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Aerosolized adrenomedullin suppresses pulmonary transforming growth factor-β1 and interleukin-1β gene expression in vivo

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Abstract

The effect of aerosolized adrenomedullin on interleukin-1β and transforming growth factor (TGF)-β1 mRNA and protein expression was studied in surfactant depleted piglets, receiving aerosolized adrenomedullin (adrenomedullin, $n=6$), aerosolized adrenomedullin plus i.v. N^G-nitro-L-arginine-methylester (adrenomedullin + L-NAME, $n=5$), or aerosolized saline solution (control, $n=6$). After 8 h of aerosol interval therapy, mRNA expression of interleukin-1β and TGF-β1 in lung tissue was quantified normalized to β-actin and hypoxanthine-guanine-phosphoribosyl-transferase by real-time polymerase chain reaction (PCR). Interleukin-1β and TGF-β1 protein concentration in lung tissue was quantified by enzyme-linked immunosorbent assay (ELISA). In the adrenomedullin group, interleukin-1β and TGF-β1 mRNA expression was lower than in controls. Reduction for interleukin-1β/β-actin was 56% ($p<0.001$), for interleukin-1β/hypoxanthine-guanine-phosphoribosyl-transferase 60% ($p<0.001$), for TGF-β1/β-actin 65.5% ($p<0.001$), and for TGF-β1/hypoxanthine-guanine-phosphoribosyl-transferase 56.2% ($p<0.001$). Mean interleukin-1β protein expression was different between the groups, $p<0.05$ (adrenomedullin 601 ± 61, Control 836 ± 88 pg/mg protein). L-NAME did not antagonize adrenomedullin effect on TGF-β1 mRNA. In conclusion, aerosolized adrenomedullin reduced pulmonary inflammatory and pro-fibrotic response.

Keywords: Adrenomedullin; Aerosol; Lung fibrosis; TGF-β_1 (transforming growth factor-β_1); Interleukin-1β

1. Introduction

Adrenomedullin, a 52-amino acid peptide, has been first described as hypotensive peptide in human pheochromocytoma (Kitamura et al., 1993). It is present in various tissues such as lung, heart, kidney, adrenal gland, duodenum, spleen, submandibular gland and plasma as well as in various cell types as endothelial cells, smooth muscle cells and macrophages (Kubo et al., 1998). In the lung, adrenomedullin has been found to be expressed in the columnar epithelium, endothelial cells, neurons of the pulmonary parasympathetic nervous system, chondrocytes, alveolar macrophages, smooth muscle cells and several glands (Jougasaki and Burnett, 2000). Cytokines such as interleukin-1α, interleukin-1β, transforming growth factor (TGF)-β, tumor necrosis factor (TNF) and lipopolysaccharide are known to stimulate adrenomedullin secretion (Jougasaki and Burnett, 2000; Kubo et al., 1998; Sugo et al., 1995). On the other side, TGF-β has been demonstrated to suppress adrenomedullin production dose-dependently in RAW 264.7 cells (Kubo et al., 1998). Adrenomedullin is inactivated by first-pass mechanism in porcine lung tissue (Sabates et al., 1996).

The predominant effect of adrenomedullin is vasodilation in systemic and pulmonary artery vessels. However, several other functions have been reported, such as natriuresis, bronchodilation and inhibition of apoptosis, cell migration, salt appetite, water drinking and gastric motion (Jougasaki and Burnett, 2000). Data concerning cell proliferation are controversial, revealing proliferative and antiproliferative effects of adrenomedullin in different tissues (Jougasaki and Burnett, 2000). There are limited data on a possible in vitro effect of adrenomedullin on inflammatory reactions (Isumi et al., 1999; Kamoi et al., 1995). A protective effect on pulmonary function could be mediated through an adrenomedullin-induced increase of the secretion of phosphatidylcholine, the predominant component of pulmonary surfactant, by type II pneumocytes (Okumura et

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al., 2000). In vivo data on (anti-)inflammatory effects of adrenomedullin, studied in the acetic acid peritonitis in rats (Clementi et al., 1999) and ocular inflammation in rabbits, are limited and indefinite (Clementi et al., 2000). In urinary-tract infections in children, adrenomedullin concentrations in urine were found to be increased (Dötsch et al., 1998).

