Midkine Ameliorates the LPS-Induced Apoptosis of Airway Smooth Muscle Cells Through the Notch2 Pathway

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Abstract

Background: Airway smooth muscle cells (ASMCs) produce several cytokines during inflammation, causing changes in extracellular matrix components, leading to airway remodeling. Midkine (MK) promotes the chemotaxis of inflammatory cells and releases proinflammatory factors. Whether Notch and Midkine jointly affect the proliferation and apoptosis of ASMCs is unknown. This research aimed to study the role of MK in ASMCs using an LPS-induced acute lung injury model.

Methods: ASMCs were cultured in vitro and divided into five groups according to treatment: control, lipopolysaccharide (LPS), non-target siRNA, MK siRNA, and g-secretase inhibitor LY411575. Cell proliferation was assessed using the Cell Counting Kit-8 assay. Apoptosis was measured by flow cytometry. Changes in the levels of cytokines related to the MK/Notch2 signaling pathway were detected by Western blotting, qPCR, and immunofluorescence.

Results: LPS increased the mRNA and protein expression of MK and Notch2. MK silencing and LY411575 reduced this effect. LPS reduced the viability and increased the rate of apoptosis of ASMCs. This effect was attenuated by exogenous MK and enhanced by MK silencing and LY411575 treatment.

Conclusions: The MK/Notch2 signaling pathway plays a regulatory role in ASMC proliferation and apoptosis in airway inflammation.

Background

Acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) is characterized by acute and progressive respiratory failure caused by various factors[1]. ARDS may occur at the end-stage of several diseases. The occurrence and development of ALI/ARDS are mainly related to microbial infections and other factors, including severe trauma, non-cardiogenic shock, poisoning, long-term cardiopulmonary resuscitation, surgery, and acute pancreatitis[2].

Midkine (MK) is a heparin-binding growth factor that attracts inflammatory cells, releases cytokines, and participates in inflammation[3, 4]. MK activates Notch signaling[5, 6, 7]. MK induces the structural recombination of actin by binding to the Notch-2 receptor, resulting in the phosphorylation and activation of STAT3, promoting cell growth and differentiation. MK activates other pathways by inducing changes in the Notch-2 receptor and stimulating the expression of Hes1 and NF- κ B[8]. MK is highly expressed in cystic pulmonary fibrosis and ARDS[9, 10]. However, the mechanisms underlying MK actions are incompletely understood.

Airway smooth muscle cells (ASMCs) have contractile activity and produce inflammatory cytokines and growth factors, and these cells are regulated by extracellular matrix components[11, 12]. The abnormal proliferation and hypertrophy of ASMCs can lead to airway wall thickening and, ultimately, airway remodeling[13]. However, it is unclear whether the MK-Notch pathway participates in ASMC proliferation.

MK may inhibit apoptosis and promote ASMC proliferation through the Notch pathway, and these effects can be reversed by MK inhibition. This study assesses the effect of MK on rat ASMCS in vitro.

Methods

Materials

Rat airway smooth muscle cells were purchased from Guangzhou Gennio Biological Technology Co., Ltd. All experiments were approved by the Research Ethics Committee of our institution.

Cell culture

Airway smooth muscle cells were cultured in DMEM containing 10% FBS in a humidified incubator with 5% CO₂ at 37 °C, and the medium was changed every 2 days for subcultivation. Mid-logarithmic-phase ASMC cells were divided into five groups according to treatment, as follows: (a) Control: untreated. (b) lipopolysaccharide (LPS): cells treated with LPS (0.2 mg/mL) for 72 h. (c) Control siRNA: cells transfected with non-target siRNA and stimulated with LPS for 72 h. (d) MK-siRNA group: cells transfected with MK siRNA and stimulated with LPS (0.2 mg/mL) for 72 h. (e) LY411575 group: cells stimulated with LPS (0.2 mg/mL) for 72 h and treated with the γ-secretase inhibitor of Notch signaling LY411575 (0.03 mg/mL) for 48 h. The nucleotide sequence of MK siRNA is 5'-CAAAGGCCAAAAGC CAAGAAA-3', 5'-GAAGAGGCTCGGTACAAT-3', 5'-CGACTGCAAATACAA GTT-3'.

