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N-acetyl cysteine inhibits lipopolysaccharide-mediated induction of interleukin-6 synthesis in MC3T3-E1 cells through the NF-kB signaling pathway

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ABSTRACT

Background: Interleukin-6 (IL-6) is a potent stimulator of osteoclastic activity. Lipopolysaccharide (LPS) has been shown to regulate the expression of potent inflammatory factors, including TNF-α and IL-6. Currently, effective therapeutic treatments for bacteria-caused bone destruction are limited. *N*-acetyl cysteine (NAC) is an antioxidant small molecule that possibly modulates osteoblastic differentiation. However, whether NAC can affect the LPS-mediated reduction of IL-6 synthesis in MC3T3-E1 cells is still unknown.

Aims: The aim of this study was to investigate the role of NAC in the LPS -mediated reduction of IL-6 synthesis by MC3T3-E1 cells and to explore the underlying molecular mechanisms. In addition, we aimed to determine the involvement of the NF-kB pathway in any changes in IL-6 expression observed in response to LPS and NAC.

Methods: MC3T3-E1 cells (ATCC, CRL-2593) were cultured in α-minimum essential medium. Cells were stimulated using NAC or LPS at various concentrations. Cell proliferation was observed at multiple time points using a cell counting kit 8 (CCK-8). IL-6 mRNA expression and protein synthesis were determined using quantitative polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assay analyses. NF-kB mRNA expression and protein synthesis was determined using qPCR and Western blots analyses.

Results: The results demonstrate that LPS induced IL-6 and NF-kB mRNA expression and protein synthesis in the cultured MC3T3-E1 cells. However, these effects were abolished following pre-treatment with NAC. Pretreatment with NAC (1 mmol/l) or BAY11-7082 (10 μmol/l) both significantly inhibited the NF-kB activity induced by LPS.

Conclusion: NAC inhibits the LPS-mediated induction of IL-6 synthesis in MC3T3-E1 cells through the NF-kB pathway.

1. Introduction

Interleukin-6 (IL-6) is a well-known multifunctional cytokine that plays an important role in the regulation of various biological processes, including hematopoiesis, the inflammatory response and the immunological reaction (Nishimoto, 2010; Savvatis et al., 2014; Walter, Mais, & Hermanns, 2015). More specifically, IL-6 is a sensitive systemic indicator of inflammation and tissue damage (Hai-lang & Guo-qiang, 2014). It has been reported that bone resorption is mediated by an increased localized production of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and IL-6 (Charatcharoenwitthaya, Khosla, Atkinson, Mccready, & Riggs, 2010). IL-6 induces the formation of osteoclasts from precursor cells and stimulates bone resorption (Nagao et al., 2017). Hence, IL-6 is a well-recognized and potent osteoclastic factor in bone metabolism (Kozawa, 2010).

Lipopolysaccharide (LPS) is a gram negative bacterial endotoxin released from the cell wall component that contributes to inflammation in the body. LPS is the main pathogenic factor of gram negative bacteria, and is positively related to clinical symptoms and the severity of bone destruction (Guha & Mackman, 2001; Moon et al., 2013; Morsczeck, Drees, & Gosau, 2012). LPS is involved in the regulation of the expression of potent inflammatory factors, including TNF-α and IL-6, through the nuclear factor-kappa B (NF-kB) pathway (Eijo et al., 2011; Li et al., 2013; Nagao et al., 2017). NF-kB pathway is considered

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to mediate unspecific host defense mechanisms and play critical roles in clearing microorganisms including bacterial, viral and fungal infections.

N-acetyl cysteine (NAC) is a novel antioxidant thiol compound, and a precursor of glutathione (Fadhel, Sabri, & Haider, 2014) that can increase the content of intracellular glutathione (Yan, Ferrari, & Greene, 1995). Scavenging oxygen free radicals can inhibit NF-kB activation, thereby inhibiting osteoclast formation and controlling osteoclastic activity and bone resorption (Amore et al., 2013; Farid, Reid, Li, Gerken, & Durham, 2005; Qiu et al., 2013; Wang et al., 2013). The effects of NAC on the LPS-mediated induction of IL-6 synthesis can be investigated through *in vitro* experiments using the osteoblast culture system. The understanding of the underlying molecular mechanisms through which the NF-kB pathway inhibits IL-6 secretion may provide a theoretical basis for further clinical applications of NAC in the prevention and treatment of LPS-induced bone resorption. Therefore, the aim of this study was to investigate the role of NAC on the LPS-mediated induction of IL-6 synthesis by MC3T3-E1 cells and the underlying molecular mechanisms. In addition, the involvement of the NF-KB pathway in the expressions of IL-6 in response to LPS and NAC was explored.

2. Materials and methods

2.1. Materials

LPS and NAC were obtained from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). Fetal bovine serum (FBS) and α-minimum essential medium (α-MEM) were purchased from HyClone Laboratories (HyClone, Logan, Utah, USA). Bay11-7082 was purchased from Biomol (Hamburg, Germany). The CCK-8 was purchased from Dojindo (Dojindo Molecular Technologies, Rockville, MD 20850, USA) and the mouse IL-6 enzyme-linked immunosorbent assay (ELISA) kit was purchased from Research and Diagnostic systems (Minneapolis, MN, USA). The anti-NF-kB and anti-phospho-NF-kB antibodies were obtained from Cell Signaling Technology (Beverly, Massachusetts, MA, USA).

