GSH loss attained at 0.1 µg/ml dose was observed. These results demonstrated that hyperoxia caused the loss of intracellular GSH in lung ECs which was protected by Ad treatment.

Hyperoxic lung injury is protected in adiponectin-overexpressing transgenic mice *in vivo*

Earlier *in vitro* experiments of the current study clearly established that hyperoxia induced cell morphology alterations, ROS formation, lipid peroxidation, loss of GSH, paracellular transport/leak, cytoskeletal reorganization, and barrier dysfunction in lung ECs, all of which were attenuated by exogenous addition of Ad. Having these *in vitro* findings as the premise, we investigated whether hyperoxia-induced lung damage *in vivo* would be attenuated in transgenic mice overexpressing Ad (AdTg). Histological analysis revealed that the lungs of WT and AdTg mice exposed to normoxia (room air) for 84 h showed similar morphology of bronchiole (Fig. 8A: I and II) and alveolar duct (Fig. 8B: I and II). However, the histological analysis of bronchiole (Fig. 8A: III) and alveolar duct (Fig. 8B: III) of lungs of WT mice exposed to hyperoxia for 84 h revealed extensive tissue damage (marked cellular necrosis, hemorrhage, and cellular infiltration) as compared to that in the bronchiole (Fig. 8A: IV) and alveolar duct (Fig. 8B: IV) of lungs of AdTg mice under identical conditions.

Neutrophil influx has been shown to play in the hyperoxic lung injury of the newborn (39). Also, we have reported that Ad inhibits NADPH oxidase-catalyzed superoxide generation in neutrophils (12.). Therefore, here, we investigated whether hyperoxia would induce neutrophil influx into the hyperoxic lung and if Ad would modulate that hyperoxic response. Using the Ly-6G neutrophil-specific antibody, we showed that hyperoxia (84 h) induced an increase of influx of neutrophils into the lung of WT animals as compared to that in the WT and AdTg mice exposed to normoxia (room air) under identical conditions (Fig. 8C: I, II, and III). However, the hyperoxia-induced increase in the neutrophil influx into the lung of AdTg mice was strikingly lower as compared to that in the WT mice under identical conditions (Fig 8C: III and IV). Overall, these results revealed that the hyperoxic-induced lung injury and neutrophil influx into lung were attenuated in the AdTg mice.

Hyperoxia-induced lung vascular leak is attenuated in adiponectin-overexpressing transgenic mice *in vivo*

In the current study, the earlier experiments revealed that Ad protected against the hyperoxia-induced lung EC paracellular permeability *in vitro* and the hyperoxic lung injury *in vivo* as observed in the histological examination, was markedly attenuated in the AdTg

mice. Therefore, here, we investigated whether the hyperoxia-induced lung vascular leak in the WT mice would be modulated in the AdTg mice overexpressing Ad. Our results revealed that the lung vascular leak induced by hyperoxia (72 h) in WT mice was significantly attenuated in the AdTg mice (Figs. 9A–9B). Also, the levels of Ad in BAL of the AdTg mice were significantly higher than that in the WT mice (Fig. 9C). These results demonstrated that the hyperoxia-induced lung vascular leak in the WT mice was attenuated in the AdTg mice which exhibited higher levels of Ad in BAL.

Hyperoxia-induced lipid peroxidation is attenuated in lungs of AdTg mice *in vivo*

From the earlier experiments of the current study, the formation of hyperoxia-induced lipid peroxidation product, 4-hydroxynonenal (4-HNE), which was attenuated by Ad in the lung ECs *in vitro* was observed. Therefore, here, we investigated whether lipid peroxidation in the lungs of WT mice exposed to hyperoxia (84 h) would be modulated in AdTg mice under identical conditions. Lipid peroxidation products (malondialdehyde, MDA, 4-hydroxyalkenals, HAE, and 4-HNE Michael adducts) were assayed here as the indices of lipid peroxidation (40, 41). Hyperoxia significantly induced the formation of higher levels of MDA and HAE in the lungs of WT animals as compared to the same in the WT animals exposed to normoxia (room air) under identical conditions. The hyperoxia-induced formation of MDA and HAE in the lungs of AdTg mice, under identical conditions, was significantly attenuated as compared to the same in the WT mice (Fig. 10A). The hyperoxia-induced formation of 4-HNE Michael adducts in the lungs of AdTg animals was markedly lower as compared to that in the lungs of WT mice under identical conditions (Fig. 10B). These results demonstrated that the hyperoxia-induced lipid peroxidation in the lungs *in vivo* was attenuated in the AdTg animals overexpressing Ad.