Cell survival

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) assay. ASMC cells were seeded in 96-well plates at 10⁵ cells/mL and 100 μL per well. The following experiments were carried out 1 day after inoculation: in experiments 1 and 2, the IC₅₀ of LPS and LY411575 was determined. The viability of ASMCS under different treatment conditions was determined by next experiments.

Western blotting

The total protein of ASMC cells from each group was extracted and prepared according to standard procedures and quantified using the BCA method. Proteins were separated on a 12% SDS polyacrylamide gel and transferred to a PVDF membrane. The membrane was incubated with primary antibodies (anti-MK, anti-Notch2, and anti-β-actin) (Abcam) at 4 °C overnight and then incubated with goat anti-rabbit IgG at room temperature for 1 h. The membrane was washed three times with TBST. Immunoreactive bands were visualized using the Tanon 5200 enhanced chemiluminescence detection system (Tanon, China) and quantified by densitometry using ImageJ. The relative expression of MK and Notch2 was measured in the four study groups.

qPCR analysis

The total RNA of ASMC cells from each group was extracted using TRizol and prepared according to standard procedures. RNA concentration and purity were determined using a NanoDrop

spectrophotometer. The following primers were used: MK, 5'-CAGACCCAGCGCATCATTG-3' (forward), 5'-TCTTGGAGGTGCAGGCTTG-3' (reverse); Notch2, 5'-GGTGGTCAAGAGCCCTGTGT-3' (forward), 5'-TGGCCTGCGTCACACAGTA-3' (reverse); GAPDH, 5'-CAGCCAGGAGAAATCAAACAG-3' (forward), 5'-GACTGAGTACCTGAACCGGC-3' (reverse). Primers were designed and synthesized by Bioengineering Co. Ltd. (Shanghai). Reverse transcription was performed using a reverse transcription kit (Thermo Fisher). Real-time quantitative PCR was carried out using a SYBR green PCR kit. The amplification conditions included a denaturation step at 95 °C for 5 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s, and an extension step at 60 °C for 30 s. The reaction mixture contained 10 µL of the Hieff® qPCR SYBR® Green Master Mix, 0.5 µL of each primer (10 µM), 1 µL of template DNA, and 8 µL of sterile ultrapure water. Gene expression was normalized to GAPDH using the $2^{-\Delta\Delta C_t}$ method. The mRNA expression of MK and Notch2 was determined in ASMCs from each group (Fig. 3).

Immunofluorescence microscopy

ASMCs (10^5 cells/mL) from each group were inoculated in dish (NEST 801002) which is used for confocal laser microscopy. Cells were washed in PBS three times (3 min each time), fixed in 4% paraformaldehyde for 15 min, and washed in PBS as described above. Cells were permeabilized with 0.5% Triton X-100 in PBS at 4°C for 15 min and washed in PBS. The slides were incubated with anti-MK and anti-Notch2 primary antibodies at 4°C overnight, washed with PBST three times (3 min each time), and incubated with fluorescein-conjugated goat anti-rabbit IgG for 1 h at room temperature. Non-specific reactions were blocked with normal goat serum for 30 min at room temperature. Nuclei were counterstained with DAPI. Sections were observed under a fluorescence microscope. The protein expression of MK and Notch2 in ASMCs of each group was evaluated by immunofluorescence microscopy.

Assessment of apoptosis by flow cytometry

ASMCs (10^5 cells/mL) were transferred to six-well plates and digested with trypsin without EDTA. Cells were resuspended in 1X binding buffer (BD Pharmingen™), incubated with 5 µL of annexin V-FITC (BD Pharmingen™) in the dark for 15 min at room temperature, and then incubated with 5 µL of propidium iodide for 15 min at room temperature.

