Original Research

Quercetin supplementation attenuates airway hyperreactivity and restores airway relaxation in rat pups exposed to hyperoxia

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Impact statement

Prolonged hyperoxic exposure of immature lungs contributes to lung injury and is manifested by increased airway hyperreactivity, features that characterize bronchopulmonary dysplasia. Treatments for airway hyperreactivity among preterm survivors are ineffective. Here, we provide the evidence on protective effect of quercetin against neonatal hyperoxia-induced airway hyperreactivity and lung inflammation. The findings of this study make quercetin as a compound that could be considered to use as a new therapy in treatment of airway hyperreactivity.

Abstract

Hyperoxia exposure of immature lungs contributes to lung injury and airway hyperreactivity. Up to now, treatments of airway hyperreactivity induced by hyperoxia exposure have been ineffective. The aim of this study was to investigate the effects of quercetin on hyperoxia-induced airway hyperreactivity, impaired relaxation, and lung inflammation. Newborn rats were exposed to hyperoxia ($FiO_2 > 95\%$) or ambient air (AA) for seven days. Subgroups were injected with quercetin ($10\,mg \cdot kg^{-1} \cdot day^{-1}$). After exposures, tracheal cylinders were prepared for *in vitro* wire myography. Contraction to methacholine was measured in the presence or absence of organ bath quercetin and/or *N*ω-nitro-L-arginine methyl ester (L-NAME). Relaxation responses were evoked in preconstricted tissues using electrical field stimulation (EFS). Lung tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β) levels were measured by enzyme-linked immunosorbent assay (ELISA). A $P < 0.05$ was considered statistically significant. Contractile responses of tracheal smooth muscle (TSM) of hyperoxic animals were significantly increased compared with AA animals ($P < 0.001$). Treatment with quercetin significantly reduced contraction in hyperoxic groups compared with hyperoxic control ($P < 0.01$), but did not have any effect in AA groups. In hyperoxic animals, relaxation of TSM was significantly reduced compared with AA animals ($P < 0.001$), while supplementation of quercetin restored the lost relaxation in hyperoxic groups. Incubation of preparations in L-NAME significantly reduced the quercetin effects on both contraction and relaxation ($P < 0.01$). Treatment of hyperoxic animals with quercetin significantly decreased the expression of TNF-α and IL-1β compared with hyperoxic controls ($P < 0.001$ and $P < 0.01$, respectively).

The findings of this study demonstrate the protective effect of quercetin on airway hyperreactivity and suggest that quercetin might serve as a novel therapy to prevent and treat neonatal hyperoxia-induced airway hyperreactivity and inflammation.

Keywords: Bronchopulmonary dysplasia, hyperoxia, airway hyperreactivity, quercetin, IL-1β, TNF-α

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Introduction

Bronchopulmonary dysplasia (BPD) is a major cause of morbidity and mortality of premature infants.[1] Oxygen therapy as a part of neonatal care contributes to neonatal lung injury characterized by alveolar simplification and airway hyperreactivity which are main features of BPD.[2] In normal conditions, there is a balance between contractile and relaxant processes of airway smooth muscle (ASM), and disturbance of this balance leads to airway hyperreactivity – a persistent phenotype of BPD patients.[3]

Exposure of newborn rodents to hyperoxia represents an established model that reproduces the BPD phenotype including airway hyperreactivity and alveolar simplification, and also induces inflammation.[4–7] Previously, we have shown that hyperoxia decreases relaxant responses due to reduced nitric oxide (NO) production, a major component of bronchodilation.[5,8] Reduced relaxant responses of ASM in an experimental model of BPD were associated with increased contractile responses which involved upregulation of Ca^{2+} sensitization signaling.[4] Moreover, neonatal hyperoxia induces upregulation of proinflammatory cytokines

such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β). Lung inflammation considered to be a risk factor for development of BPD and also contributes to increased, airway hyperresponsiveness in rats.[7,9–11] Although there are many known pathophysiological mechanisms that lead to airway hyperreactivity, treatments for airway hyperreactivity among preterm survivors are surprisingly ineffective. Therefore, a therapy that could prevent neonatal hyperoxia-induced airway hyperreactivity and lung injury is imperative.

Quercetin, a natural flavonoid, is widely distributed in plant products such as red wine, fruits, and vegetables.[12] Quercetin has attracted a significant attention in both scientific and community due to its pharmacological activities that have been reported. It has been shown that quercetin could relax human-isolated bronchus, and potentiates β-agonist-induced relaxation of ASM.[13,14] In addition, quercetin exerts many beneficial health effects, such as attenuation of lung injury, improvement of cardiovascular health, and decrease in blood pressure.[15,16] In rat aortic ring segments, quercetin showed both endothelium-dependent and endothelium-independent vasodilatory effect. The endothelium-dependent pathway involves increase in NO production and subsequently increased cyclic-guanosine-monophosphate (cGMP) levels, while the endothelium-independent pathway involves increasing levels of cGMP via inhibition of phosphodiesterase 4 (PDE4) activity.[17,18] However, the effect of quercetin on restoring the balance between relaxant and contractile responses of ASM under hyperoxic condition remains unstudied. We hypothesized that quercetin supplementation will reverse the airway hyperreactivity and restore impaired relaxation induced by neonatal hyperoxia. We also hypothesized that quercetin will prevent the hyperoxia-induced upregulation of proinflammatory cytokines.