Lungs of AdTg mice have higher levels of GSH and thiols

GSH and other thiols contribute to the thiol-redox-regulated antioxidant protection against oxidative stress (42). Also, the earlier experiments of the current study showed that Ad protected the hyperoxia-induced loss of GSH in the lung ECs *in vitro* and the hyperoxia-induced lung and lung vascular damage were attenuated in the AdTg mice. Therefore, here, we determined the endogenous basal levels of GSH and total thiols in the lungs of WT and AdTg mice. The results revealed that the levels of both GSH and total thiols were significantly higher in the lungs of AdTg mouse as compared to the same in the WT mice (Figs. 11A–11B).

DISCUSSION

Hyperoxia exposure *in vivo* has been shown to cause severe lung and parenchymal inflammation, fibrin deposition, and pulmonary edema (13). Despite its potential to cause the lung damage, oxygen therapy is necessary for the survival of many patients. However, effective and safer strategies to counteract the oxygen toxicity during hyperoxic therapy are imminent. The utilization of *in vitro* lung EC system and *in vivo* mouse model, as used in the current study offered a platform for detailed insights into probing the biochemical and molecular mechanisms of protection against the hyperoxic lung injury by Ad. Therefore, we hypothesized that Ad would protect against the hyperoxic lung and vascular damage *in vitro*

and *in vivo*. Overall, for the first time, our study demonstrated that Ad protected against the hyperoxia-induced cell morphology alterations, ROS production, GSH loss, lipid peroxidation, barrier dysfunction, and cytoskeletal reorganization in ECs *in vitro*. On the other hand, our study also revealed that the hyperoxia-induced oxidative stress, lung vascular leak, and lung injury were attenuated in AdTg mice overexpressing Ad *in vivo*.

Among several adipokines secreted by the adipose tissue, Ad stands out as a unique metabolic regulator in normal physiological and pathophysiological conditions (1). Ad has pleiotropic actions including anti-diabetic, anti-atherogenic, and anti-inflammatory properties (43, 44). Ad has also been shown to decrease the expression of vascular cell adhesion molecules which are known to modulate endothelial inflammatory responses (8). Studies have also revealed that Ad inhibits oxidized low-density lipoprotein-induced ROS production in ECs (10). Earlier, we have reported that Ad inhibits the NADPH oxidase-catalyzed formation of ROS in human neutrophils (12). These studies prompted us to further explore the anti-oxidative actions of Ad in hyperoxic lung injury. Ad, exclusively synthesized by the adipocyte, exists in circulation (serum) as multimers ranging from low-molecular-weight (LMW) trimers to high-molecular-weight (HMW) dodecamers (Schema 1). The oligomers of Ad (hexamers and HMW species) are formed through the disulfide bond formation between individual homotrimers (45). Although experimental evidences reveal that the HMW complex is the most metabolically active Ad complex (45), the biological effects of LMW forms of Ad can not be ruled out. The molecular structure of Ad shows an N-terminal signal sequence, a variable domain, a collagen-like domain, and a C-terminal globular domain that is homologous to TNF- α (5). Knowledge of this structure has led to the development of a transgenic mouse model overexpressing Ad that contains the structural integrity of the circulating adipokine (24). This AdTg mouse model is unique in that Ad elevation is within the physiological levels (3-fold) and accurately recapitulates the changes in endogenous levels during maturation of the animal. Ad in this model is therefore under the same developmental control as in WT mice, except that the basal set-point is approximately 3-fold higher in the former. These AdTg mice were utilized in the current study as an *in vivo* model, with high circulating levels of Ad, in order to determine the modulation or protection of the hyperoxic lung injury by Ad.