Statistical analysis

Data were expressed as means and standard deviations and analyzed using SPSS software version 20.0. Inter-group and intra-group differences were evaluated by one-way analysis of variance and independent-sample t-test, respectively. P-values of less than 0.05 were considered statistically significant.

Results

Half maximal inhibitory concentration of LPS and LY411575

The effects of LPS and LY411575 on the viability of ASMC cells were evaluated using the CCK-8 assay. The IC₅₀ of LPS and LY411575 was 0.2 mg/mL and 0.03 mg/mL, respectively (Fig. 1).

Western blot analysis

LPS significantly increased MK expression ($P < 0.05$), and MK siRNA reduced this effect (Fig. 2). LPS significantly increased Notch2 expression ($P < 0.05$), and MK siRNA and LY411575 attenuated this effect (Fig. 2).

qPCR analysis

LPS significantly increased the relative mRNA expression of MK ($P < 0.05$), and MK siRNA reduced this effect (Fig. 3A). LPS significantly increased the relative mRNA expression of Notch2 ($P < 0.05$), and MK siRNA and LY411575 decreased this effect (Fig. 3B).

Immunofluorescence analysis

LPS significantly increased the expression of MK ($P < 0.01$), and both MK siRNA and non-target siRNA reduced this effect ($P > 0.05$) (Fig. 4A). LPS significantly increased the expression of Notch2 ($P < 0.05$), and MK siRNA and LY411575 abrogated this effect ($P < 0.05$) (Fig. 4B).

Effect of LPS and MK-siRNA on cell viability

LPS significantly decreased the viability of ASMCs ($P < 0.05$), and this effect was potentiated by MK siRNA transfection and LY411575 treatment (Fig. 5).

Effect of rMK on cell viability

Recombinant midkine(rMK) did not significantly affect the survival of ASMCs (Fig. 6).

Effect of rMK on cell viability in the presence of LPS

rMK significantly increased the survival of ASMCs treated with LPS for 48 and 72 h, and this increase was dose-independent (Fig. 7).

Effect of LPS and rMK on the rate of apoptosis

The apoptosis rate of ASMCs was determined by flow cytometry (Fig. 8A). LPS significantly increased the rate of apoptosis, and this effect was enhanced by MK silencing and reduced by rMK treatment (Fig. 8B).

Effects of LPS, MK silencing, and LY411575 on the rate of apoptosis

The apoptosis rate of ASMC cells was detected by flow cytometry (Fig. 9A). LPS increased the rate of apoptosis, and MK silencing and LY411575 potentiated this effect (Fig. 9B).

Discussion

ALI is a clinical disease with high mortality and high treatment burden. Despite great progress in diagnosis and treatment, disease prognosis is poor. ALI is characterized by the apoptosis and necrosis of vascular endothelial cells and impaired vascular integrity, leading to increased permeability and pulmonary edema. LPS is a glycolipid of the outermost membrane of Gram-negative bacteria. This glycolipid induces inflammatory reactions and cytokine secretion and has been shown to trigger ALI in animal models[14, 15]. In our study, the effect of LPS-induced ALI was assessed using a cell model. Some studies have found that TNF- α expression is increased in LPS-induced ALI. TNF- α reduces vascular permeability, leading to pulmonary vascular endothelial cell injury and pulmonary edema [16, 17]. MK promotes the release of MCP-1, MIP-2, and other chemokines, and the aggregation of macrophages and neutrophils[18]. MK expression is low in healthy organs and increases during oxidative stress, inflammation, and tissue repair[19, 20]. In this study, LPS increased the protein expression of MK in rat airway smooth muscle cells. However, the role of MK and related signaling pathways in ASMCs in LPS-induced ALI is incompletely understood.