Materials and methods

Animals and experimental design

Wistar-Rattus norvegicus pups (P4) from two different litters were randomly mixed and assigned to either hyperoxia ($FiO_2 > 95\%$) or AA groups for seven days. Food and water were available *ad libitum* for mothers, and a 12 h on/12 h off light cycle was maintained. Hyperoxic groups were housed with their mothers in a commercial rat cage placed into a plexiglass box (38 L) with continuous O_2 (2 L/min), and its concentration was monitored with an oximeter (MiniOX-1, Ohio Medical Corp, IL, USA). To avoid hyperoxic toxicity, mothers were swapped each day between groups. Subsets from each group were treated by intraperitoneal (i.p.) injection with quercetin ($10\ mg \cdot kg^{-1} \cdot day^{-1}$; Sigma, Germany) during exposure time. Control animals received equal volumes of saline as vehicle. Both sexes were included in this study. This study was conducted in compliance with the rules described in guidelines for the use of laboratory animals, and the protocol was approved by the ethical research committee and doctoral studies of the Faculty of Medicine, University of Prishtina where the study was performed.

TSM preparation

After exposures, animals were euthanized with CO_2, and the trachea was removed and freed of serosal connective tissue in an ice-cold oxygenated Krebs–Henseleit (KH) buffer (mM: 118.2 NaCl, 25 $NaHCO_3$, 4.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, and 10% D-glucose, pH = 7.4; [all from Sigma-Aldrich, Germany]). Rings of 3 mm length were isolated from the mid-trachea and transferred into a tissue-organ bath containing KH buffer (10 mL) at 37°C, for *in vitro* force contraction measurements, as previously described.[4]

Tracheal rings were suspended between a stainless-steel wire triangle at the bottom of the tissue bath and a force-displacement transducer. TSM tension was measured by the four-channel organ bath system (DMT-750TOBS, Danish Myo Technology, Denmark) connected to Power Lab/8SP (AD Instruments Inc., CO, USA) and interfaced with computer-monitored recordings (Chart 8.0 software). TSM tension was expressed in grams (g). An initial load of 0.3 g was applied and then equilibrated for 45 min. During the equilibration time, KH buffer was changed every 15 min and continuously aerated with a 95% O_2 + 5% CO_2 gas mixture.

Methacholine-induced contraction of TSM

Airway reactivity was studied by constructing a dose–response curve to methacholine (MCh, 10^{-8}–10^{-4} M; Sigma-Aldrich, Germany). After recording the control responses of TSM to MCh, the tracheal rings were rinsed three to four times every 5 min with warm KH solution until the TSM tension returned to baseline and then were allowed to rest for an additional 45 min. During the equilibration time, preparations were incubated in a single concentration of quercetin (100 μM) for 10 min before a second dose–response curve to MCh was recorded. In addition, the effect of *in vivo* supplementation of animals with quercetin on contractile responses to MCh was studied in TSM obtained from animals treated with quercetin or vehicle.

To explain the mechanisms of quercetin effects to counteract the ASM hyperreactivity, tracheal rings in organ baths were incubated with a nitric oxide synthase (NOS) inhibitor – L-NAME (100 μM) *alone* for 30 min or co-incubated with L-NAME and quercetin (100 μM), followed by MCh challenging. All records for every MCh dose–response for every condition were obtained in duplicate.

EFS-induced relaxation of TSM

To show the effect of hyperoxia on relaxant responses of TSM, tracheal rings obtained from both hyperoxia- and AA-exposed animals were placed in organ baths as described above. After equilibration, a cumulative dose–response curve of bethanechol (Sigma-Aldrich, Germany) induced contraction in TSM was built, followed by washing outs. A concentration 3×10^{-5} μM of bethanechol was found to be the optimal dose to elicit 75% of maximal response. Following the equilibration time, TSM was preconstricted using a single concentration of bethanechol (3×10 μM); then, incremental EFS was applied through platinum electrodes (5–60 V alternating current [AC] at 50 Hz) for 10 s at 2-min intervals to induce relaxation. The relaxation of the TSM was

expressed as percentage (%) of preconstricted level for each preparation. In addition, in subsets of TSM after the first recordings of EFS-induced relaxation responses, followed by washing outs, and equilibration, tracheal rings in organ baths were incubated in a single concentration of quercetin (100 μM) for 10 min, or L-NAME (100 μM) for 30 min, or co-incubated with L-NAME and quercetin; then, EFS was applied in preconstricted TSM.

TNF-α and IL-1β ELISA assay

Lung tissue obtained from quercetin or vehicle-treated animals exposed to hyperoxia or AA were homogenized, and concentrations were measured in duplicate with commercially available ELISA assay kits for TNF-α and IL-1β (BMS622 and BMS630, respectively) (Bender MedSystems GmbH, Vienna, Austria) according to the manufacturer's instructions. The plate was read at 450 nm wavelength with a spectrophotometer (Bio Rad Laboratories, CA, USA), and the results were expressed as pg/mg protein.

Statistical analysis

Results are expressed as mean ± SEM. Statistical significance was determined by two-way analysis of variance (ANOVA) with repeated measurements for TSM responses with post hoc Tukey–Kramer multiple comparison test for group and dose differences. For TNF-α and IL-1β levels, one-way ANOVA was used to determine statistical significance. A $P < 0.05$ was considered statistically significant.

Results

Effect of quercetin on the contraction of TSM

MCh induced a dose–response contraction of TSM, and the neonatal exposure to hyperoxia significantly increased contractile responses ($n = 10$) compared with AA (overall, $P < 0.001$; $n = 10$) (Figure 1(a)). The TSM responses in hyperoxic rat pups were significantly higher at concentrations $10^{-5.5}$–10^{-4} M of MCh, and the maximal values of responses in hyperoxic and AA groups were 1.93 ± 0.12 and 1.28 ± 0.17 g, respectively (Figure 1(a)).