Oxygen therapy can be life-saving for the critically ill patients with respiratory failure, but prolonged exposure to high concentrations of oxygen results in hyperoxic lung injury (14). Newborns and preterm infants, with respiratory distress, are often exposed to high concentrations of oxygen (hyperoxia) supported by mechanical ventilation that leads to infant chronic lung disease, which is also called bronchopulmonary dysplasia (46, 47). Clinical hyperoxic exposure likely leads to disturbances in the development of the alveoli (47). Mechanical ventilation along with hyperoxia is crucial in the treatment of patients with respiratory distress (48). One noteworthy feature of clinical hyperoxia therapy is the generation of ROS which contribute to the dysfunction and injury of lung (46–49). Hyperoxic lung injury involves both the lung epithelial and vascular endothelial cell damage mediated by ROS, oxidative stress, and intricate signaling cascades (48, 50). Oxygen toxicity, including that is encountered during hyperoxia therapy, is known to cause lipid peroxidation of lung, which further leads to the pulmonary tissue and vascular damage (16,

51). Membrane lipid peroxidation products such as MDA, 4-HNE, and 4-HAE arising from oxygen toxicity are known to activate cellular signaling leading to the tissue damage during hyperoxia (52–54). Hyperoxic lung is not an exception to this and has been shown to undergo peroxidative degradation of membrane lipids (16, 54). Along those lines, the current study revealed that hyperoxia induced lipid peroxidation (formation of MDA, 4-HNE, and 4-HAE) in lung ECs *in vitro* and lung tissue *in vivo*. Furthermore, the current study also demonstrated that Ad attenuated the hyperoxia-induced lipid peroxidation in lung ECs in culture and also AdTg mice overexpressing Ad exhibited decreased extent of lipid peroxidation *in vivo*. Earlier, we have reported that NAD[P]H oxidase is activated during hyperoxia in lung ECs leading to the generation of ROS through mitogen-activated protein kinase (MAPK) signaling (15). EC NAD[P]H oxidase, through the generation of ROS and oxidant signaling has been shown to be associated with physiological and pathophysiological states (55). Also, NAD[P]H oxidase has been demonstrated to play a critical role in the hyperoxic lung injury in mice (56). Oxidants have been established to cause EC barrier dysfunction and hyperpermeability (paracellular leak) regulated by MAPKs (21). Vascular EC permeability is regulated by cytoskeleton, tight junctions, and intricate signaling cascades (21, 57). EC hyperpermeability has been shown to be induced by oxidants including the ROS (58). Cytoskeleton (actin and cortactin) of pulmonary ECs is known to regulate the vascular EC function (34, 59). Pulmonary vascular endothelial barrier integrity and microvascular permeability have also been shown to redox-regulated (60). Lung microvascular EC dysfunction induced by ROS has been identified as a thiol-redox-sensitive and p38 MAPK-regulated phenomenon (61). Hyperoxia has also been reported to cause reorganization of actin cytoskeleton and cortactin in lung ECs that is associated with NADPH oxidase activation and ROS generation (20). Concurring with these reports, our current study revealed that (i) hyperoxia induced ROS generation, loss of GSH, lipid peroxidation, cytoskeletal rearrangement, and paracellular leak in lung ECs *in vitro* and (ii) lipid peroxidation, vascular leak, and tissue injury in lungs of mice *in vivo* (Schema 2). Furthermore, the current study also demonstrated that exogenous Ad protected against the hyperoxia-induced EC dysfunction as well as attenuation of the hyperoxia-induced lung tissue damage and vascular leak in AdTg mice overexpressing Ad *in vivo*. It is also conceivable to surmise that the observed protective effects of Ad against the hyperoxic *in vitro* EC damage and *in vivo* lung injury could be mediated by both the LMW and HMW complexes of Ad.