MK promotes cell proliferation[21]. MK is expressed in many tumors and inflammatory diseases and has anti-apoptotic effects[22]. The present study assessed the effect of rMK on the proliferation and apoptosis of ASMCs. In a mouse model of acid- and ventilator-induced lung injury, an increase in collagen deposition and hydroxyproline levels and a decrease in lung compliance were attenuated in MK^{-/-} mice vs. wild-type mice[25]. The inhibition of Nox1, MK, and Notch2 attenuated epithelial-to-mesenchymal transition (EMT), demonstrating that MK plays an important role in airway remodeling [10, 23]. MK enhances the hypoxia-induced proliferation and differentiation of human lung epithelial cells [24]. Our results showed that ASMC survival was lower in the MK siRNA group than in the LPS group, indicating that MK reduced LPS-induced ALI. In addition, LPS decreased the viability and rate of apoptosis of ASMCs, whereas rMK attenuated these effects. These results show that MK participates in the repair of inflammatory tissues, increases ASMC survival, and inhibits apoptosis, leading to airway remodeling, and these effects are reversed by MK inhibition.

Notch2 is an MK receptor and an evolutionarily conserved pathway that regulates many cell-fate decisions during development. Notch signaling activity is regulated by receptor proteolysis. Upon proteolysis, the Notch intracellular domain travels to the nucleus and interacts with a transcription factor complex to regulate gene expression [25, 26]. The γ -secretase inhibitor of Notch signaling LY411575 inhibits osteoclast differentiation and bone destruction through the Notch/HES1/MAPK/Akt pathway[27]. Notch2 causes tumor invasion and metastasis by regulating EMT and is downregulated by Notch inhibitors and endogenous compounds, resulting in mesenchymal-to-epithelial transition[28]. Notch2 interacts with MK in human lung epithelial cells, and MK-induced EMT is dictated by mechanical stretching. Notch2 signaling inhibition blocks EMT more effectively than endogenous MK. Silencing MK or Notch2 reduces pulmonary fibrosis[29]. Notch2 is required for inflammatory cytokine-driven goblet cell metaplasia in the human lung, and Notch2 inhibition can be a therapeutic strategy for preventing this pathological process in airway diseases[30]. Antisense oligonucleotides (ASOs) downregulated Jag1 and

Notch2 in goblet cell metaplasia associated with allergen-induced asthma and upregulated the ciliated cell marker FoxJ1. Moreover, the ASO-mediated decrease in Jag1 and Notch 2 expression inhibited goblet cell metaplasia, mucus production, and airway remodeling [31]. Our results showed that LPS increased the mRNA and protein expression of MK and Notch2, consistent with previous studies. Tian et al. found that MK promoted the proliferation of gastric cancer cells through Notch signaling, whereas the cisplatin-induced apoptosis of these cells was reduced by rMK and enhanced by MK silencing. Apoptosis may be induced in MK siRNA-transfected cells by an apoptosome-dependent mechanism, with the downregulation of Bcl-2 expression, upregulation of Bax expression, reduction in mitochondrial membrane potential, release of cytochrome c, and the activation of caspases 3, 8, and 9[32, 33]. This study found similar results in airway smooth muscle cells. The inhibition of MK and Notch2 expression repressed the proliferation of ASMCs and promoted apoptosis, demonstrating that MK and Notch2 jointly participate in lung tissue remodeling.

Conclusion

Our results demonstrate that MK has a role in ALI. MK promotes the proliferation of ASMCs and reduces cell apoptosis through Notch2 signaling. This study helped elucidate the role of ASMCs in a rat model of airway remodeling. The results confirmed that MK plays a role in ASMC proliferation in vitro in an LPS-induced ALI model through Notch2 signaling. However, the functions of proteins downstream of the MK/Notch2 signaling pathway are unclear, and the role of MK can be further investigated using other gene-delivery technologies, such as lentiviral vectors.

