Telocytes Reduce Oxidative Stress by Downregulating DUOX2 Expression in Inflamed Lungs of Mice

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Research

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Abstract

Background: Telocytes (TCs), a newly identified interstitial cell type, have been found to participate in tissue protection and repair. This study investigated the antioxidative effects of TCs in inflamed lungs of mice.

Methods: Acute respiratory distress syndrome mice were used as model of inflamed lungs of mice. Gene sequencing was used to screen for differentially expressed miRNAs in TCs after lipopolysaccharide (LPS) stimulation. AntagomiR-146a-5p-pretreated TCs were first injected into mice, and the antioxidant activity of TCs was estimated. TCs, RAW264.7 cells, and MLE-12 cells were collected for mRNA, miRNA, and protein measurements after LPS stimulation. Silencing miRNAs were delivered to examine the involved signaling pathways in the experiments. Oxidative stress was examined in cultured cells, supernatants, and lung tissue by measuring malondialdehyde (MDA) levels.

Results: MiR-146a-5p and miR-21a-3p were upregulated in TCs after LPS stimulation. Compared with ARDS mice, TC-preinfused ARDS mice had reduced lung tissue injury scores, lung wet-dry ratios, white blood cell counts in alveolar lavage fluid and MDA concentrations in lung tissue. However, ARDS mice preinfused with antagomiR-146a-5p TCs did not exhibit any of the corresponding changes. DUOX2 was downregulated in TCs at both the gene and protein levels, and the concentration of MDA was decreased in TCs and their culture medium after LPS stimulation. DUOX2 expression was restored when TCs were treated with antagomiR-146a-5p. CREB1 was downregulated by miR-146a-5p, as confirmed by a dual-luciferase reporter assay, and the protein expression of DUOX2 was downregulated by CREB1, which was further confirmed by treating TCs with a specific CREB1 inhibitor.

Conclusion: LPS stimulation increases miRNA-146a-5p expression in TCs, which downregulates the CREB1/DUOX2 pathway, resulting in a decrease in oxidative stress in cultured TCs. TCs reduce LPS-induced oxidative stress by decreasing DUOX2 expression in inflamed lungs of mice.

Introduction

Acute respiratory distress syndrome (ARDS) is an acute clinical symptom characterized by non-cardiogenic pulmonary edema and hypoxic dyspnea (1–4). Enhance oxidative stress is an important mechanism underlying the development of ARDS(5, 6). It is reported that ARDS patients have higher levels of hydrogen peroxide in their breath than healthy subjects(7, 8). In pulmonary tissue, oxidative stress reduces the formation of alveolar surfactants, leading to a collapse of alveoli. Antioxidant treatments reduce leakage in bronchoalveolar lavage and mitigate pulmonary edema in LPS-induced lung injury (9).

TCs is an interstitial Cajal-like cell featured with its typical telopodes (10). The presence of TCs has been reported in the atrial and ventricular myocardium(11), gastrointestinal(12), and kidneys (13). TCs exert protective effects in acute kidney injury(14), infected lung(15) as well as infarcted myocardium(16). In our previous studies, exogenous TCs protected against LPS-induced pulmonary injury by reducing

cytokine levels (15) and promoting angiogenesis (17). In in vitro experiments cocultured with primary human dermal microvascular endothelial cells (HDMECs) as well as mouse fibroblast cells (L929), TCs significantly reduced LPS-induced oxidative stress (18), indicating that they have antioxidative features. However, the antioxidant role of TCs in LPS-induced lung injury has been less reported.

MicroRNAs (miRNAs), which are small noncoding ribonucleic acid molecules, regulate target genes by inhibiting mRNA translation or promoting mRNA degradation. It has been reported that miRNAs regulate reactive oxygen species (ROS) in cardiovascular disease[19]. MiRNA-146a-5p regulates DUOX2 expression through both signal transducer and activator of transcription 1 (STAT1)[20] and cAMP-response element-binding protein 1 (CREB1)[21].

