High oxygen concentrations predispose mouse lungs to the deleterious effects of high stretch ventilation

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Mechanical ventilation is a necessary therapeutic intervention for patients with acute lung injury. However, mechanical ventilation can propagate acute lung injury and increase systemic inflammation. The exposure to >21% oxygen is often associated with mechanical ventilation yet has not been examined within the context of lung stretch. We hypothesized that mice exposed to >90% oxygen will be more susceptible to the deleterious effects of high stretch mechanical ventilation. C57B1/6 mice were randomized into 48-h exposure of 21 or >90% oxygen; mice were then killed, and isolated lungs were randomized into a nonstretch or an ex vivo, high-stretch mechanical ventilation group. Lungs were assessed for compliance and lavaged for surfactant analysis, and cytokine measurements or lungs were homogenized for surfactant-associated protein analysis. Mice exposed to >90% oxygen + stretch had significantly lower compliance, altered pulmonary surfactant, and increased inflammatory cytokines compared with all other groups. Our conclusion is that 48 h of >90% oxygen and high-stretch mechanical ventilation deleteriously affect lung function to a greater degree than stretch alone.

pulmonary surfactant; inflammatory cytokines

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tribute to decreased lung stability, decreased oxygenation, and increased inflammation in the injured lung.

The purpose of this study was to examine the effect of high oxygen exposure on lung function within a model of lung stretch. We hypothesized that exposure to high oxygen enhances the damaging effects of lung stretch through alterations of the pulmonary surfactant system and increases in proinflammatory cytokines.

MATERIALS AND METHODS

A total of 106 C57B1/6 mice (Charles River Laboratory) weighing 18–32 g were used for these experiments. The mice were group housed and allowed access to standard rodent chow and water ad libium with an automatically controlled 12-h light-dark cycle. All mice were allowed a minimum 4-day acclimatization period before randomization into the experiments described below. All procedures performed on the mice were approved by the animal use subcommittee of the University of Western Ontario.

Four groups of animals were investigated: 1) 48-h exposure to 21% oxygen (21% nonstretch); 2) 48-h exposure to >90% oxygen (>90% nonstretch); 3) 48-h exposure to 21% oxygen followed by 2 h of high stretch, ex vivo mechanical ventilation (21% + stretch); and 4) 48-h exposure to >90% oxygen followed by 2 h of high stretch, ex vivo mechanical ventilation (>90% + stretch). Because of the low amounts of material obtained from each animal, four separate subgroups of animals from these experimental groups were used for 1) surfactant analysis of bronchoalveolar lavage (BAL), 2) determination of surfactant protein (SP)-A and D protein levels in homogenized lung tissue, 3) measurements of mRNA of surfactant-associated proteins, and 4) analysis of TNF-α and IL-6 in the BAL.

Animal procedures. Initially, mice were randomized into one of two groups: normal air (21% oxygen) or high oxygen (>90% oxygen). Oxygen concentrations were controlled by a gas mixer (model 3500HL, Scherst Medical Products Division, Anaheim, California) that was supplied by the hospital's medical air and oxygen lines. A sealed Plexiglas box (32.5 cm, Parker Plastic, Ontario, Canada) was filled with the appropriate gas mixture, and, after an initial body weight was recorded, mice were placed inside for a duration of 48 h. Oxygen levels inside the box were verified every 12 h with a MiniOx-I oxygen analyzer (MSA Medical Products, Pittsburgh, PA).

After the 48-h exposure, mice were again weighed and then killed with an intraperitoneal overdose injection (0.5 ml of pentobarbital sodium (65 mg/ml) and exsanguinated via cutting of the descending dorsal aorta. After death, 2-0 surgical silk was used to secure a catheter in the trachea (18 gauge). The lung was then carefully extracted from the thoracic cavity and randomized into one of two groups: nonventilation (nonstretch) and ex vivo mechanical ventilation (stretch). Briefly, isolated lungs were placed inside a 37°C humidified chamber and mechanically ventilated with a volume cycle Voltek R5 rodent ventilator (Voltek Enterprises, Toronto, Canada), with an inspired oxygen fraction of 1.0 for 2 h with a tidal volume of 20 ml/kg and zero end-expiratory pressure at a rate of 40 cycles/min (31). Peak inspiratory pressures (PIP) were monitored every 30 min throughout the 2-h ventilation period. At the completion of ventilation (immediately after lung isolation for the nonstretch group), a static pressure-volume curve was determined to assess the compliance of the lung at a maximum pressure of 25 cmH₂O (31).