Incubation of tissues with quercetin significantly reduced contractile responses in hyperoxic TSM toward MCh compared with the responses obtained in the absence of quercetin (overall, $P < 0.001$; $n = 10$) (Figure 1(b)). The difference was significant at concentrations 10^{-6}–10^{-4} M of MCh, and the maximal contractile force in hyperoxic incubated tissue with quercetin was 0.94 ± 0.15 g. Although the presence of quercetin showed a tendency to reduce the contraction of TSM toward MCh in AA animals, the difference was not significant compared with AA controls ($P = 0.334$; $n = 10$) (Figure 1(b)). The maximal contractile force in AA control and AA-quercetin was 1.21 ± 0.16 and 0.97 ± 0.13 g, respectively.

In vivo supplementation of animals with quercetin during hyperoxic exposure prevented airway hyperreactivity. The contractile responses of TSM were significantly decreased in supplemented hyperoxic animals compared with hyperoxic controls ($P < 0.001$; $n = 7$, each condition) (Figure 1(c)). Supplementation of quercetin normalized the responses in hyperoxic animals such that they did not significantly differ from AA control ($P = 0.514$). The maximal values of responses

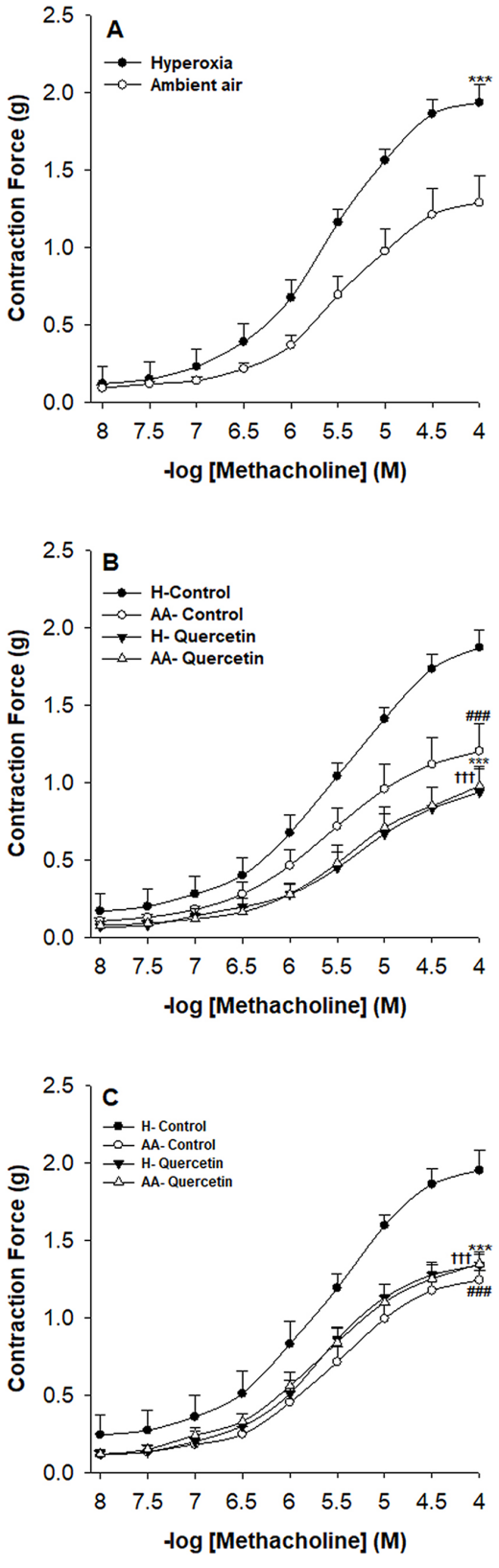

Figure 1. The effect of *in vitro* and *in vivo* quercetin supplementation on methacholine-induced contraction of TSM of rat pups: (a) contractile responses of TSM in hyperoxic and AA control groups (***$P < 0.001$; $n = 10$, each condition), (b) contractile responses of TSM obtained from hyperoxic and AA groups in the absence or presence of quercetin supplemented *in vitro* (100 μM) ($n = 10$, each condition), and (c) contractile responses of TSM obtained from hyperoxic and AA groups supplemented with quercetin *in vivo* (10 mg·kg^{-1}·day^{-1}) ($n = 7$, each condition). (***$P < 0.001$ – *H-quercetin versus H-control;* †††$P < 0.001$ – *AA-quercetin versus H-control;* ###$P < 0.001$ – *AA control versus H-control*). H: hyperoxia; AA: ambient air. Values are mean ± SEM.