Idiopathic, radiation- or drug-induced pulmonary fibrosis in adults as well as bronchopulmonary dysplasia in premature infants resulting from intensive respiratory support with volu- and barotrauma to immature lungs are still of poor prognosis and high morbidity. Therapeutic options based on pathophysiologic concepts are rare. TGF-β is supposed to be the most important cytokine in terms of the direct stimulation of lung matrix expression, which precedes fibrosis (Zhang and Phan, 1996). Transient overexpression of active TGF-β1 caused severe progressive fibrotic reactions in rat lung (Sime et al., 1997). Lung epithelial cells, alveolar macrophages, eosinophils and myofibroblasts could be identified as important cellular sources of TGF-β (Zhang and Phan, 1996). TGF-β acts pro-inflammatory, for example, by induction of interleukin-1α and interleukin-8 mRNA and proteins in rat lung alveolar epithelial cells (Kumar et al., 1996) or by stimulation of interleukin-8 release and cyclooxygenase-2 expression in human airway smooth muscle cells (Fong et al., 2000). Interleukin-1β is mainly produced by alveolar macrophages and has been shown to be among the very early pro-inflammatory and pro-fibrotic cytokines (Zhang and Phan, 1996). Transient overexpression of interleukin-1β in rat lungs induced acute inflammatory tissue response, long lasting elevated TGF-β1 concentrations and severe progressive tissue fibrosis (Kolb et al., 2001). Interleukin-1β has been shown to up-regulate gene expression of collagen types I and III and fibronectin (Zhang et al., 1993). We therefore examined, whether a possible anti-inflammatory effect of adrenomedullin might be detected in vivo in a porcine model of surfactant depletion by quantifying TGF-β1 and interleukin-1β mRNA expression and protein formation.

2. Materials and methods

Seventeen piglets with a body weight of 3.5–4.3 kg were included in the study. The animal experiments were approved by the local university Animal Care Committee and the government of Mittelfranken, Germany and performed according to the European community guidelines for use of experimental animals.

2.1. Protocol

Anesthesia and operative management of the piglets was performed as published previously (Kandler et al., 2001; von der Hardt et al., 2002). Briefly, after tracheotomy (endotracheal tube, diameter 4.5 mm, Mallinckrodt™ Hennef, Germany), a sheath was placed into the right jugular vein and an arterial catheter into the left femoral artery. Intermittent mandatory ventilation was performed with a time cycled pressure controlled neonatal respirator (Infant Star 950, Mallinckrodt™) starting with a peak inspiratory pressure of 20 cm H_2O, a positive end expiratory pressure of 4 cm H_2O, an inspiratory oxygen concentration of 100% and a frequency of 40 breaths/min, augmented to 50 breaths/min before lavage. Respiratory gas was humidified and temperature was kept at 39 °C (MR 700 AGM Fisher and Paykel®, Welzheim, Germany). Lung injury was induced by repeated saline lung lavage (Lachmann et al., 1980) (NaCl 0.9% at 39 °C) using 30 ml/kg per side. Lung injury was considered successful when the arterial PaO_2 remained below 80 mm Hg for a period of 60 min. During lavage, peak inspiratory pressure and positive end expiratory pressure were increased to 32 and 8 cm H_2O, respectively.

The animals were randomized to three different therapy groups (adrenomedullin, adrenomedullin + N^G-nitro-L-arginine-methylester (L-NAME) and control). In all animals, respiratory support was maintained constant at identical respiratory settings (positive end expiratory pressure 8 cm H_2O, peak inspiratory pressure 32 cm H_2O, inspiratory oxygen concentration of 100%, 50 breaths/min). Piglets in the adrenomedullin group received aerosolized adrenomedullin via an aerosolization catheter (Trudell Medical, London, Canada) (MacIntyre et al., 1996) at the distal end of the endotracheal tube. Adrenomedullin (Bachem, Germany) was applied in saline solution for five times 30 min with 30-min inhalation-free intervals over a total period of 5 h (volume rate of 4 ml/kg/h). The total amount of adrenomedullin applied was 5.8 μg/kg. Piglets in the adrenomedullin + L-NAME group additionally received the inhibitor of the nitric oxide synthases L-NAME (Sigma, Steinheim, Germany) at a dose of 25 mg/kg/h intravenously 30 min before and continuously during the 8 h of the experiment. Piglets in the control group received aerosolized saline solution at equal volume. After an additional observation period of 3 h (8 h after the establishment of lung injury) with constant respiratory parameters, animals were sacrificed. The trachea was clamped at a positive end expiratory pressure of 8 cm H_2O and lungs and heart were removed en bloc. Anterior and posterior tissue specimens were taken from the right lung. As the inferior lobe was larger than the superior and middle lobes, four tissue specimens were taken from the inferior lobe (central and basal), two from the superior and two from the middle lobe. Samples were frozen in liquid nitrogen and kept at −80 °C for up to 8 weeks until analysis.