Previous work in our lab has reported that LPS stimulation increases the expression of miR-146a-5p and miR-21a-3p in TCs[17], which takes part in the regulation of target genes by inhibiting mRNA translation or promoting mRNA degradation[22, 23]. Thus, the present study aimed to investigate the antioxidant effects of TCs in the inflamed lungs of mice, with a special focus on the underlying mechanisms of noncoding RNAs on the enzymes involved in oxidative stress in TCs.

Previous work of our lab has reported that LPS stimulation increases expressions of miR-146a-5p and miR-21a-3p in TCs(17), which takes part in the regulation of target genes by inhibiting mRNA translation or promoting mRNA degradation(19, 20). Thus, the present study aimed to investigate the antioxidant effects of TCs in the inflammation lung of mouse, and special attention was paid to the underlying mechanisms of non-coding RNA on enzymes involved in oxidative stress in TCs.

Methods

Animal preparation

Twenty-four male C57BL/6 mice were obtained from the Shanghai SLAC animal Company (SLAC, Shanghai, China PRC) and kept in a pathogen-free condition. The mice had free access to food and water. In the present study, mice were randomly divided into the control group, LPS stimulation group, TCs treated group in mice challenged with LPS, and TCs silencing miRNA-146-5p in mice challenged with LPS. The mouse ARDS model was set up by instilling LPS (5 mg/kg) via the trachea. TCs (1×10^6 cells) transfected with antagomir-146a-5p or not were administrated via the tail vein two hours before the LPS stimulation. The animals were sacrificed, and the lungs and bronchoalveolar lavage fluid were collected twenty-four hours after the establishment of the mouse ARDS model.

All animal procedures were performed by bioethics guidelines and were approved by the animal committee of Zhongshan Hospital, Fudan University, Shanghai, China PRC.

Assessment of lung injury

To collect white blood cells in bronchoalveolar lavage fluid, mouse lungs were perfused with ice-cold phosphate-buffered saline. After centrifugation, white blood cells were counted under microscopy. The left lung was fixed in 10% formalin solution and embedded in paraffin for [hematoxylin and eosin staining](#) [H&E stain]. The right lung was collected to assess edema by the ratio of wet weight/dry weight (W/D) of the lung after incubating in a 60°C oven for 48 hours. The lung injury was evaluated by a modified lung injury histological scoring system(21). In brief, lung injury was scored on a scale of 0-4 using the average score of the following items: (I) alveolar capillary congestion, (II) hemorrhage, (III) infiltration of neutrophils into the airspace or the vessel wall, and thickness of the alveolar wall, and (IV) alveolar wall thickness/hyaline membrane formation. A score of 0 represented normal findings, and scores of 1, 2, 3, and 4 represented mild (<25% lung involvement), moderate (25-50% lung involvement), severe (50-75% lung involvement), and very severe (>75% lung involvement) lung injury. The cytokines levels of IL-1, IL-6, TNF- α in bronchoalveolar lavage fluid were detected by Bio-Rad Bio-Plex Pro Mouse Cytokine 23-Plex Assay on the Bio-Rad MAGPIX Multiplex Reader

Cell Culture

Mouse lung TCs were kindly provided by Dr. Wang X (Biomedical Research Center, Zhongshan Hospital, Fudan University, Shanghai, China). Mouse RAW264.7 and MLE-12 cells were bought from Zhongqiao Tech (Zhongqiao, Shanghai, China PRC). All the cells were cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12, Hyclon, Gaithersburg, USA).

HEK293T cells were cultured in DMEM high glucose medium (Hyclon, Gaithersburg, USA) for the Dual-luciferase reporter assay.