Thiol-redox has been established as a critical player in the protection against the cellular and tissue oxidative stress and injury. NAC has been shown to partly protect against the hyperoxic lung injury in guinea pigs (62). GSH supplementation attenuates the hyperoxia-induced lung injury in preterm rabbits (63). GSH of mitochondria has been identified to play a protective role in the pulmonary oxygen toxicity encountered in premature infants through GSH-dependent antioxidant systems (64). The current study clearly revealed that hyperoxia caused loss of GSH in lung EC *in vitro* which was attenuated by Ad treatment. Also, in the current study, elevated basal levels of GSH and total thiols were observed in the lungs of AdTg mice overexpressing Ad. This enhancement of thiol-redox status appeared to play a critical role in the protective action of Ad leading to the attenuation of hyperoxic lung and vascular dysfunction. Furthermore, our earlier report on the inhibition of NADPH oxidase-

catalyzed ROS generation in neutrophils by Ad (12) also prompted us to surmise the possible action of Ad in attenuating the hyperoxia-induced neutrophil activation in lungs, possibly leading to the protection against hyperoxic lung injury *in vivo*. However, the detailed mechanisms of how Ad offers modulation of neutrophil infiltration and behavior in hyperoxic lung injury warrants further investigation. Whether neutrophil infiltration in hyperoxic lung injury is a cause or effect, definitely, it should be given importance because it signifies lung inflammation and damage due to hyperoxia. On the other hand, neutrophil infiltration during hyperoxic lung injury is also indicative of lung microvascular leak during hyperoxia, which sets the stage for the infiltration of neutrophils in the lung tissue. Henceforth, the inflammation of the lung, the ROS formation, and the damage of lung could be initiated by neutrophil infiltration in hyperoxic lung. Nevertheless, as revealed from the current study, that the AdTg mice exhibited marked attenuation of lung injury and decreased neutrophil infiltration in lung during hyperoxia, highlighted the role of neutrophils in hyperoxic lung injury and its amelioration by high levels of Ad. Henceforth, it could be argued that Ad protects against hyperoxic lung injury by also suppressing the neutrophil infiltration and activity, which warrants further detailed study. This is also further supported by our earlier reported finding that Ad inhibits NADPH oxidase-catalyzed ROS production in human neutrophils. Ad has been shown to offer protection against the alcoholic liver steatosis through the Ad receptor action (Adipo R1/R2) and associated signaling mechanisms (65). Antidiabetic actions of Ad have been shown to be associated with the Ad receptors (66). Therefore, the antioxidant-modulating effects of Ad towards protection against the hyperoxic lung and vascular damage might be mediated by the Ad receptors and associated signaling cascades.

Findings of the current study may shed light on the therapeutic role of Ad in not only the hyperoxic lung injury, but also in the other inflammatory conditions affecting the lungs. Many antioxidants have been used to treat pulmonary oxidative stress, and administration of small antioxidants has shown modest protection against pulmonary oxidative stress in animals and humans (17). The antioxidant actions of NAC in model systems have been well established. However, its protection against oxygen toxicity such as the hyperoxic lung injury is questionable (67). Although antioxidant treatments have been shown to protect against the oxidant-mediated lung damage, the antioxidants used in clinical settings tend to act as pro-oxidants (e.g. NAC) that could possibly exacerbate the hyperoxic lung injury. Animal and human studies show that an effective delivery of antioxidant drugs to cells under oxidative stress is needed for improved protection (17). Ad, a naturally present adipokine in circulation and tissues, appears as a promising molecule to offer protection against the hyperoxic lung injury and vascular dysfunction through the attenuation of ROS generation and oxidative stress and the modulation of thiol-redox antioxidant system (Schema 3).

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Figure 1. Adiponectin protects against hyperoxia-induced alterations in lung EC morphology

[A] BPAECs were exposed to 24 h of normoxia and hyperoxia, following which cell morphology was examined under light microscope at 100 $\times$ magnification. The loss of cell morphology, random enlargement of some cells, extensive formation of filipodia and lamellipodia, and loss of tight junctions in hyperoxia-exposed ECs were evident. **[B]** Cells were exposed to normoxia and hyperoxia (24 h), in absence and presence of Ad (0.1 μ g/ml), and cell morphology was examined under light microscope at 63 $\times$. Protection of hyperoxia-induced cell morphology alternations and loss of tight junctions by Ad were shown.