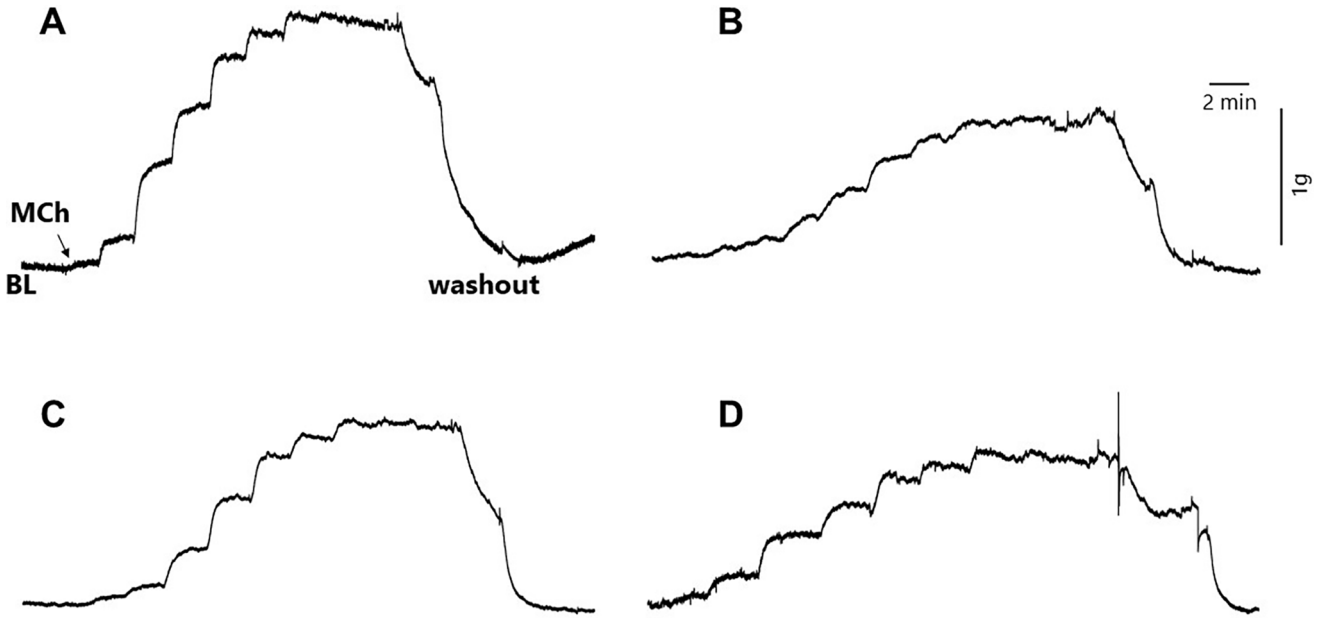

Figure 2. Tracings of representative recordings showing the methacholine-induced TSM contraction: (a) H-control, (b) H-quercetin, (c) AA control, and (d) AA-quercetin. MCh: methacholine; BL: baseline.

in hyperoxic groups treated with vehicle or quercetin were 1.95 ± 0.13 and 1.34 ± 0.08 g, respectively. The contractile responses in supplemented AA animals with quercetin didn't significantly change compared with AA controls ($n = 7$, each condition) (Figure 1(c)). The maximal values of contractile responses in AA groups treated with vehicle or quercetin were 1.24 ± 0.10 and 1.35 ± 0.10 g, respectively. The tracings of representative TSM contractile responses to MCh are presented in Figure 2.

Effect of NOS inhibition on contractile responses of TSM supplemented with quercetin

Inhibition of NOS by L-NAME did not affect the contractile responses of TSM in the hyperoxic control group, but altered the effect of quercetin. The presence of L-NAME reduced the effect of quercetin, such that the contractile responses in TSM preparations from hyperoxic animals supplemented with quercetin in the presence of L-NAME were significantly higher compared with those when quercetin was administered alone ($P < 0.05$; $n = 11$, each condition) (Figure 3(a)). The difference was significant at concentrations 10^{-6}–10^{-4} M of MCh, and the maximal values of responses in hyperoxia-quercetin + L-NAME and hyperoxia-quercetin were 1.25 ± 0.15 and 1.00 ± 0.12 g, respectively. Even though the inhibition of NOS reduced the protective effect of quercetin on airway hyperreactivity, it did not abrogate, and again, the contractile responses in the hyperoxia-quercetin + L-NAME group were significantly lower compared with both hyperoxia control and hyperoxia + L-NAME ($P < 0.05$; $n = 11$, each condition) (Figure 3(a)). The difference was significant at concentrations $10^{-5.5}$–10^{-4} M of MCh.

NOS inhibition significantly increased contractile responses in the AA + L-NAME group compared with both AA control and AA-quercetin ($P < 0.01$; $n = 6$, each condition) (Figure 3(b)), and the maximal values of responses were 1.69 ± 0.16, 1.05 ± 0.08, and 1.06 ± 0.13 g, respectively. The difference was significant at concentrations $10^{-6.5}$–10^{-4} M of MCh. Inhibition of NOS enzyme dominated the effect of quercetin in AA conditions, such that contractile responses of TSM incubated with L-NAME regardless of quercetin (AA-quercetin + L-NAME) were significantly enhanced compared with both controls (AA control) and those incubated with quercetin (AA-quercetin) ($P < 0.01$; $n = 6$, each condition) (Figure 3(b)). The difference was significant at concentrations 10^{-5}–10^{-4} M of MCh, and the maximal contractile force in the AA-quercetin + L-NAME group was 1.49 ± 0.11 g.

Effect of quercetin on hyperoxia-impaired relaxation of TSM

Neonatal hyperoxic exposure significantly decreased the EFS-induced relaxation of TSM compared with AA controls (overall, $P < 0.001$; $n = 9$ and $n = 6$, respectively). The relaxation was significantly reduced at 20–60 V, and the maximal relaxant responses in hyperoxic and AA control groups were 19.75 ± 1.15 and $46.54 \pm 3.39\%$, respectively (Figure 4(a)). *In vitro* quercetin supplementation restored the impaired relaxation in hyperoxic TSM. The relaxant responses induced by EFS were significantly increased compared with hyperoxic controls ($P < 0.001$; $n = 9$). The differences were significant at 10–60 V, and the maximal value of relaxation in hyperoxic preparations treated with quercetin was $73.23 \pm 3.53\%$ (Figure 4(b)). Inhibition of NOS by L-NAME didn't have any effect in relaxation of TSM in hyperoxic exposed rat pups when administered alone, but diminished the relaxant effects of quercetin. The EFS-induced relaxation of TSM was significantly reduced in hyperoxic preparations incubated in L-NAME and quercetin (hyperoxia-quercetin + L-NAME) compared with hyperoxia-quercetin without L-NAME ($P < 0.001$; $n = 9$, each condition), and the difference