BAL and surfactant analysis. After compliance measurements, a BAL was performed by using 1-ml wash of 0.15 M NaCl saline three times. Each lavage comprised instilling and withdrawing the saline via the tracheal catheter three times by using a 1-ml syringe. All lavages were combined, and total volumes were recorded. BAL material was centrifuged at 150 g for 10 min to remove any cellular debris. The supernatant was termed total surfactant (TS) and stored at −20°C, with the exception of a 1-ml aliquot that was further centrifuged at 40,000 g for 15 min to separate the SA in the supernatant from the LA in the pellet. LA were resuspended in 300 μl of saline, and both LA and SA were similarly frozen and stored at −20°C.

Phosphorous measurements were used to determine the amount of surfactant in the TS, SA, and LA fractions within each group. Aliquots of each fraction were lipid extracted, as described by Bligh and Dyer (4). The phospholipid-containing chloroform phase was utilized for determination of phosphorous by using a modification of the method by Duck-Chong (10). A Lowry protein assay was used to assess the total protein concentrations in the TS fraction (18).

Electrospray ionization mass spectroscopy was performed to assess the molecular species of the phosphatidylcholine (PC) and phosphatidylglycerol (PG) phospholipid species in the lung tissue fraction. The analysis was carried out in the positive and negative ion mode on a triple quadrupole instrument (model api 365, Sciex, Concord, Canada). TS samples were lipid extracted as mentioned above, and duplicates of 40 μl were aliquoted for phosphorous measurement via the Duck-Chong method (10). From the remaining sample, 80 μg of phospholipid was dried and resuspended in 500 μl of chloroform-methanol-sodium hydroxide (3:1:0.04). Each sample was injected into the spectroscope at 10 μl/min. The peak intensities from the positive mass spectrum were used to calculate the intensities of 16:0/0 PC, 16:0/14:0 PC, 16:0/16:1 PC, 16:0/16:0 PC, and 16:0/18:2 PC relative to each other. The peak intensities from the negative mass spectrum were used to calculate the relative intensities of 16:0/16:0 PG, 16:0/16:2 PG, 16:0/18:1 PG, and 18:1/18:2 PG.

The ability of the surfactant LA to adsorb at an air-liquid interface was measured by using a pulsating bubble surfactometer (ElectroSupports, Amherst, NY) (11). For this analysis, LA were reconstituted and subsequently resuspended at 1 mg phospholipid/ml in 0.15 M NaCl, 1.5 mM CaCl₂. Adsorption was measured for 15 min after creating a bubble in the surfactometer, which was set at a pulsation rate of 0. The amount of surfactant adsorbed was calculated as % of the total surfactant present in the LA suspension.

The quantification of mRNA levels of the surfactant-associated proteins in the RNA pellets isolated from the homogenized lungs was performed by using a RNA protection assay. For this experiment, the multiple template, labeled with radioactive sulphur (α-35S), probed for SP-A, SP-B, SP-C, and...
SP-D, and the housekeeping genes L32 and GAPDH, which were used as internal loading controls. The RNA protection assay gel was absorbed onto filter paper and exposed to X-ray film (Eastman Kodak, Rochester, NY) for 72 h at $-70^\circ$C. The film was subsequently analyzed by densitometry by using GAPDH as an internal loading control.