Reverse transcription-polymerase chain reaction (RT-PCR)

Cultured TCs were collected in TRIzol reagent (Takara, Shiga Japan). MicoRNAs were reversely transcribed with a Bulge-Loop miRNA qRT-PCR Starter Kit (Ribobio, Guangzhou, China), and mRNAs were reversely transcribed to complementary DNA (cDNA) with a PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Shiga, Japan). Both miRNA and mRNA were amplified on an ABI 7500 sequence detection system (Applied Biosystems, Inc., Foster, USA). The expression levels of miR-21a-3p and miR-146a-5p were normalized with U6. and mRNAs expressions were normalized with Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH). Primer set of miR-21a-3p and miR-146a-5p were provided by Ribobio (Ribobio, Guangzhou, China). Primers of mRNA used in the present study were synthesized by Sangon Biotech Co., Ltd (Shanghai, China) (Table 1).

Western Blotting

Cultured cells were lysed in RIPA buffer containing protease inhibitors (Roche, Indianapolis, IN, USA), phosphatase inhibitors (Beyotime Shanghai, China), and phenylmethanesulfonyl fluoride (PMFS) (Beyotime Shanghai, China). Total protein concentration was determined using the BCA assay (Beyotime, Shanghai, China) Samples (30 μ g) were loaded for protein electrophoreses. Polyvinylidene Fluoridewere

(PVDF) membranes were blocked with 5% bovine serum albumin in tris-buffered saline mixed with 0.1% tween 20. The membranes were then incubated with primary antibody against JAK1 (1:1000), phosphorylated JAK1(1:500), STAT1 (1:1000), phosphorylated STAT1(1:500) CREB (1:1000), phosphorylated CREB (1:500), DUOX2 (1:1000), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:10000) at 4°C for overnight. On the second day, the membranes were further incubated with an HRP-conjugated anti-rabbit IgG secondary antibody (1:2000) for one hour. Target protein signals were visualized with Omni-ECL Femto light chemiluminescence kit (EpiZyme, Shanghai, China) in a fluorescent image detection system (Tanon-52Multi, Tanon, Shanghai, China). The related signals were quantified using ImageJ software and normalized with GAPDH.

Cell transfection

Antagomir-146a-5p was bought from Ribo Biotech (Ribo, Guanzhou, China) and delivered by Lipofectamine RNAiMAX (Thermo Fisher, Carlsbad, MA, USA) according to the manufactory's protocol. In brief, 50 nM antagomiR-146a-5p was prepared in a serum-free and antibiotic-free medium. After transfection, TCs were supplied with full growth medium until confluence. For animal experiments, TCs (1×10^6) were freshly prepared in 100 μ l phosphate-buffered saline.

Dual-luciferase reporter assay

The PHY-881 reporter vector (Hanyin Biotechnology, Shanghai, China) was used to construct the luciferase reporter vector plasmids PHY-881-WT-CREB1-3'-UTR and PHY-881-Mut-CREB1-3'-UTR. The plasmid CREB1-WT or CREB1-Mut were co-transfected with miR-146a-5p mimic or negative control into human embryonic kidney (HEK293T) cells using Lipofectamine 2000 reagent (ThermoFisher, Carlsbad, MA, USA). A dual-luciferase reporter assay system (Promega, Madison, WI, USA) was used to assess luciferase activity after 24 h of transfection. Relative firefly luciferase activity was normalized to renilla luciferase activity.

Malondialdehyde(MDA) assay

Malondialdehyde levels in TCs and culture medium were measured according to the manufacturer's instructions. Briefly, culture medium or cell lysates were incubated in MDA working solution at boiling temperature for 15 min. The MDA concentration was measured at absorbance 532 nm and normalized with protein concentration in culture medium or cell lysates, respectively.