Figure 2. Adiponectin attenuates hyperoxia-induced ROS production in lung ECs
 BPAECs, without/with Ad (0.05 µg/ml) pretreatment for 2 h, were subjected to normoxia or hyperoxia for 3 h in absence and presence of Ad (0.05 µg/ml). At the end of treatment, intracellular ROS production was determined by measuring the fluorescence of oxidized DCFDA. Data represent mean $\pm$ S.D. of three independent experiments. *Significantly different from normoxic control cells at $P = 0.05$. **Significantly different from hyperoxic cells at $P = 0.05$.

Figure 3. Adiponectin attenuates hyperoxia-induced lipid peroxidation in lung ECs

[A] BPAECs, without/with Ad (0.1 µg/ml) pretreatment for 2 h, were subjected to normoxia or hyperoxia (24 h) in absence and presence of Ad (0.1 µg/ml), following which the intracellular formation of 4-HNE was detected by confocal immunofluorescence microscopy under 63× magnification. Each micrograph is a representative of three independent experiments. [B] Fluorescence intensities of 4-HNE immunostaining in BPAECs were presented. Data represent mean ± S.D. of three independent experiments. *Significantly different from normoxic control cells at $P = 0.05$. **Significantly different from hyperoxic cells at $P = 0.05$.

Figure 4. Adiponectin attenuates hyperoxia-induced paracellular permeability in lung ECs

[A] BPAEC monolayers, without/with Ad (0.1 µg/ml) pretreatment for 2 h, were subjected to normoxia or hyperoxia (24 h) in absence and presence of Ad (0.1 µg/ml). At the end of treatment, the leak of FITC-dextran through the EC monolayer was measured fluorometrically. Data represent mean $\pm$ S.D. of three independent experiments. *Significantly different from normoxic control cells at $P = 0.05$. **Significantly different from hyperoxic cells at $P = 0.05$. **[B]** Light microscopy pictures captured under 20 $\times$ magnification reveal the morphology of BPAECs in monolayers under identical conditions. Each micrograph is a representative of three independent observations under identical conditions.

Figure 5. Adiponectin protects against hyperoxia-induced cytoskeletal reorganization in lung ECs

[A] Ad attenuates hyperoxia-induced actin cytoskeletal rearrangement (stress fiber formation) in BPAECs. BPAECs, without/with Ad (0.1 µg/ml) pretreatment for 2 h, were subjected to normoxia or hyperoxia (24 h) in absence and presence of Ad (0.1 µg/ml), following which actin cytoskeletal rearrangement (stress fibers) was visualized by rhodamine-phalloidin staining under confocal fluorescence microscope at 63× magnification. **[B]** Ad attenuates hyperoxia-induced cortactin rearrangement in BPAECs. BPAECs were subjected to normoxia or hyperoxia (24 h) under identical conditions as described before, following which cortactin was visualized by confocal immunofluorescence microscopy at 63× magnification. Each micrograph is a representative of three independent observations under identical conditions.

Figure 6. Adiponectin protects against hyperoxia-induced tight junction alterations in lung ECs

[A] Ad protection against hyperoxia-induced ZO-1 tight junction alterations in BPAECs. BPAECs, without/with Ad (0.1 µg/ml) pretreatment for 2 h, were subjected to normoxia or hyperoxia (24 h) in absence and presence of Ad (0.1 µg/ml), following which ZO-1 proteins were examined by confocal immunofluorescence microscopy at 63× magnification. [B] Ad protection against hyperoxia-induced occludin rearrangement in BPAECs. BPAECs were subjected to normoxia or hyperoxia (24 h) under identical conditions as described before, following which occludins were examined by confocal immunofluorescence microscopy at 63× magnification. Each micrograph is a representative of three independent observations under identical conditions.