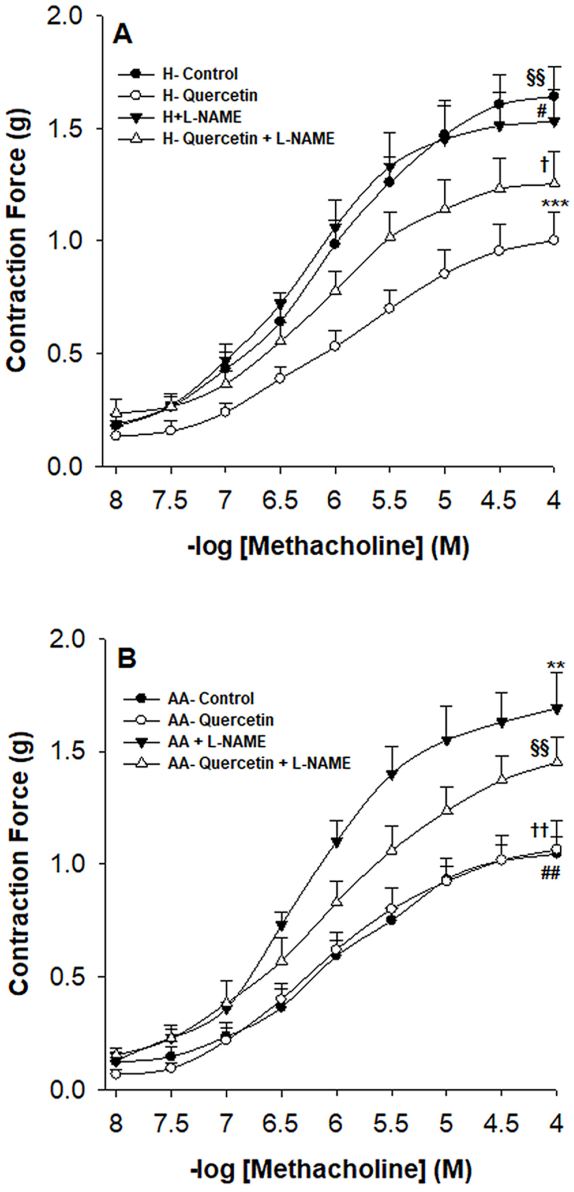

Figure 3. Effect of NOS blockage on quercetin-reversed airway hyperreactivity: (a) contractile responses of TSM in hyperoxia-exposed rat pups in the absence or presence of L-NAME (100 μM) and/or quercetin (100 μM) ($n = 11$, each condition; $^{***}P < 0.001$ – *H-quercetin versus H + L-NAME/H-control;* $^{\dagger}P < 0.05$ – *H-quercetin + L-NAME versus H-quercetin;* $^{\S\S}P < 0.01$ – *H-control versus H-quercetin + L-NAME*; $^{\#}P < 0.05$ – *H + L-NAME versus H-quercetin + L-NAME*). (b) Contractile responses of TSM of AA-exposed rat pups in the absence or presence of L-NAME (100 μM) and/or quercetin (100 μM) ($n = 6$, each condition; $^{**}P < 0.01$ – *AA + L-NAME versus AA control;* $^{\S\S}P < 0.01$ – *AA-quercetin + L-NAME versus AA control;* $^{\#\#}P < 0.01$ – *AA-quercetin versus AA + L-NAME;* $^{\dagger}P < 0.05$ – *AA-quercetin versus AA-quercetin + L-NAME.* H: hyperoxia; AA: ambient air. Values are mean ± SEM.

became significant at 10–60 V (Figure 4(b)). The maximal value of relaxation in hyperoxia-quercetin + L-NAME was 52.55 ± 3.5%. Despite NOS inhibition attenuation of the relaxant effect of quercetin, the measured relaxation in hyperoxia-quercetin + L-NAME was significantly higher compared with hyperoxia control and hyperoxia + L-NAME (overall, $P < 0.001$).

Quercetin supplementation didn't have any effect on EFS-induced relaxation of TSM in the AA group, and the maximal value of relaxation in quercetin-supplemented preparations in AA group was 48.50 ± 1. 55%. Inhibition of NOS significantly reduced the relaxation compared