Abbreviations

COPD: Chronic obstructive pulmonary disease; LPS: Lipopolysaccharide; MK: Midkine; rMK: recombinant midkine; IC50: Half-maximal inhibitory concentration; PTP ζ : Protein tyrosine phosphatase- ζ ; ALK: Anaplastic lymphoma kinase; ALI: Acute lung injury; WB: Western blotting; RAS: Renin-angiotensin system; MAPK: Mitogen-activated protein kinase; Bcl-2: B-cell lymphoma 2; NADPH: Nicotinamide adenine dinucleotide phosphate; EMT: Epithelial-to-mesenchymal transition; TNF- α : Tumor necrosis factor alpha; TGF- β : Transforming growth factor beta; ASO: Antisense oligonucleotide; ASMC: Airway smooth muscle cells.

Declarations

Declaration of interest

The authors declare that they have no conflict of interest related to the present study.

Acknowledgements

Not applicable.

Authors' contributions

LX and SY contributed conception, design, and quality control of the study. , DT, LL, QJ, LQ, LK, XS, WH and WX performed the experiments. HQ, DT managed data analysis and manuscript writing. LX and HQ edited the language. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate

This study received permission from the ethics committee of Zhongshan hospital, Fudan University. Written informed consent was obtained from all patients.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest

References

1. Siempos II , Choi A M K . Midkine: In the Middle of the Pathogenesis of Acute Respiratory Distress Syndrome-associated Lung Fibrosis?. American Journal of Respiratory & Critical Care Medicine.2015;192(3):271-2.
2. Lambris,John,D.and K Ritis.Method of Treating Acute Respiratory Distress Syndrome . US.2011.
3. Krzystek-Korpacka M, Mierzchala M, Neubauer K, Durek G, Gamian A. Midkine, a multifunctional cytokine, in patients with severe sepsis and septic shock: A pilot study. Shock. 2011; 35(5):471–477.
4. Salaru, Delia Lidia, Arsenescu-Georgescu C , Chatzikyrkou C, J Karagiannis,A Fischer,et al. Midkine, a heparin-binding growth factor, and its roles in atherogenesis and inflammatory kidney diseases.Nephrol Dial Transplant.2016;(11):1781-1787.
5. Kishida S, Mu P,Miyakawa S,Fujiwara M, Abe T,Sakamoto K,et al.Midkine promotes neuroblastoma through notch2 signaling. Cancer Research.2013;73(4), 1318-1327.

6. Liedert A, Mattausch L, Rontgen V, Blakytyn R, Vogele D, Pahl M, et al. Midkine-deficiency increases the anabolic response of cortical bone to mechanical loading. *Bone*. 2011;48, 945-951.
7. K Shimizu, S Chiba, T Saito, K Kumano, Y Hamada, H Hirai. Functional diversity among notch 1, notch 2, and notch 3 receptors. *Biochemical & Biophysical Research Communications*. 2002;291(4), 775-779.
8. S Kamakura, K Oishi, T Yoshimatsu, M Nakafuku, N Masuyama, Y Gotoh. Hes binding to STAT3 mediates crosstalk between Notch and JAK-STAT signalling. *Nature Cell Biology*. 2004;6(6):547-554.
9. SL Nordin, S Jovic, A Kurut, C Andersson, A Gela, A Bjartell, et al. High expression of midkine in the airways of patients with cystic fibrosis. *American Journal of Respiratory Cell & Molecular Biology*. 2013;49(6), 935-942.
10. Zhang R, Pan Y, Fanelli V, Wu S, Luo AA, Islam D, et al. Mechanical stress and the induction of lung fibrosis via the midkine signaling pathway. *American Journal of Respiratory and Critical Care Medicine*. 2015; 192:315–323.
11. B G Dekkers, Dedmer Schaafsma, S Adriaan Nelemans, Johan Zaagsma, Herman Meurs. Extracellular matrix proteins differentially regulate airway smooth muscle phenotype and function. *AJP: Lung Cellular and Molecular Physiology*. 2007;292(6), L1405-L1413.
12. Postma D S, W Timens. Remodeling in asthma and chronic obstructive pulmonary disease. *Proc Am Thorac Soc*. 2006;3:434–439.
13. Martinez F D. The origins of asthma and chronic obstructive pulmonary disease in early life. *Proceedings of the American Thoracic Society*. 2009;6(3), 272-277.
14. MS Khan, T Ali, MW Kim, MH Jo, MG Jo, H Badshah, et al. Anthocyanins protect against LPS-induced oxidative stress-mediated neuroinflammation and neurodegeneration in the adult mouse cortex. *Neurochemistry International*. 2016;100:1-10.
15. MC Oliveira, FR Greiffo, NC Rigonato-Oliveira, RWA Custódio, VR Silva, NR Damaceno-Rodrigues, et al. Low level laser therapy reduces acute lung inflammation in a model of pulmonary and extrapulmonary LPS-induced ARDS. *Journal of Photochemistry & Photobiology B Biology*. 2014;134:57-63.
16. Mukhopadhyay S, Hoidal J R, Mukherjee T K. Role of TNF α in pulmonary pathophysiology[J]. *Respiratory research*, 2006, 7(1):125
17. Suat, Erdogan, Kader, Turkekul, Ilker. Dibirdik, et al. Midkine silencing enhances the anti-prostate cancer stem cell activity of the flavone apigenin: cooperation on signaling pathways regulated by ERK, p38, PTEN, PARP, and NF- κ B. *Investigational New Drugs*. 2019.
18. T Kosugi, Y Yuzawa, W Sato, H Arata-Kawai, N Suzuki, N Kato, et al. Midkine is involved in tubulointerstitial inflammation associated with diabetic nephropathy. *Laboratory investigation; a journal of technical methods and pathology*. 2007;87(9), 903-13.
19. S Taskinen, O Leskinen, J Lohi, M Koskenvuo, M Taskinen. Effect of Wilms tumor histology on response to neoadjuvant chemotherapy. *Journal of Pediatric Surgery*. 2018;54(4).