Reagents and antibodies

LPS was purchased from Sigma (Sigma-Aldrich, St. Louis, MO). Antibodies against JAK1 (CST-3344), phosphorylated JAK1(CST-74129), STAT1 (CST-14994), phosphorylated STAT1(CST-7649), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, CST-5558) were purchased from [Cell Signaling Technology](#) (CST, Danvers, MA, USA). Antibodies against CREB1 (ab31387) and phosphorylated CREB1 (ab32096) were purchased from Abcam (Abcam, Cambridge, MA, USA). Antibody against DUOX2 (NB110-

61576ss) was purchased from NOVUS (NOVUS, Centennial, CO, USA). Specific inhibitors of CREB1, KG-501(HY-103299), and 666-15(HY-101120) were purchased from MCE (MedChem Express, Monmouth Junction, NJ, USA), Lipofectamine RNAiMAX was purchased from Thermo Scientific (Thermo, Rockford, MA, USA). Bicinchoninic acid assay (BCA) kit, RIPA lysis buffer, and loading buffer were purchased from Beyotime Biotechnology (Beyotime Shanghai, China PRC).

Statistical analysis

Data were presented as mean $\pm$ standard error of the deviation. The data were analyzed using the GraphPad Prism software (GraphPad Software, LaJolla, CA). One-way analysis of variance (ANOVA) was used for comparison. $P < 0.05$ was considered statistically significant.

Results

Exogenous TCs ameliorate LPS-induced ARDS, and antagomir-146a-5p pretreated TCs lost the lung protection role in ARDS

LPS significantly increased expressions of miR-146a-5p and miR-21a-3p in TCs (Fig. 1A). The ARDS model was set up by LPS instillation(22, 23).TCs reduced score of lung tissue injury (Fig. 1B & C), W/D ratio of the lung, white blood cells, and cytokine concentration in alveolar lavage fluid in ARDS mice (Fig. 1C). However, when silenced with miR-146a-5p, TCs failed to exert any protective effects in LPS-stimulated ARDS mice(Fig. 1B & C).

Antagomir-146a-5p pretreated TCs decrease oxidative stress both in vitro and vivo

MDA is the product of lipid oxidative stress, and it correlates with the severity of oxidative stress. MDA levels in the lung tissue of ARDS mice were increased,but decreased in the TCs-treated group and increased again when infused TCs pretreated with antagomir-146a-5p(Fig. 2A). MDA levels in the TCs and its culture media were decreased when treated with LPS, and MDA level was restored when treated with antagomir-146a-5p(Fig. 2B & C). Both KG-501 and 666 – 15 reduced the MDA level in TCs pretreated with antagomir-146a-5p (Fig. 2B and C).

The miR-146a-5p decreased oxidative stress through DUOX2 in TCs

To screen out the enzymes involved in TCs protection, both RAW264.7 and MLE-12 cells were used in the present study. RAW264.7 were accumulated in the lung while ARDS and MLE-12 cells were the pulmonary epithelial cells that were destroyed in ARDS. In cultured RAW247.6, LPS stimulation significantly increased mRNA expressions of oxidative enzyme NADPH oxidases 1 (NOX1), NOX2, Dual oxidase 1 (DUOX1), and DUOX2, but significantly decreased mRNA expressions of antioxidant enzymes Superoxide dismutase 3 (SOD3), Glutathione peroxidase 1(GPX1), GPX2, and Catalase (CAT) (Fig. 3A). In MLE-12 cells, LPS significantly increased mRNA expressions of DUOX2 and SOD3, but significantly reduced of NOX1 and NOX2 (Fig. 3A). In TCs, LPS significantly increased SOD2, SOD3 mRNA expression, but significantly reduced NOX2, NOX3, NOX4, and DUOX2 mRNA expression (Fig. 3A). MDA levels increased

in both RAW247.6 and MLE-12 cells but reduced in TCs after LPS stimulated(Fig. 3B). Since DUOX2 was the candidate gene distinguishing TCs from RAW247.6 and MLE-12, DUOX2 protein expression was measured in the three cells stimulated with LPS. DUOX2 protein level was significantly increased in RAW247.6 and MLE-12 cells but significantly reduced in TCs after LPS treated(Fig. 3C). Silencing miR-146a-5p, but not miR-21a-3p upregulated DUOX2 mRNA expression and protein presence in TCs stimulated with LPS (Fig. 3D).