**RESULTS**

Quantification of SP-A and SP-D via Western blot. Lungs were homogenized in 1 ml of saline with a polytron PT2100. The homogenate was then diluted another 10x with saline and stored at $-20^\circ$C until analysis. A Lowry protein assay (18) was performed on the diluted homogenate, and subsequently 100 µg of homogenate protein were loaded on a 12% polyacrylamide gel containing 0.01% sodium dodecyl sulphate. Proteins were separated via 2 h of 100-V electrophoresis (Bio-Rad Laboratories, Mississauga, Canada), after which the proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories). Nitrocellulose membranes for SP-A analysis were blocked at 4°C overnight in 3% Blotto in PBS (pH 7.4). The nitrocellulose was then incubated in rabbit anti-rat SP-A IgG primary antibody in a 1:700 dilution with TBS, followed by a 5-min wash in 5% Blotto in 1× TBS. Nitrocellulose membranes for SP-D analysis were blocked at 4°C overnight in 5% Blotto in Tris-buffed saline (TBS; pH 7.6). The nitrocellulose was then incubated in rabbit anti-rabbit horseradish peroxidase-linked secondary antibody diluted 1:1,000 in 1× PBS. Nitrocellulose membranes for SP-D analysis were then washed 3× 5 min in 1× PBS with 0.01% Tween 20 followed by incubation with goat anti-rabbit horseradish peroxidase-linked secondary antibody diluted 1:1,000 in 1× PBS. Nitrocellulose membranes for SP-D analysis were blocked at 4°C overnight in 5% Blotto in 1× TBS. The nitrocellulose was washed 5 min in 1× TBS, 5 min in TBS with 0.01% Tween 20, 3× 5 min in 1× TBS, followed by a 5-min wash in 5% Blotto in 1× TBS. After the washing, the nitrocellulose membrane was incubated in goat anti-rabbit horseradish peroxidase-linked secondary antibody diluted 1:1,000 in 5% Blotto in 1× TBS. Finally, substrates for enhanced chemiluminescent (Amersham Life Science, Little Chalfont Bucks, UK) reaction were applied to all nitrocellulose membranes, which were exposed to X-ray film (Eastman Kodak) and developed by using standard techniques. Densitometry was performed on the X-ray films, and immunoreactivity was expressed as a percentage of the 21% nonstretch group.

**TNF-α and IL-6 cytokine analysis.** After compliance measurements, the lungs for cytokine analysis were lavaged with 2× 1 ml of 0.15 M NaCl. Each lavage was instilled and withdrawn three times and centrifuged at 200 g for 10 min at 4°C. The supernatant was divided into four equal aliquots, immediately snap frozen in liquid nitrogen, and stored at $-70^\circ$C until further use. The concentration of TNF-α and IL-6 were measured in all samples by using separate opti-eia ELISA kits specific for each cytokine, following the instructions provided by the manufacturer (PharMingen, San Diego, CA).

**Statistical analysis.** All data are presented as means ± SE. Statistical analysis were performed by using a two-way ANOVA, with a Tukey’s post hoc test, to explore any interactive effects. A one-way ANOVA, with a Tukey’s post hoc test, was used to explore differences among specific groups. SPSS statistical software package for Windows, version 9.0.0 (SPSS Chicago, IL), was used to perform the statistical comparisons. Differences between groups were considered statistically significant at probability values of $<0.05$.

**RESULTS**

All mice exposed to either 21% or >90% oxygen survived the entire 48-h exposure period. There were no significant differences observed between the initial and death masses of mice exposed to 21% oxygen ($n = 50$) compared with the mice exposed to >90% oxygen ($n = 56$), nor were there any differences observed between the initial and death masses within each of the 48-h exposure groups. The BAL volumes used to analyze the surfactant for the 21% nonstretch group was 2.4 ± 0.1 ml, which was not different from all other groups. Similarly, BAL volumes recovered from lungs used to analyze the inflammatory cytokines were not significantly different among all groups (21% nonstretch = 1.6 ± 0.1 ml).

The PIP measurements obtained from both stretch groups are presented in Fig. 1A. At the time 0 point, the 21% + stretch group and the >90% + stretch group had PIP values that were not statistically different. However, after 30 min of ventilation, the >90% + stretch group had a significantly higher PIP compared with the 21% + stretch group (Fig. 1A); this difference remained significant until the end of the ventilation period.

Analysis of static pressure-volume curves, shown in Fig. 1B, indicated no significant differences between the 21% nonstretch group and the >90% nonstretch group (Fig. 1B). The 21% + stretch group exhibited a significantly reduced compliance, as noted by the vol-

![Fig. 1. Measures of ex vivo lung compliance.](http://jap.physiology.org/)
ume at a maximal pressure of 25 cmH2O, compared with both nonstretch groups. Furthermore, the >90% + stretch group had a significantly reduced compliance compared with all other groups (Fig. 1B).