20. Sato W , Sato Y.Midkine in nephrogenesis, hypertension and kidney diseases. *British Journal of Pharmacology*. 2014;171(4):879-87.
21. Takashi M.Midkine and Pleiotrophin: Two Related Proteins Involved in Development, Survival, Inflammation and Tumorigenesis. *Journal of Biochemistry*. 2002;132:359–371.
22. Yazhan N , Kocak M K , Akcl E . Midkine in Inflammation and Inflammatory Diseases. Springer Netherlands, 2012.
23. LT Weckbach,L Groesser,J Borgolte,JI Pagel,B Walzog.Midkine acts as proangiogenic cytokine in hypoxia-induced angiogenesis. *AJP Heart and Circulatory Physiology*. 2012;303(4):H429-38.
24. Zhang H,Okamoto M,Panzhinskiy E,Zawada WM,Das M.PKCδ/midkine pathway drives hypoxia-induced proliferation and differentiation of human lung epithelial cells. *Am J Physiol Cell Physiol*. 2014;306(7):648-58.
25. Fortini M E . Notch Signaling: The Core Pathway and Its Posttranslational Regulation. *Developmental Cell*, 2009, 16(5):633-647.
26. Kopan R, Ilagan MX.The canonical notch signaling pathway: unfolding the activation mechanism. *Cell*.2009;137:216–33.
27. Chen Xinwei,Chen Xuzhuo,Zhou Zhihang et al. LY411575, a potent γ-secretase inhibitor, suppresses osteoclastogenesis in vitro and LPS-induced calvarial osteolysis in vivo.[J] .*J Cell Physiol*, 2019, 234: 20944-20956.
28. Anderberg J, Gray J,Paul M,Kevin N.Methods and compositions for diagnosis and prognosis of renal injury and renal failure. 2014.
29. F Zhu,L Tang,F Qiu,J Fan,Q Zhou,X Ding,et al. Preventive Effect of Notch Signaling Inhibition by a γ-Secretase Inhibitor on Peritoneal Dialysis Fluid-Induced Peritoneal Fibrosis in Rats, *The American Journal of Pathology*. 2010;176(2):650-659.
30. Danahay H, Pessotti AD,Julie C,E Brooke,Montgomery,et al.Notch2 is required for inflammatory cytokine-driven goblet cell metaplasia in the lung.*Cell Rep*. 2015;10(2):239–52.
31. M CarrerJR CrosbyG SunC ZhaoTR Grossman. Antisense Oligonucleotides Targeting Jagged 1 Reduce House Dust Mite-Induced Goblet Cell Metaplasia in the Adult Murine Lung,*Am J Respir Cell Mol Biol*. 2020.
32. Slutsky A S , Ranieri V M.Ventilator-Induced Lung Injury. *New England Journal of Medicine*. 2013;369(22):2126-2136.
33. Tian W Y,Shen J Q,Chen W C.Suppression of midkine gene promotes the antitumoral effect of cisplatin on human gastric cancer cell line AGS in vitro and in vivo via the modulation of Notch signaling pathway. *Oncology Reports*. 2017;38:745-754.