2.2. RNA extraction and reverse transcription

Total RNA was extracted from the tissue using guanidine-thiocyanate acid phenol (RNAzol, WAK Chemie®, Medical, Bad Homburg, Germany). The RNA concentration was

determined spectrophotometrically. One microgram of RNA was reversely transcribed in a volume of 20 μl at 39 °C for 60 min (all chemicals were obtained from Boehringer®, Mannheim, Germany).

2.3. TaqMan real time polymerase chain reaction (PCR)

Efficiency and reliability of this method have been shown earlier (Dötsch et al., 2000; Heid et al., 1996). The approach for the measurement of gene expression by TaqMan real time PCR is based upon the 5′ exonuclease activity of the Taq polymerase. Within the amplicon defined by a gene specific oligonucleotide primer pair, an oligonucleotide probe labeled with two fluorescent dyes is designed. As long as the probe is intact, the emission of a reporter dye (i.e. 6-carboxy-fluorescein, FAM) at the 5′ end is quenched by the second fluorescent dye (6-carboxy-tetramethyl-rhodamine, TAMRA) at the 3′ end. During the extension phase of the PCR, the Taq polymerase cleaves the probe releasing the reporter dye. An automated photometric detector combined with a special software (ABI Prism® 7700 Sequence Detection System, Perkin-Elmer®, Foster City, CA) monitors the increasing reporter dye emission. The algorithm normalizes the signal to an internal reference (ΔRn) and calculates the threshold cycle number (C_T) when the ΔRn reaches 10 times the standard deviation of the baseline. The C_T values of the probes are interpolated to an external reference curve constructed by plotting the relative amounts of a serial dilution of a known template vs. the corresponding C_T values. Commercial reagents (TaqMan PCR Reagent Kit, Perkin-Elmer) and conditions according to the manufacturer's protocol were employed. cDNA (reverse transcription mixture) and oligonucleotides of 2.5 μl with a final concentration of 300 nM of primers and 200 nM of TaqMan hybridization probe were added to 25 μl reaction mix. The oligonucleotides of each target of interest were designed by the Primer Express software (Perkin-Elmer®) using uniform selection parameters that allowed the application of the same cycle conditions confirmed by primer optimization. Primers and probes were purchased from Eurogentec®, Belgium. The thermocycler parameters were 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Serial dilutions of one of the samples served as reference providing relative quantification of the unknown samples. Gene expression was related to the housekeeping genes β-actin and hypoxanthine-guanine-phosphoribosyl-transferase.

The following primers and TaqMan probes were used:

Hypoxanthine-guanine-phosphoribosyl-transferase:
Forward: 5′-TGGAAAGAATGTCTTGATTGTT-GAAG-3′
Reverse: 5′-ATCTTTGGATTATGCTGCTTGACC-3′
TaqMan probe: 5′ (FAM)-ACACTGGCAAAACAA-TGCAAACCTTGCT-(TAMRA)3′

β-Actin:
Forward: 5′-TCATCACCATCGGCAACG-3′
Reverse: 5′-TTCCTGATGTCCACGTCGC-3′
TaqMan probe: 5′ (FAM)-CCTTCCTGGGCATG-GAGTCCTGC-(TAMRA)3′

Interleukin-1β:
Forward: 5′-GGTTTCTGAAGCAGCCATGG-3′
Reverse: 5′-GATTTGCAGCTGGATGCTCC-3′
TaqMan probe: 5′ (FAM)-AAAGAGATGAAGTGC-TGCACCCAAAACCTG-(TAMRA)3′

TGF-β1:
Forward: 5′-TACGCCAAGGAGGTCACCC-3′
Reverse: 5′-CAGCTCTGCCCGAGAGAGC-3′
TaqMan probe: 5′ (FAM)-CTAATGGTGGAAAGCG-GCAACCAAATCTA-(TAMRA)3′.