Mir-146a-5p regulated the expression of DUOX2 through CREB1 in TCs

The expression of CREB1 in TCs was decreased when treated with LPS, and increased when TCs were pre-treated with antagomiR-146a-5p. LPS significantly reduced CREB1 protein expression, both phosphorylated and total protein. Antagomir-146-5p restored CREB expression in the presence of LPS (Fig. 4A). Dual-luciferase reporter assay revealed that miR-146a-5p mimic significantly reduced CREB1 promoter activity in wild type HEK293T cells, but not in HEK293T cells mutated CREB1(Fig. 4B). Antagomir-146-5p significantly increased DUOX2 protein expression in TCs when compared with cells transfected with the negative control. In the presence of antagomir-146-5p and LPS, pharmacological inhibitors of CREB1, KG-501, and 666 – 15, significantly reduced DUOX2 protein expression (Fig. 4C).

JAK1 and STAT1 did not take part in the expression of DUOX2 in TCs

LPS significantly increased phosphorylated levels of JAK1(Fig. 5A), however, did not change after treated with antagomir-146a-5p(Fig. 5B). LPS stimulation increased phosphorylated levels of STAT1 on tyrosine 701 residue(Fig. 5A), which was restored by antagomir-146a-5p incubation, but it failed to change the expression of DUOX2(Fig. 5B).

Family members of JAK/STAT1 did not take part in the expression of DUOX2 in TCs

Phosphorylated levels of JAK2, STAT3 and STAT5 were significantly increased, while other did not change after LPS stimulation (Fig. 6), however, all the members did not change after treated with antagomir-146a-5p, and failed to change the expression of DUOX2(Fig. 7).

Discussion

The present study reports that oxidative enzyme, dual oxidase 2, plays a vital role in the protective effect of TCs in a mouse ARDS model. In LPS stimulation, DUOX2 protein expression is decreased in mouse TCs, which is regulated by transcription factor CREB1. Enhanced expression of miRNA-146-5p inhibits CREB1 transcription, resulting in reduced expression of DUOX2 protein as well as a decrease in oxidative stress(Fig. 8).

The enzyme DUOX2, a member of the nicotinamide adenine dinucleotide phosphate (NADPH) family, has a peroxidase homology domain and a gp91phox domain. Increased expression of DUOX2 has been reported in human pancreatic cancer cells when exposed to IFN- γ (24). In the present study, DUOX2

expression was reduced in pulmonary TCs, but increased in RAW264.7 and MLE-12, suggesting that TCs exert distinguished antioxidative effects in response to LPS as well as in the mouse ARDS model.

DUOX2 protein is regulated at a transcriptional level. In cultured human pancreatic cells, interferon-gamma activates signal transducer and activator of transcription 1 (STAT1), shown as increased phosphorylation on tyrosine 701 residue, leading to the upregulation of DUOX2 protein(24, 25). In the present study, LPS stimulation increased phosphorylated levels of STAT1 in TCs, but reduced DUOX2 expression. Therefore it indicates that STAT1 does not regulate DUOX2 protein in the TCs. Cyclic AMP response element protein 1(CREB1) binds to the promoter of the DUOX2 gene. In the present study, LPS stimulation reduced CREB1 protein expression, both phosphorylated and total protein. Inhibition of CREB1, in the presence of antagomiR-146-5p in which CREB1 and DUOX2 expressions were restored, downregulated DUOX2 protein supporting the note that CREB1 regulates DUOX2 protein expression.