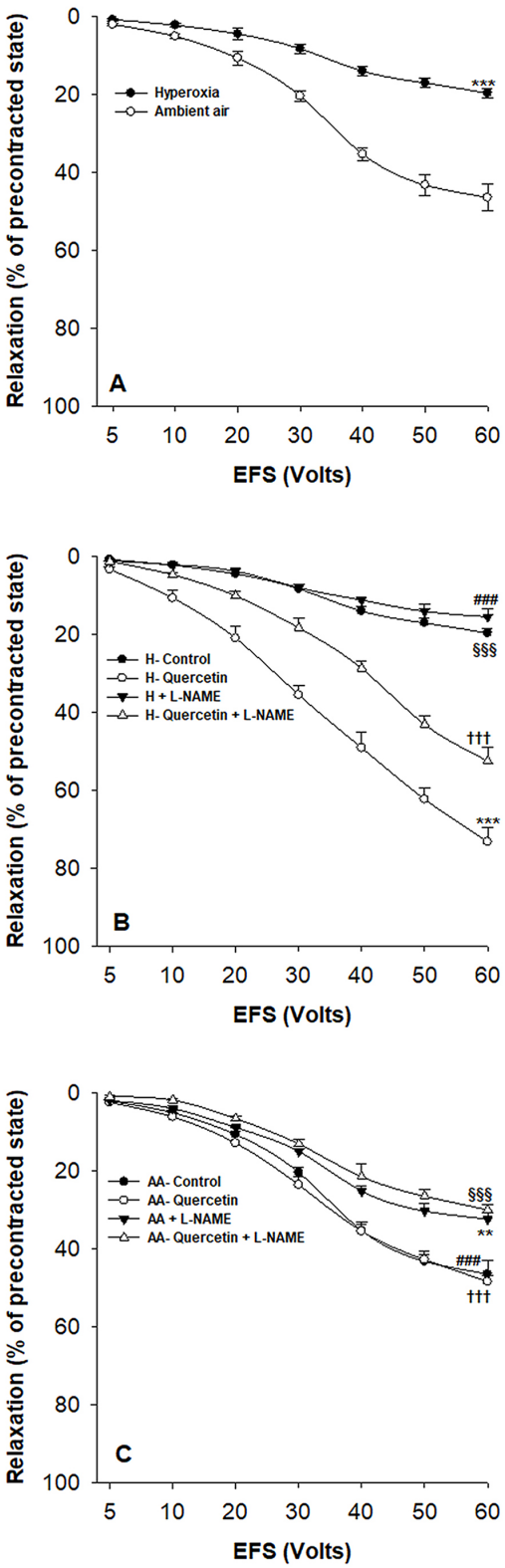

Figure 4. Effect of *in vitro* quercetin supplementation and NOS inhibition on EFS-induced relaxation of TSM of rat pups: (a) relaxant responses of TSM in hyperoxic and AA control groups ($^{***}P < 0.001$; $n = 9$ and $n = 6$, respectively) and (b) relaxant responses of TSM of hyperoxia-exposed rat pups in the absence or presence of quercetin (100 μM) and/or L-NAME (100 μM) ($n = 9$, each condition; $^{***}P < 0.001$ – *H-quercetin versus H-control/H + L-NAME;* $^{\dagger\dagger\dagger}P < 0.001$ – *H-quercetin + L-NAME versus H-quercetin;* $^{\S\S\S}P < 0.001$ – *H-control versus H-quercetin + L-NAME*; $^{\#\#\#}P < 0.001$ – *H + L-NAME versus H-quercetin + L-NAME*). (c) Relaxant responses in AA groups in the absence or presence of quercetin (100 μM) and/or L-NAME (100 μM) ($n = 6$, each condition; $^{**}P < 0.01$ – *AA + L-NAME versus AA control;* $^{\S\S\S}P < 0.001$ – *AA-quercetin + L-NAME versus AA control;* $^{\#\#\#}P < 0.001$ – *AA-quercetin versus AA + L-NAME;* $^{\dagger\dagger\dagger}P < 0.001$ – *H-quercetin versus AA-quercetin + L-NAME.* H: hyperoxia; AA: ambient air. Values are mean ± SEM.

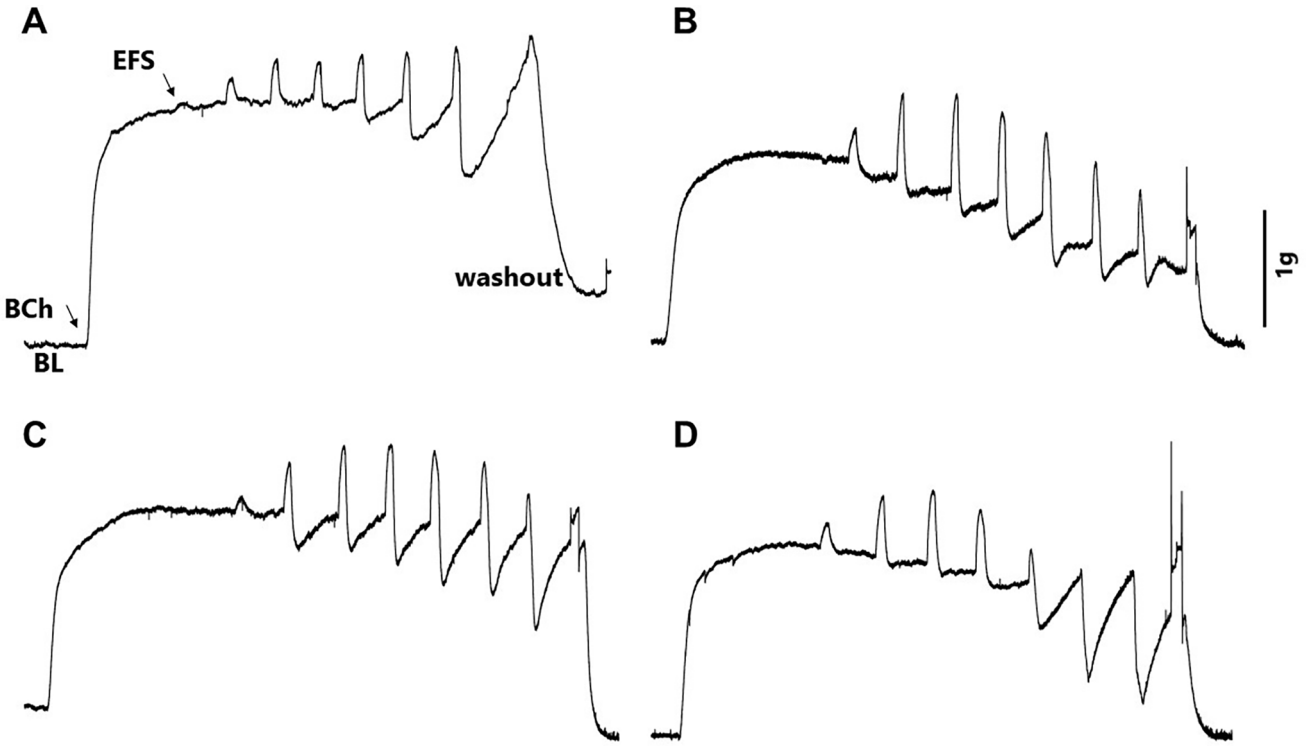

Figure 5. Tracings of representative recordings showing the EFS-induced relaxation of preconstricted TSM: (a) H-control, (b) H-quercetin, (c) AA control, and (d) AA-quercetin. BCh: bethanechol, BL: baseline.