2.4. Interleukin-1β and TGF-β1 protein expression

Porcine lung tissue was homogenized by mixer mill (Retsch, Haan, Germany) in lysis buffer, sonicated and centrifuged. In the supernatant, interleukin-1β was quantified by a commercially available porcine sandwich enzyme-linked immunosorbent assay (ELISA, BioSource, Nivelles, Belgium). TGF-β1 was quantified by a commercially available human sandwich enzyme-linked immunosorbent assay (Quantikine®, R&D Systems, Minneapolis, USA), cross-reacting with porcine TGF-β1. Total protein was determined by the Bio-Rad assay (Bio-Rad, Hercules, USA). Interleukin-1β and TGF-β1 protein expression was given as pg/mg total protein.

2.5. Data analysis

Data analysis was performed with Microsoft ACCESS®, Microsoft EXCEL® and Graph Pad PRISM®. Values are expressed as mean ± S.E.M. Depending on the presence of Gaussian distribution, either the ANOVA (analysis of variance) or Kruskal–Wallis test was used for comparison of the groups. In case of significance, Bonferroni or Dunns post-hoc test was applied, respectively. A p-value of less than 0.05 was considered significant. A difference between the groups was only considered as significant, if a p-value of less than 0.05 was found for normalization to both housekeeping genes. For analysis of two groups (ELISA for TGF-β1), Mann–Whitney test was used.

3. Results

3.1. mRNA expression

TGF-β1 and interleukin-1β mRNA expression was significantly lower in the adrenomedullin group compared to the control group. Gene expression was reduced by 65.5% for TGF-β1/A ($p < 0.001$), by 56.2% for TGF-β1/hypoxanthine-guanine-phosphoribosyl-transferase ($p < 0.001$), by

Table 1
Pulmonary TGF-β1 and interleukin-1β mRNA expression (relative units, RU) (mean ± S.E.M.) normalized to β-actin and hypoxanthine-guanine-phosphoribosyl-transferase (HPRT) in surfactant depleted neonatal piglets treated either with aerosolized adrenomedullin (adrenomedullin), with aerosolized adrenomedullin and additionally intravenous L-NAME (adrenomedullin + L-NAME) or with aerosolized saline solution (Control)

	Adrenomedullin	Adrenomedullin + L-NAME	Control
n (piglets)	6	5	6
n (samples)	48	40	48
TGF-β1/hypoxanthine-guanine-phosphoribosyl-transferase (HPRT) (RU)	0.67 ± 0.03*	0.50 ± 0.04*	1.53 ± 0.11
Interleukin-1β/hypoxanthine-guanine-phosphoribosyl-transferase (HPRT) (RU)	0.96 ± 0.21*,**	1.39 ± 0.19	2.40 ± 0.57

* $P < 0.001$ compared to control.
** $P < 0.001$ compared to adrenomedullin + L-NAME.

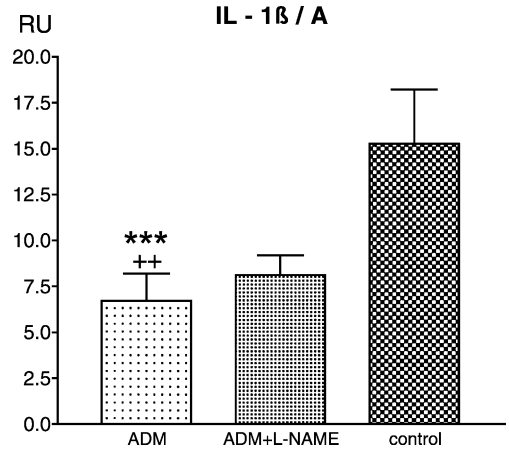

Fig. 2. Pulmonary interleukin-1β/β-actin mRNA expression (mean ± S.E.M., relative units, RU) in the lung of surfactant depleted piglets after an interval therapy period of 5 h with aerosolized adrenomedullin without (adrenomedullin, $n = 6$) or with additional intravenous application of L-NAME (adrenomedullin + L-NAME, $n = 5$), or with aerosolized saline solution (control, $n = 6$) during intermittent mandatory ventilation and an additional observation period of 3 h. ***$P < 0.001$ compared to control. $^{++}P < 0.01$ compared to adrenomedullin + L-NAME.