Figure 7. Adiponectin protects against hyperoxia-induced GSH depletion in lung ECs

BPAECs (100% confluent in 96-well plates), following pre-treatment with MEM alone or MEM containing Ad (0.1, 0.5, and 1 μg/mL) for 2 h, were exposed to normoxia or hyperoxia (24 h) in absence and presence of Ad (0.1, 0.5, and 1 μg/mL). At the end of experiment, the intracellular GSH concentrations were determined by GSH-Glo assay. Data represent mean $\pm$ S.D. of three independent experiments. *Significantly different from normoxic control cells at $P = 0.05$. **Significantly different from hyperoxic cells at $P = 0.05$.

Figure 8. Hyperoxic lung injury is protected in adiponectin-overexpressing transgenic mice

Formalin fixed lung sections of WT and AdTg mice exposed to normoxia and hyperoxia (84 h) were stained with hematoxylin and eosin and Ly-6G neutrophil-specific antibody to examine the lung tissue histology and neutrophil infiltration, respectively. Images were captured under a light microscope at 100× magnification. The histology images of bronchiole [A: I–IV] alveolar duct [B: I–IV] and [C] neutrophil infiltration revealed that after hyperoxic exposure, lungs of WT mice showed more tissue damage with marked cellular necrosis, hemorrhage, and cellular infiltration and striking neutrophil infiltration into lungs as compared to that in AdTg mice. Each micrograph is a representative of three independent observations under identical conditions.

Figure 9. Hyperoxia-induced lung vascular leak is attenuated in adiponectin-overexpressing transgenic mice

WT and AdTg mice were exposed to hyperoxia (72 h), following which, *ex vivo* Evan's blue-albumin (EBA) extravasation in lungs was photographed and also determined spectrophotometrically. **[A]** Gross EBA extravasation was visualized in the lungs and attenuation of EBA leak in the lungs of hyperoxia-exposed AdTg mice was evident. **[B]** Spectrophotometric determination of EBA extravasation in lungs. Attenuation of EBA leak in lungs of AdTg mice was evident. **[C]** Ad levels were determined by ELISA assay in BAL fluid of WT and AdTg mice after exposure to hyperoxia (72 h). Data represent mean $\pm$ S.D. of three independent experiments. *Significantly different from WT animals exposed to hyperoxia at $P = 0.05$.

Figure 10. Hyperoxia-induced lipid peroxidation is attenuated in lungs of AdTg mice

[A] WT and AdTg mice were exposed to normoxia and hyperoxia (84 h), following which [A] the extent of lipid peroxidation as the formation of MDA and HAE in lungs was determined by spectrophotometric method. [B] Lipid peroxidation in lungs was also assessed by analyzing the formation of 4-HNE Michael adducts by SDS-PAGE and Western blotting. Data represent mean $\pm$ S.D. of three independent experiments. *Significantly different from WT mice exposed to normoxia at $P = 0.05$.

**Significantly different from WT mice exposed to hyperoxia at $P = 0.05$.

Figure 11. Lungs of AdTg mice have higher levels of GSH and thiols

Levels of GSH and total thiols in lungs of WT and AdTg mice were determined by spectrophotometric method and GSH-Glo assay. *Significantly different from WT animals at $P = 0.05$.

Schema 1.

Adipose-derived adipocytokines and their pleiotrophic effects. The background includes illustrations of Ad complexes. Low-mol. wt. (LMW) Ad dimer assembles into medium-mol. wt. (MMW) hexamers and high-mol. wt. (HMW) oligomers through disulfide linkage. Under reduction, MMW and HMW Ad complexes can dissociate into LMW Ad.

Schema 2.

Illustration of hyperoxia-induced generation of ROS by neutrophils, macrophages, lung epithelial cells, and vascular ECs, which contribute to oxidative stress-mediated (lipid peroxidation) lung vascular EC barrier dysfunction, leak, and injury.

Schema 3.

Schematic representation of probable mechanism of Ad-mediated protection against hyperoxic lung vascular leak and injury through modulation of thiol-redox status, antioxidant enzyme activity, protective signaling cascades, and modulation of oxidative stress.