with both AA control and AA-quercetin (overall, $P < 0.01$ and $P < 0.001$, respectively; $n = 6$) (Figure 4(c)). The difference became significant at 30–60 V, and maximal values of responses in AA-quercetin + L-NAME and AA + L-NAME were 30.10 ± 1.5 and 32.50 ± 1.8 %, respectively. The tracings of representative recordings of EFS-induced TSM relaxation are presented in Figure 5.

Effect of quercetin on TNF-α and IL-1β in lung tissue exposed to hyperoxia

Neonatal hyperoxic exposure significantly increased expression of TNF-α compared with AA-exposed animals ($P < 0.001$; $n = 6$, each group). The mean values of TNF-α levels in lung tissue were 45.9 ± 2.5 and 28.8 ± 2.0 pg/mg, respectively. Supplementation of animals with daily quercetin during hyperoxic exposure significantly reduced expression of TNF-α ($P < 0.001$), but didn't have a significant effect in AA-exposed animals ($n = 6$, each group) (Figure 6(a)). The mean values of TNF-α levels in lung tissue were 26.6 ± 2.9 and 25.3 ± 1.9 pg/mg, respectively.

Hyperoxia also significantly increased expression of IL-1β levels in lung tissue compared with AA-exposed animals ($P < 0.01$; $n = 6$, each group), and supplementation of animals with daily quercetin during hyperoxic exposure reduced expression of IL-1β ($P < 0.01$), while didn't have effect on expression in AA-exposed animals ($n = 6$, each group) (Figure 6(b)). The mean values of IL-1β levels in lung tissue of hyperoxic animals treated with vehicle or quercetin as well as in AA-exposed animals treated with vehicle or quercetin were 98.8 ± 8.1, 66.3 ± 6.8, 62.1 ± 3.5, and 59.7 ± 2.8 pg/mg, respectively.

Discussion

To the best of our knowledge, this is the first study to report the protective effect of quercetin against neonatal hyperoxia-induced airway hyperreactivity. An experimental model of BPD was developed in rat pups by hyperoxic exposure. In this study, quercetin-supplemented *in vitro* or *in vivo* reduced ASM reactivity to MCh. In addition, hyperoxia-impaired EFS-induced relaxation of ASM, while *in vitro* supplementation with quercetin completely restored the lost relaxant responses.

Inhibition of NOS attenuated quercetin benefits, indicating the NO-cGMP signaling pathway is involved in the quercetin mechanisms of action either to inhibit contractility or to promote relaxation of TSM. Furthermore, hyperoxia increased expression of proinflammatory cytokines – TNFα and IL-1β in lung tissue. Supplementation of animals with quercetin during hyperoxia exposure prevented this increase in levels of TNFα and IL-1β in lungs.

BPD remains an important complication of premature birth with long-term consequences.[19] The pathogenesis of BPD is multifactorial, with hyperoxia as a key factor that is associated with airway hyperreactivity.[20,21] While oxygen therapy remains clinically important for preterm survival, finding new therapies to treat or prevent airway hyperreactivity is imperative. Consistent with previous studies, here we demonstrate that neonatal hyperoxia leads to airway hyperreactivity. The airway hyperreactivity due to hyperoxia involves Ca^{2+} sensitization via upregulation of Rho/Rho-kinase signaling which potentiates contractile state of smooth muscle, and is associated with decreased