MicroRNAs (miRNAs), small non-coding ribonucleic acid molecules, regulate target genes by inhibiting mRNA translation ,or promoting mRNA degradation(26, 27). It is proposed that miRNAs are potential therapeutic targets in acute lung injury(28), since mir-216a level was decreased in ARDS patients, and the reduced expression of mir-216a was negatively correlated with the mortality of ARDS patients, overexpressing mir-216a decreases TNF α , IL-6, and IL-1 β levels in adenocarcinomic human alveolar basal epithelial A549 cells stimulated with LPS (29). Inhibition of miR-26a disrupts human lung microvascular endothelial cells barrier via increasing the expression of Eph receptors EA2(30). Neutrophil-derived miR-223 accelerates acute lung injury by downregulating poly(ADP-ribose)polymerase-1(PARP-1) protein (31). MicroRNA-146a alleviates LPS-mediated inflammation in a mouse ARDS model by inhibiting protein expressions of interleukin-1 receptor-associated kinase 1 and TNF receptor-associated factor 6(32, 33). MicroRNA-21-3p promotes angiogenesis in endothelial cells under inflammatory conditions by regulating E2F8-Notch2 protein expressions (15, 17). In the present study, miR-146a-5p regulated CREB1 protein expression by binding its promoter, leading to a decreased expression of DUOX2 protein, indicating that miR-146-5p exerts beneficial effects by downregulating oxidative enzymes in TCs.

In conclusion, miR-146a-5p in TCs downregulates the expression of DUOX2 via the CREB1 pathway and white blood cell administration owned protect the function of the lung when undergone oxidative stress damage.

Abbreviations

ARDS, acute respiratory distress syndrome; ROS, reactive oxygen species; DUOX2, dual oxidase 2; CREB1, cAMP-response element-binding protein 1; MDA, malondialdehyde; NADPH, nicotinamide adenine dinucleotide phosphate; NOX, NADPH oxidases; SOD, superoxide dismutase; GPX, glutathione peroxidase; CAT, catalase; STAT, signal transducer and activator of transcription.

Declarations

Authors' contributions

Tao Liang and Nu Zhang designed the study and completed the experimental process, literature search, and generation of figures, Huihui Ju, Yile Zhou, Yajie Yang, and Haihong Tang completed the experimental process and generation of figures. Tao Liang, Nu Zhang, Dongli Song, and Hao Fang wrote and edited the manuscript. All authors reviewed the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Availability of data and material

All data generated or analysed during this study are included in this published article.

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Table

Table 1.

Sequences of PCR primers		
Genes	Forward	Reverse
GAPDH	GTTCAACGGCACAGTCAAG	GCCAGTAGACTCCACGACAT
NOX1	GGTTGGGGCTGAACATTTTTTC	TCGACACACAGGAATCAGGAT
NOX2	TGTGGTTGGGGCTGAATGTC	CTGAGAAAGGAGAGCAGATTTTCG
NOX3	CAACGCACAGGCTCAAATGG	CACTCTCGTTCAGAATCCAGC
NOX4	CCAAATGTTGGGCGATTGTGT	CAGGACTGTCCGGCACATAG
DUOX1	AAAACACCAGGAACGGATTGT	AGAAGACATTGGGCTGTAGGG
DUOX2	AAGTTCAAGCAGTACAAGCGAT	TAGGCACGGTCTGCAAACAG
SOD1	GGAACCATCCACTTCGAGCA	CCCATGCTGGCCTTCAGTTA
SOD2	TTCTGGACAAACCTGAGCCC	GTCACGCTTGATAGCCTCCA
SOD3	CCTTCTTGTTCTACGGCTTGC	TCGCCTATCTTCTCAACCAGG
GPX1	AGTCCACCGTGTATGCCTTCT	GAGACGCGACATTCTCAATGA
GPX2	AAGTGTGACGTCAATGGGCA	TGAGGGAGAACGGGTCATCA
CAT	AGCGACCAGATGAAGCAGTG	TCCGCTCTCTGTCAAAGTGTG

Figures

Figure 1

MiR-146a-5p in TCs played an essential lung protection role in the mouse ARDS model. (A) LPS significantly increased the expression of miR-146a-5p and miR-21a-3p in TCs. (B,C) Compared with ARDS mice, TCs-treated ARDS mice had a reduced lung tissue injury score, a lower W/D ratio of the lung, fewer white blood cells, and lower concentration of cytokines in alveolar lavage fluid. However, when silenced with miR-146a-5p, TCs failed to exert any protective effects in LPS-stimulated ARDS mice. The experiment was repeated six times, and the results are shown as the mean $\pm$ standard deviation. * $P < 0.05$. Mice in each group $n=6$.