56.0% for interleukin-1β/β-actin ($p < 0.001$) and by 60.0% for interleukin-1β/hypoxanthine-guanine-phosphoribosyl-transferase ($p < 0.001$). In the adrenomedullin + L-NAME group, reduction of TGF-β1 mRNA expression was 77.8% for TGF-β1/β-actin ($p < 0.001$) and 68.0% for TGF-β1/hypoxanthine-guanine-phosphoribosyl-transferase ($p < 0.001$) compared to the control group. Similarly, interleukin-1β mRNA expression was lower compared to the control group (reduction 49.8% for interleukin-1β/A and 40.4% for interleukin-1β/hypoxanthine-guanine-phosphoribosyl-transferase), but the difference did not reach statistical significance. L-NAME treatment did not influence the adrenomedullin effects on TGF-β1 mRNA expression. However, for interleukin-1β mRNA expression, a significant reduction was found in the adrenomedullin group when compared to the adrenomedullin + L-NAME group. Reduction was 12.4% for interleukin-1β/β-actin ($p < 0.01$) and 32.9% for interleukin-1β/hypoxanthine-guanine-phosphoribosyl-transferase ($p < 0.001$).

For further details, see Table 1 and Figs. 1 and 2.

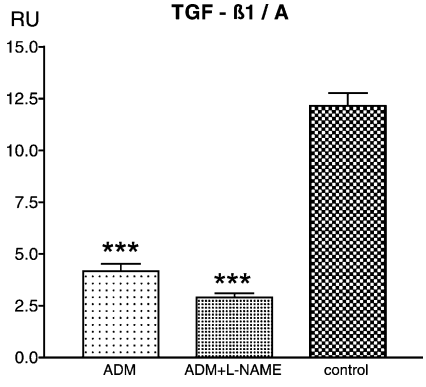

Fig. 1. Pulmonary transforming growth factor-β1/β-actin mRNA expression (mean ± S.E.M., relative units, RU) in the lung of surfactant depleted piglets after an interval therapy period of 5 h with aerosolized adrenomedullin without (adrenomedullin, $n = 6$) or with additional intravenous application of L-NAME (adrenomedullin + L-NAME, $n = 5$), or with aerosolized saline solution (control, $n = 6$) during intermittent mandatory ventilation and an additional observation period of 3 h. ***$P < 0.001$ compared to control.

3.2. Protein expression

Means of interleukin-1β concentration were significantly different between the three treatment groups. However, no significance was achieved with Bonferroni post-hoc test. No difference was shown for TGF-β1 protein expression. Further information is given in Table 2.

Table 2
Pulmonary TGF-β1 and interleukin-1β protein expression (pg/mg total protein) (mean ± S.E.M.) in surfactant depleted neonatal piglets treated either with aerosolized adrenomedullin (adrenomedullin), with aerosolized adrenomedullin and additionally intravenous L-NAME (adrenomedullin + L-NAME) or with aerosolized saline solution (Control)

	Adrenomedullin	Adrenomedullin + L-NAME	Control
Interleukin-1β* (pg/mg total protein)	601 ± 61	614 ± 52	836 ± 88
TGF-β1 (μg/mg total protein)	9.15 ± 0.50	not done	8.28 ± 0.43

* $P < 0.05$ difference of means (one-way ANOVA).

4. Discussion

Therapy with aerosolized adrenomedullin is a new therapeutic approach, which we intended to contribute to a reduction of pulmonary inflammatory and fibrotic reactions.