2.2. Cell culture

The osteoblastic cell line, MC3T3-E1, was provided by the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). In order to examine the effects of LPS and NAC on osteoblast proliferation, MC3T3-E1 cells (5×10^4 cells/cm^2) were grown inα-MEMCCK supplemented with 10% (v/v) FBS and incubated at 37 °C in 5% CO_2 humidified air. After 24 h, cells were treated with variable concentrations of LPS (1, 5, 10 and 20, μg/ml), NAC (0.5, 1, 2.5 and 5 mmol/L) or left untreated (control) in the α-MEM medium for the indicated times.

Experiments were divided into six groups: control (without LPS and NAC), NAC (contained only NAC), LPS (contained only LPS), NAC and LPS (N + L; in this group MC3T3-E1 cells were treated with NAC, followed by LPS after one hour). The BAY11-7082 and LPS (B + L; in this group MC3T3-E1 cells were treated with BAY 11-7082, followed by LPS after one hour) and BAY11-7082 (contained only BAY 11-7082). The groups are displayed in Table 1.

Table 1
The experimental groups.

Groups	Control	NAC	LPS	NAC + LPS	BAY11-7082 + LPS	BAY11-7082
NAC	−	+	−	+	−	−
LPS	−	−	+	+	+	+
BAY11-7082	−	−	−	−	+	+

2.3. Cell counting kit 8 (CCK-8) assay

Cell proliferation was measured using the CCK-8 assay. Cell proliferation assays were separated into three groups: blank, control and experimental group (NAC and LPS groups). A series of variable concentrations of NAC (0.5, 1, 2.5 and 5 mmol/L) and LPS (1, 5, 10 and 20 μg/ml) were applied to the NAC and LPS groups respectively. MC3T3-E1 cells (density; 2.0×10^4 cells/well) were seeded in a 96-wells plate after being cultured in 5% CO_2 at 37 °C for variable time intervals (24, 48 and 72 h) and the cell proliferation rate was measured using the CCK-8 assay, for which the cells were incubated for 4 h in 5% CO_2 at 37 °C. The absorbance was measured at 450 nm using a MCC 340 multi-scan microplate reader (Thermo Fisher Scientific Inc, Pittsburgh, USA). All experiments were performed in triplicates.

2.4. Real-time quantitative polymerase chain reaction (RT-qPCR)

The levels of IL-6 and NF-kB P65 mRNA were measured quantitatively through RT-qPCR. The total cellular RNA was isolated using a Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The total RNA from cells was reverse-transcribed using a PrimeScriptTM RT reagent kit (Takara, Osaka, Japan) in the presence of an oligo-(dT) primer at 42.8 °C for 1 h, to produce cDNA. Briefly, the cDNA synthesized from the total RNA was amplified in a 10 mL volume with SYBR1 Premix Ex TaqTM II (Takara, Osaka, Japan), 0.1 mM dNTPs, 0.4 mM each primer, and 1 U Taq DNA polymerase (Takara, Osaka, Japan) using an ABI Prism 7300 sequence detection PCR system (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol. The primer sequences are displayed in Table 2. The internal reference control was glyceraldehyde-phosphate dehydrogenase (GAPDH).

2.5. Protein expression by enzyme-linked immunosorbent assay (ELISA)

ELISA was performed using the osteoblast culture supernatants, according to the manufacturer's instructions from the IL-6 ELISA kit (R &D systems, Minneapolis, MN, USA). Cell culture supernatants (100 μL were pipetted into the provided 96-well plate and incubated for 2 h followed by three washes using the washing buffer. Wells were dried and 200 μL of substrate (tetramethylbenizidine) was added into each well for 20 min in the dark at room temperature. The plate was read at a wavelength of 450 nm using a MCC 340 multi-scan microplate reader (Thermo Fisher Scientific Inc, Pittsburgh, USA). The levels of IL-6 in the samples were determined by comparison with the standard curve generated using the standards supplied by the manufacturer. The standard curve was generated by plotting the average O.D. (450 nm) obtained from six solutions of standard concentrations on the vertical (Y) axis versus the corresponding concentration on the horizontal (X) axis.

2.6. Protein isolation and Western blot analysis

Cultured MC3T3-E1 cells were washed with ice-cold phosphate buffer saline (PBS) and lysed for 30 min in ice-cold lysis buffer (containing 1% Triton X-100, 20 mM Hydroxyethyl piperazine ethyl sulfonic

Table 2
Sequences of PCR Primers used for Quantitative Real-time PCR.

Gene name	Gene symbol	Primer sequence (5'-3')	Amplicon length
Interleukin-6	IL-6	5'-CCGGAGAGGAGACTTCACAG-3'	134
Nuclear Factor	NF-kB	5'-CAGAATTGCCATTGCACAAC-3'	122
Kappa B	GAPDH	5'-TCTGGTGCATTCTGACCTTG-3'	84
Glyceraldehyde-		5'-TCAGGTCCATCTCCTTGGTC-3'	
3-phosphate		5'-GGACCTCATGGCCTACATGG-3'	
dehydrogenase		5'-TAGGGCCTCTCTTGCTCAGT-3'	