Figure 2

MiR-146a-5p in TCs decreased oxidative stress both in vitro and in vivo. (A) MDA levels in the lung tissue of ARDS mice were increased, and decreased in the telocyte-treated group, MDA levels increased again when infused telocytes were treated with antagomir-146a-5p, (B, C) MDA levels in the TCs and culture media were decreased when treated with LPS, and increased again when treated with antagomir-146a-5p. Both KG-501 and 666-15, CREB1 inhibitors, reduced the MDA level in TCs silenced with miR-146-5p. The

experiment was repeated six times, and the results are shown as the mean $\pm$ standard deviation. *P < 0.05. Mice in each group n=6.

Figure 3

MiR-146a-5p in telocytes decreased oxidative stress through the DUOX2 pathway. (A) The screening results of the enzymes involved in TC protection. (B) LPS increased MDA levels in both RAW264.7 and MLE-12 cells but reduced MDA levels in TCs. (C) DUOX2 was downregulated in TCs after LPS stimulation at both the gene and protein levels compared with in RAW264.7 and MLE-12 cells. (D) Silencing miR-146a-5p, but not miR-21a-3p, upregulated DUOX2 mRNA expression and protein expression in TCs stimulated with LPS. The experiment was repeated six times, and the results are shown as the mean $\pm$ standard deviation. *P < 0.05.

Figure 4

MiR-146a-5p regulated the expression of DUOX2 through the CREB1 pathway. (A) The expression of CREB1 in telocytes was decreased when treated with LPS and increased when TCs were treated with antagomiR-146a-5p. Antagomir-146-5p restored CREB expression in the presence of LPS. (B) A dual-luciferase reporter assay revealed that the miR-146a-5p mimic significantly reduced CREB1 promoter activity. (C) Antagomir-146-5p significantly increased DUOX2 protein expression in TCs compared with cells transfected with the negative control. In the presence of antagomir-146-5p and LPS, pharmacological inhibitors of CREB1, KG-501 and 666-15, significantly reduced DUOX2 protein expression. The experiment was repeated six times, and the results are shown as the mean $\pm$ standard deviation. * $P < 0.05$ vs Control.

Figure 5

Phosphorylated JAK1/STAT1 was upregulated after LPS stimulation in telocytes but was not related to the expression of DUOX2. (A) LPS significantly increased the phosphorylated levels of JAK1 and STAT1. (B) The phosphorylated levels of JAK1 failed to be restored by antagomir-146a-5p incubation, but the phosphorylated levels of STAT1 were restored. The experiment was repeated six times, and the results are shown as the mean $\pm$ standard deviation. * $P < 0.05$ vs Control.

Figure 6

Family members of JAK/STAT revealed different response to the LPS stimulation. LPS significantly increased the phosphorylated levels of JAK2, STAT3 and STAT5, while others did not change significantly. The experiment was repeated six times, and the results are shown as the mean $\pm$ standard deviation. *P < 0.05 vs Control.

Figure 7

Family members of JAK/STAT were not related to the expression of DUOX2. The phosphorylated levels of family members of JAK/STAT failed to be restored by antagomir-146a-5p incubation. The experiment was repeated six times, and the results are shown as the mean $\pm$ standard deviation. *P < 0.05 vs Control.

Figure 8

Schematic pathway of DUOX2 production controlled by miR-146a-5p. TCs induced by LPS decreased ROS production through the miR-146a-5p/CREB1/DUOX2 signaling pathway in TCs. TC, telocyte; ROS, reactive oxygen species; LPS, lipopolysaccharide; JAK, Janus Kinase; STAT, signal transducer and activator of transcription; DUOX2, dual oxidase 2; CREB1, cAMP-response element binding protein 1.