The significant and profound reduction of interleukin-1β and TGF-β1 gene expression by adrenomedullin in this in vivo model of surfactant depletion points to an important role adrenomedullin may have in the complex cytokine network of pulmonary inflammation and fibrosis. These findings are consistent with in vitro data on anti-inflammatory effects of adrenomedullin, which showed adrenomedullin to inhibit cytokine-induced neutrophil chemoattractant secretion from lipopolysaccharide-stimulated rat alveolar macrophages (Kamoi et al., 1995), to suppress TNF and interleukin-6 secretion from lipopolysaccharide-stimulated RAW 264.7 cells (Kubo et al., 1998) and to suppress TNF production in interleukin-1β stimulated Swiss 3T3 cells (Isumi et al., 1999). A possible anti-fibrotic effect of adrenomedullin and its effect on TGF-β have not been examined so far to our knowledge. As TGF-β is the major cytokine involved in pulmonary fibrosis (Zhang and Phan, 1996), the significant reduction of TGF-β1 mRNA expression is suggestive of a possible anti-fibrotic effect. Several studies reveal a close interaction of adrenomedullin with the NO system: adrenomedullin increases the activity of the constitutive (Shimekake et al., 1995) and the expression of the inducible (Ikeda et al., 1996) nitric oxide synthases. On the other hand, adrenomedullin secretion, gene expression and receptor function is stimulated by NO (Dötsch et al., 2002a,b). A decrease in exudate volume in the acetic acid peritonitis in rats by treatment with adrenomedullin could be abolished by additional treatment with L-NAME (Clementi et al., 1999). Therefore, the adrenomedullin effect seems to be exerted at least partially via mechanisms activating the NO system. However, in our study, the reduction of TGF-β1 mRNA was not modified by the additional application of L-NAME, suggesting that TGF-β1 mRNA suppression by aerosolized adrenomedullin does not predominantly involve the NO system. The intravenous dose of 25 mg/kg/h L-NAME led to a significant and severe increase in systemic arterial blood pressure, indicating efficient blocking of NO synthases in this model.

The exact regulatory mechanisms of adrenomedullin on pulmonary cytokine gene expression cannot be elucidated by our study. As TGF-β is stimulated by interleukin-1β (Isumi et al., 1998; Sugo et al., 1995), the decrease in TGF-β1 mRNA may be partially caused by the decrease in interleukin-1β. However, different mechanisms of TGF-β1 and interleukin-1β suppression may be present in this model, as the additional administration of L-NAME modified interleukin-1β-but not TGF-β1 mRNA expression.

Housekeeping genes are used for normalization of mRNA expression. Gene expression of the housekeeping genes used has been proven to be not regulated under conditions applied. To securely avoid any risk of bias due to possible minimal variances of housekeeping genes in the experimental setting, we normalized target gene expression to two housekeeping genes.

The significant difference in interleukin-1β protein expression in lung tissue shows that mRNA findings are reflected on protein level after an experimental time of not more than 8 h. This underlines the significance of the mRNA results for protein formation. TGF-β protein expression in bronchoalveolar lavage fluid has been described to parallel TGF-β mRNA expression (Yi et al., 1996). However, our data did not show a reduction of TGF-β1 on protein level with adrenomedullin compared to control. This might be due to the relatively short observation period, as TGF-β1 is one of the late cytokines, peaking several days after interleukin-1β in the bronchioalveolar fluid of infants developing bronchopulmonary dysplasia (Özdemir et al., 1997). Also, a possible reduction of histologically confirmed pulmonary fibrosis by aerosolized adrenomedullin cannot be expected after the considerably short treatment period.

Adrenomedullin is reported to be inactivated in the lung of neonatal piglets (Sabates et al., 1996). Nevertheless, our data show aerosolized adrenomedullin to be biologically active in reducing cytokine gene expression. The pulmonary site of adrenomedullin inactivation is unknown. Adrenomedullin applied as aerosol appears to have a sustained effect before inactivating mechanisms begin to become efficient. Possibly, an anti-inflammatory and anti-fibrotic potential of adrenomedullin might open new therapeutic perspectives in lung conditions, where chronic inflammatory and fibrotic processes lead to severe and irreversible structural lung damage, as seen for example in bronchopulmonary dysplasia or lung fibrosis.

In summary, our study demonstrates a significant suppression of pulmonary TGF-β1 and interleukin-1β mRNA expression by aerosolized adrenomedullin in vivo, suggesting adrenomedullin to play a role in the complex network of pulmonary cytokines. We speculate that therapy with aerosolized adrenomedullin exerts anti-inflammatory and anti-fibrotic effects.

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