Table 1 shows the amount of total protein in the BAL. There were no significant differences in the total protein recovered from BAL (µg/g body wt) among the 21% nonstretch, >90% nonstretch, and 21% + stretch groups. The amount of total protein was significantly higher in the >90% + stretch group compared with all other groups (Table 1).

Surfactant analysis. No significant difference was observed when comparing the amount of TS between the 21% nonstretch and >90% nonstretch groups (Fig. 2A). The 21% + stretch group had a significant increase in TS compared with all other groups. The >90% + stretch group had a significantly greater amount of TS compared with both nonstretch groups, but this value was significantly less than the 21% + stretch group (Fig. 2A). Statistical comparison of the amounts of LA indicates that LA was significantly higher in the 21% + stretch group compared with the three other groups (Fig. 2A). There was no significant difference in the amount of SAs recovered in the BAL among all groups (Fig. 2A). Figure 2B shows the LA expressed as a percentage of the TS to determine the contribution of LA. The results show that both >90% groups had significantly lower percent LA compared with both 21% groups (Fig. 2B).

Results from the electrospray ionization mass-spectroscopy analysis of PC species are shown in Fig. 3. There were no statistical differences among 21% nonstretch, >90% nonstretch, and 21% + stretch groups for all PC species measured. The >90% + stretch group had significantly greater amounts of 16:0/0 PC (lyso-PC) and 16:0/18:2 PC. Furthermore, the >90% + stretch group had significantly less 16:0/16:0 (DPPC) compared with all other groups. Analysis of the relative amounts of PG species indicated that there were no significant differences among the four groups (data not shown).

Figure 4 represents the adsorption measurements of the isolated LA from each group. There were no significant differences among the 21% nonstretch, >90% nonstretch, and the 21% + stretch groups. However, the >90% + stretch group had a significantly greater surface tension after just 30 s of adsorption, and this remained significantly greater than all other groups for the remainder of the 15-min experimental time course (Fig. 4).

Table 1. Total protein in BAL

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>n</th>
<th>Total Protein in BAL, µg/g body wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>21% nonstretch</td>
<td>5</td>
<td>14.8 ± 2.2</td>
</tr>
<tr>
<td>&gt;90% nonstretch</td>
<td>4</td>
<td>20.4 ± 4.0</td>
</tr>
<tr>
<td>21% + stretch</td>
<td>5</td>
<td>62.5 ± 5.8</td>
</tr>
<tr>
<td>&gt;90% + stretch</td>
<td>5</td>
<td>229.3 ± 28.1*</td>
</tr>
</tbody>
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*Values are means ± SE. BAL, bronchoalveolar lavage. *P < 0.05 vs. all groups.

Results from the normalized densitometry values for the relative levels of surfactant-associated protein mRNA are shown in Table 2. For all four SPs, there were no differences observed between the 21% non-
Mechanical ventilation is an essential but potentially harmful supportive therapy for patients with ALI and ARDS (3, 9). Numerous investigations into optimal ventilation strategies have been reported (6, 7, 27, 29). One specific component of mechanical ventilation, namely the stretch associated with lung inflation, has recently been proven to be a significant contributor to the damaging effects of this intervention (9, 28). In the study reported by the ARDS Network (6), it was demonstrated that ventilation with tidal volumes of 6 ml/kg (low stretch) compared with 12 ml/kg (high stretch) resulted in significantly lower mortality and increased ventilator-free days in patients with ALI/ARDS (9). The concentration of TNF-\(\alpha\) was not significantly different among the 21% nonstretch, >90% nonstretch, and 21% + stretch groups (Fig. 6A). The >90% + stretch group had a significantly higher concentration of TNF-\(\alpha\) compared with all other groups (Fig. 6A). The concentration of IL-6 was not significantly different between the two nonstretch groups. Similarly, the IL-6 concentration in the 21% + stretch and the >90% + stretch groups were not significantly different. Examination of the effects of stretch revealed that the two stretch groups had significantly higher concentration of IL-6 compared with both nonstretch groups (Fig. 6B).