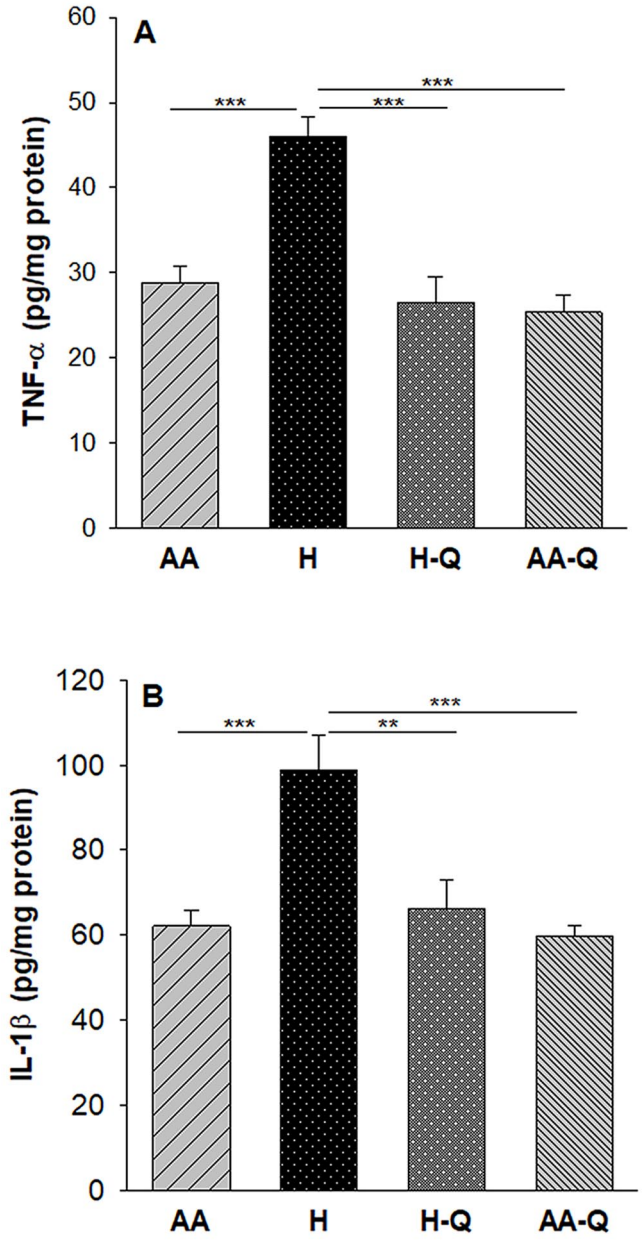

Figure 6. Effect of quercetin on proinflammatory cytokines in lung tissue of rat pups: (a) TNF-α level in lung tissue of rat pups exposed to hyperoxia or AA ($n=6$, each condition) and (b) IL-1β level in lung tissue of rat pups exposed to hyperoxia or AA ($n=7$, each condition). $^{**}P<0.01$; $^{***}P<0.001$. H: hyperoxia; AA: ambient air; H-Q: hyperoxia + quercetin; AA-Q: ambient air + quercetin. Data reported as mean ± SEM.

NO production, a major component of ASM relaxation.[4,8] Flavonoids like quercetin were shown to have an inhibitory effect on contractility of TSM in a rat model of asthma.[22] In this study, supplementation of quercetin either *in vitro* or *in vivo* reduced contractile responses of TSM toward MCh, confirming quercetin's capability to reverse or prevent airway hyperreactivity induced by hyperoxia. Inhibition of NOS by L-NAME reduced the reversal effect of quercetin on TSM contractility. Our results are in line with other studies that demonstrate the modulatory role of NO in quercetin-induced reduction in contractility of ASM and other tissues as well, like aorta and intestine.[18,22–24]

Furthermore, our study demonstrates that quercetin restores the hyperoxia-impaired relaxation of TSM induced by EFS. The relaxant effect of quercetin was also reported by other studies in isolated tissues such as human bronchial smooth muscle, murine airways, rat aortic rings, and pulmonary arteries.[13,14,25] Sopi *et al*.[5] demonstrated that neonatal hyperoxia impairs relaxant responses of rat parenchymal strips due to disruption of NO-cGMP signaling pathway. Inhibition of NOS in this study reduced the promoting relaxant effect of quercetin on airways, indicating involvement of NO in this signaling cascade of quercetin. Quercetin enhances NOS activity by stimulating NOS phosphorylation, hence increasing NO and cGMP production, and subsequently inducing relaxation of rat aortic rings.[17] Incubation of tissues in L-NAME reduced but didn't abrogate the relaxant effect of quercetin. We speculate that this is because quercetin may act through other mechanisms in addition to the NO-cGMP signaling pathway to promote relaxation. In mouse tracheal rings, quercetin was shown to inhibit PDE4, increasing the level of cGMP, thereby potentiating isoproterenol-induced relaxation.[14] Another study revealed that quercetin reduces myocardial Rho-kinase activity in rats, while in previous study we found that hyperoxia increases Rho-kinase expression and activity, and the administration of Rho-kinase inhibitors was protective against hyperoxia-induced airway hyperreactivity.[4,26] However, future studies are needed to undertake to clarify the involvement of quercetin on Rho/Rho-kinase signaling in its protective effect under hyperoxic conditions.

Besides airway hyperreactivity, supplemental high fraction of inhaled oxygen also induces inflammation which has a considerable role in pathogenesis of BPD and contributes to airway hyperresponsiveness too.[27] There is an imbalance between pro- and anti-inflammatory cytokines in preterm babies due to inability to regulate inflammation, and an increase in levels of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) has been shown in tracheal aspirate and serum from premature infants with BPD.[28,29] In this study, we demonstrated that hyperoxia increased the levels of TNF-α and IL-1β in lung tissue, while quercetin supplementation played a protective role and reduced proinflammatory cytokines levels to that of controls. An anti-inflammatory and antioxidative effect of quercetin has been reported in lungs of neonatal mice.[15]

In summary, this study provided novel findings that quercetin attenuates airway hyperreactivity induced by neonatal hyperoxia, restores airway relaxation, and prevents the accumulation of proinflammatory cytokines in the lung. These data suggest that quercetin might serve as a therapy to protect against adverse effects of hyperoxia.

AUTHORS' CONTRIBUTIONS

I.K: conceptualization and design, funding acquisition, resources, investigation, formal analysis, validation, visualization and interpretation of data, writing – original draft of the article, and final approval of the version to be published; S.R.: investigation and formal analysis, and reading the article; Q.T.: investigation and reading the article; N.H.P.: investigation and reading article; F.K.: data interpretation, and revising and

editing the article; M.B.S.: funding acquisition, data interpretation, and revising and editing the article; M.M.: conceptualization and design, interpretation of data, and reviewing and editing the article; R.B.S.: conceptualization and design, funding acquisition, supervision, resources, data acquisition, interpretation of data; data curation, writing – original draft, and reviewing and editing the article; and final approval of the version to be published. All authors have read and approved the final version of the article.

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