Figures

Figure 1

The half-maximal inhibitory concentration of LPS (A) and the γ -secretase inhibitor of Notch signaling LY411575 (B) on airway smooth muscle cells in a rat model of acute lung injury. Values are means and standard deviations of six independent experiments.

Figure 2

Western blot analysis of the expression of MK (A and B) and Notch2 (C and D) in airway smooth muscle cells in a rat model of acute lung injury.* $P < 0.05$ vs. the control. # $P < 0.05$ vs. the LPS group. Data are means and standard deviations of three independent experiments.

Figure 3

Effect of LPS treatment, LPS + non-target siRNA transfection, LPS + MK siRNA transfection, and treatment with LPS and the γ -secretase inhibitor LY411575 on the relative mRNA expression of midkine and Notch2 in airway smooth muscle cells in a rat model of acute lung injury. * $P < 0.05$ vs. the control. # $P < 0.05$ vs. the LPS group. Data are means and standard deviations of three independent experiments.

Figure 4

Immunohistochemical expression of midkine and Notch2 in airway smooth muscle cells in a rat model of acute lung injury. (A) Midkine expression in the control, LPS, non-target siRNA transfection, and MK siRNA transfection groups ($\times 400$). (B) Notch2 expression in the control, LPS, MK-siRNA, and LY411575-treated groups ($\times 400$). Images are representative of three biological replicates. Cell nuclei (blue) were stained with DAPI. Midkine and Notch2 (green) localized to the cytoplasm and cell membrane, respectively.

Figure 5

Effect of LPS, non-target siRNA transfection, MK siRNA transfection, and γ -secretase inhibitor LY411575 on the viability of airway smooth muscle cells in a rat model of acute lung injury. * $P < 0.05$ vs. the control.

#P<0.05 vs. the LPS group. Data are means and standard deviations of six independent experiments.

Figure 6

Survival of airway smooth muscle cells treated with different concentrations of rMK. Data are means and standard deviations of six independent experiments.

Figure 7

Effect of rMK on the viability of airway smooth muscle cells treated with LPS. Cells were treated with 0.1 mg/mL (A and B), 0.2 mg/mL (C and D), or 0.3 mg/mL of LPS (E and F) for 48 or 72 hours in the presence and absence of midkine. * $P < 0.05$ vs. the untreated group. # $P < 0.05$ vs. the LPS group. Data are means and standard deviations of six independent experiments.

Figure 8

Effect of LPS, MK siRNA transfection, and rMK treatment on the rate of apoptosis of airway smooth muscle cells in a rat model of acute lung injury. (A) Representative FACS. (B) Rate of apoptosis. * $P < 0.05$ vs. the untreated group. # $P < 0.05$ vs. the LPS group. Data are means and standard deviations of three independent experiments.

Figure 9

Effect of LPS, MK siRNA transfection, and LY411575 treatment on the rate of apoptosis of airway smooth muscle cells in a rat model of acute lung injury. (A) Representative FACS. (B) Rate of apoptosis. * $P < 0.05$ vs. the control. ## $P < 0.01$ vs. the LPS group. Data are means and standard deviations of three independent experiments.