### DISCUSSION

Cytokine analysis in BAL. The concentration of TNF-\(\alpha\) was not significantly different among the 21% nonstretch, >90% nonstretch, and 21% + stretch groups (Fig. 6A). The >90% + stretch group had a significantly higher concentration of TNF-\(\alpha\) compared with all other groups (Fig. 6A). The concentration of IL-6 was not significantly different between the two nonstretch groups. Similarly, the IL-6 concentration in the 21% + stretch and the >90% + stretch groups were not significantly different. Examination of the effects of stretch revealed that the two stretch groups had significantly higher concentration of IL-6 compared with both nonstretch groups (Fig. 6B).

### Table 2. Densitometry measurements of surfactant-associated proteins at the mRNA level

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>(n)</th>
<th>SP-A</th>
<th>SP-B</th>
<th>SP-C</th>
<th>SP-D</th>
</tr>
</thead>
<tbody>
<tr>
<td>21% nonstretch</td>
<td>3</td>
<td>100.0 ± 11.1</td>
<td>100.0 ± 7.3</td>
<td>100 ± 10.0</td>
<td>100 ± 26.0</td>
</tr>
<tr>
<td>&gt;90% nonstretch</td>
<td>3</td>
<td>67.3 ± 7.8</td>
<td>68.6 ± 16.7</td>
<td>79.6 ± 22.8</td>
<td>54.0 ± 7.7</td>
</tr>
<tr>
<td>21% + stretch</td>
<td>3</td>
<td>86.6 ± 6.2</td>
<td>97.2 ± 5.7</td>
<td>107.0 ± 14.4</td>
<td>65.1 ± 8.9</td>
</tr>
<tr>
<td>&gt;90% + stretch</td>
<td>4</td>
<td>45.3 ± 2.7</td>
<td>40.2 ± 16.0</td>
<td>53.5 ± 21.1</td>
<td>31.3 ± 4.5</td>
</tr>
</tbody>
</table>

Values are means ± SE and are expressed relative to the 21% nonstretch group and normalized to GAPDH density. SP, surfactant protein. *\(P < 0.05\) vs. 21% nonstretch group.
ARDS. In this clinical study as well as in other smaller trials, oxygen concentrations delivered to the patient were titrated to maintain blood-gas values (5, 6, 27). However, exposures to higher concentrations of oxygen have also been shown to cause damage to the lung (16). This information was our rationale to examine the effects of prolonged exposure to high oxygen concentrations on the lung in the context of lung stretch. On the basis of previous observations in mice, we hypothesized that alterations of the pulmonary surfactant system were propagated (16, 31).

Our experimental approach was to first expose mice to room air or high oxygen and subsequently expose the isolated lung to either nonstretch or stretch. We utilized 48 h of exposure to >90% oxygen because this condition by itself does not result in lung dysfunction (2). The advantages of this approach in mice include the possibility to utilize transgenically modified animals in the future. The ex vivo approach allows for an extreme high-stretch protocol (tidal volume of 20 ml/kg) to potentially observe differences over the relatively short timeframe, which also minimized the 21% + stretch group exposure to the inspired oxygen fraction of 1.0. Obvious disadvantages include the lack of physiological measurements, the potentially contributing effects of ischemia of the lung, and the discrepancy between normal mouse breathing patterns and those used in the study (31). To overcome these limitations, additional studies approaching the hypothesis by utilizing an in vivo approach are therefore warranted.

The main conclusion from our experiments was that mild oxidative stress and high-stretch mechanical ventilation deleteriously affects lung function to a greater degree than stretch alone. Lung compliance was unaffected by oxygen exposure alone compared with air exposure. However, when oxygen-exposed lungs were subsequently ventilated, compliance was significantly decreased compared with the ventilated air-exposed lungs. A similar conclusion that oxygen exposure and ventilation together contribute to progressive lung damage was reported by Davis et al. (8). In our experiment, subsequent to compliance measurements, we analyzed the pulmonary surfactant system and concentrations of TNF-α and IL-6 in the lung on the basis of previous studies that implicated these components in the development of lung dysfunction (16, 31).

The biophysical function of pulmonary surfactant is to reduce the surface tension at the air-liquid interface of the alveoli (22). The active subfraction of surfactant, the LA, accomplishes this function by forming a DPPC-containing lipid film at the air-liquid interface. Impairment of this process results in decreased lung compliance. Compared with all other groups, the surfactant from lungs exposed to oxygen and stretch had a decreased amount of DPPC, decreased amounts of immunoreactive SP-D, and impaired adsorptive properties. This >90% + stretch group also had significantly greater amounts of non-SPs in the lavage that are known to inhibit surfactant function. The overall conclusion from those measurements is that, compared with all other groups, the >90% + stretch group had a significantly impaired surfactant system that was likely responsible for the decreased compliance.

A limitation of the present study is that prolonged oxygen exposure and stretch were administered separately rather than together, as would occur in mechanically ventilated patients (5, 6, 27). We would still suggest that our results reflect the clinical situation in which high oxygen is administered to a patient for a prolonged period of time and is combined with potential areas of overdistension of the lung due to the heterogeneous nature of the lung injury (13). Furthermore, our experimental design allowed us to observe individual effects of the two aspects of mechanical ventilation. In this regard, it was interesting that the oxygen exposure resulted in relative minor alterations of surfactant, such as a decreased percentage of LA and changes in protein and mRNA level of SP-A, but without significant lung injury. SP-A is important in the formation of tubular myelin and stabilization of LA structures by counteracting the protein inhibition of surfactant and in-host defense functions (20). These functions imply a beneficial role for SP-A in situations of lung stress. Thus, in our experiments, the reduced SP-A protein in the lung because of oxygen exposure may have been responsible for making the lung more
susceptible to the effects of lung stretch. One specific example of this potential mechanism is through counteracting protein inhibition. Lung stretch was shown to induce an increase in protein in the lavage. It is possible that in the 21% + stretch group SP-A was able to counteract the inhibitory effects of these proteins, whereas in the >90% + stretch group lower amounts of SP-A allowed for an inhibition of surface tension reduction.

Whereas the above discussion focuses on SP-A, other SPs may also have been involved. Analysis of mRNA levels of the SPs revealed similar patterns for the four proteins with respect to the effects to oxygen exposure and stretch, and, although only SP-A and SP-D reached statistical significance, this was likely due to the low n values used in this analysis (n = 3/group). The overall decrease in mRNA levels of the SPs and the decreased amounts of SP-A and SP-D in lung homogenate may implicate general effects of oxygen exposure and stretch on type II cell metabolism. We did not determine whether the effects of hyperoxia and stretch on SPs in the lung homogenate were associated with decreased amounts of these proteins in the lavage. We did, however, observe a relative decrease in DPPE in the lavage from the >90% + stretch group, which could also be related to altered, type II cell metabolism. On the basis of the relative increase in lysophosphatidylcholine in this >90% + stretch group, increased phospholipase A2 activity may be an alternative explanation for this specific finding (15).

It has been suggested that mechanical ventilation could be a contributor to multiorgan dysfunction (26). Although some controversy exists, mechanical ventilation has been reported to significantly affect inflammatory mediator release (25, 28). To test whether the combination of high oxygen and high stretch impacts host defense, we measured two major cytokines in the BAL, TNF-α and IL-6. Interestingly, the responses to the experimental conditions differed between these two cytokines. IL-6 was detected in the BAL after stretch regardless of oxygen exposure. The stretch-induced increase in IL-6 concentration is consistent with clinical studies in which patients were ventilated with low- and high-stretch strategies. In these studies, increased IL-6 concentrations were observed in the BAL fluid (24) and in plasma (6). Although it did not reach statistical significance, stretch increased TNF-α concentrations to a similar extent as previously reported (31). The concentrations of TNF-α were not increased by hyperoxia, but the combination of hyperoxia and stretch resulted in a significance increase in the lavage values of this cytokine. Injection of TNF-α into the lung can cause lung dysfunction and alterations of the pulmonary surfactant system (21, 23). It is thus possible that the increased TNF-α concentrations in the >90% + stretch group may have contributed to the decreased compliance, although this seems unlikely considering the short period of stretch utilized.

Overall, we propose that the combinatorial effects of high oxygen concentration and high stretch mechanical ventilation can propagate ALI by altering pulmonary surfactant and possibly culminate to multiple organ failure syndrome via the release of proinflammatory mediators. Future in vivo and clinical studies are warranted to examine this concept further.

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