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In vitro effect of dexmedetomidine on the respiratory burst of neutrophils

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ABSTRACT. The immunosuppressive effects of dexmedetomidine, a highly selective and widely used α_2-adrenoceptor agonist for sedation, analgesia, and stress management, are investigated *in vitro*. In the present study, the respiratory burst of human neutrophils separated from venous blood was evaluated with dexmedetomidine treatment after *Escherichia coli* stimulation. The effects of five concentrations of dexmedetomidine (1, 5, 10, 50, 100 µg/mL) were evaluated by rhodamine in a flow cytometer. The nitric oxide (NO) production and nitric oxide synthase (iNOS) activity were also determined by using commercial kits. The results were compared to the positive control responses (respiratory burst without drug). We found that dexmedetomidine significantly suppressed respiratory burst, NO production, and iNOS activity after stimulation with *E. coli*, in a dose-dependent manner. The suppressive effects of dexmedetomidine

on phagocytic activity of human neutrophils were associated with respiratory burst coupled with NO production.

Key words: Dexmedetomidine; Respiratory burst of neutrophils; NO production; iNOS activity

INTRODUCTION

Neutrophils, also known as polymorphonuclear leukocytes (PMN), are the major cell type of the innate immune system. They contain approximately 50-70% of leukocytes and predominantly eliminate pathogens, inducing acute inflammation (Cowburn et al., 2008; Kantari et al., 2008; Raffaghello et al., 2008). Elimination of pathogens by neutrophils involves a series of physiological processes including chemotaxis, phagocytosis, and microbial killing. The success of pathogen elimination by neutrophils depends on respiratory burst, which is a major process that mediates microbial killing through formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Cowburn et al., 2008). Phagocytosed microbes are internalized into phagosomes, in which ROS are used as microbicides. ROS production is initiated by the NADPH phagosome oxidase (phox) enzyme complex, which produces various types of ROS such as hydrogen peroxide, superoxide anion, oxygen cations, and free radicals (Sheppard et al., 2005). Moreover, RNS results from the catalyst of L-arginine by nitric oxide synthase (NOS) to produce nitric oxide (NO) reactive species. ROS can cause severe bacterial infection when overproduction triggers vascular damage in chronic diseases such as hypertension and arthrosclerosis (Elbim and Lizard, 2009).

Spontaneous activation of neutrophils releases proinflammatory factors and ROS, which exhibits negative effects on vascular tone and their adhesion to the endothelium. Furthermore, a current study has indicated that an excessive load of free radicals secreted by neutrophils at vascular sites aggravated inflammation by limiting the bioavailability of NO secreted by endothelial cells (Chatterjee et al., 2007). Oxidative stress in the neutrophils of hypertensive patients has been shown to associate with increased NADPH oxidase production and lipid peroxidation and decreased cytosolic and mitochondrial superoxide dismutase concentrations (Delles et al., 2008; Hopps et al., 2009; Sedeek et al., 2009). Furthermore, the overexpression of adhesion molecules, such as β_2-integrin, can promote PMN adhesion and leukocyte-endothelium interactions, which may contribute to vascular damage and exacerbate the initiation or complication of arterial hypertension (Tung et al., 2009).

Dexmedetomidine (DEX), a highly selective and potent α_2-adrenoceptor agonist, is widely used for conscious sedation, analgesia, and stress management in the operating room and the intensive care unit (Chrysostomou and Schmitt, 2008; Zhang et al., 2013). Previous studies have shown that DEX can be administered orally, nasally, transmucosally, or intramuscularly for premedication (Ghali et al., 2011; Özcengiz et al., 2011). However, DEX is not well tolerated hemodynamically (Talke et al., 2003; Farag et al., 2012). High doses of DEX or combinations with other medications often cause bradycardia, hypotension, and serious side effects, which are usually thought to result from the negative feedback modulation of the catecholamine release through the activation of presynaptic α_2-adrenoceptors expressed throughout the central nervous system (Funai et al., 2014).

Therefore, the main goal of this study was to investigate the respiratory burst activity impairment of DEX in human neutrophils. Respiratory burst was induced from phagocytosis

of *Escherichia coli*. An approximate quantity of *E. coli* was used to produce submaximal stimulation of respiratory burst. Thus, potential suppression or augmentation of the effects of DEX could be verified.

MATERIAL AND METHODS

After the ethics approval from the Medical Research Ethics Committee of the Second Affiliated Hospital of Nanchang University was received, 5 mL peripheral blood was drawn from healthy donors (N = 10) and heparinized (10 U/mL, Liquemin N, Hoffmann-La Roche, Grenzach-Wyhlen, Germany), immediately before the beginning of the experiments.

Tested agents

DEX was purchased from Waterstone Technology (Carmel, IN, USA). All other chemical reagents used were of analytical grade, and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Five concentrations (1, 5, 10, 50, and 100 μg/mL) of the tested agents, which were dissolved with phosphate-buffered saline (Dulbecco's without Ca^{2+} and $MgCl_2$; GIBCO BRL, Eggenstein, Germany), were used for treatment.

Neutrophil isolation

The following methods were performed to optimize neutrophil isolation. Venous blood (10 mL) was collected and diluted in 1X Hank's balanced salt solution (HBSS) (Flowlab, Irvine, Scotland) medium at a 1:1 ratio. Diluted blood (10 mL) was then layered over 5 mL Lymphoprep (Nycomed, Zürich, Switzerland) and centrifuged at 2000 *g* for 40 min at room temperature. Plasma and the mononuclear cell layer were then discarded. The red cell pellet with PMN and red blood cells (RBC) was suspended in 5 mL HBSS. The red cell suspension was layered over 3% Dextran and left at room temperature for 45-60 min to further sediment the RBC. The supernatant from the Dextran sedimentation was collected and the remnant RBC was lysed using a hypotonic lysing procedure to obtain a pure PMN population. Morphological examination and cell count were performed to determine the purity of the PMN.

Sample preparations

The effects of DEX on respiratory burst were measured by evaluation of the intracellular oxidation of dihydrohodamine 123 (DHR; MoBiTec, Göttingen, Germany) by a flow cytometer. The assay is dependent on the incorporation of DHR into the cell. Subsequently, DEX of different concentrations was added to the samples. After an incubation period of 10 min at 37°C, respiratory burst was induced with either 50 μL *E. coli* (1 x 10^9/mL, HB 101, Sigma, St. Louis, MO, USA). Stimulation was terminated by transferring the samples onto ice after 20 min. Viability discrimination was performed by adding 10 μM propidium iodide (PI; Serva, Heidelberg, Germany) just before measurement. Internal positive controls (submaximal stimulation by *E. coli*) and negative controls (no stimulation) for each sample were carried out without adding the tested substances. The above-mentioned amounts of *E. coli* that induced submaximal stimulation of neutrophils in the positive control were evaluated in a percentage of inhibition compared to the positive controls in the absence of the tested

drugs. Pre-stimulation of neutrophils could be excluded by negative controls: respiratory burst without stimuli and agents.

Flow cytometry analyses

The samples were analyzed using a flow cytometer (FACScan, Becton Dickinson, Heidelberg, Germany). For each sample, 15,000 events were measured. The flow cytometer was equipped with an argon ion laser adjusted to a wavelength of 488 nm. Rhodamine emission was filtered and measured within the spectrum of 515-545 nm by the corresponding photomultiplier (FL 1). The photomultiplier for FL 3 was used for measuring the PI emission in excess of 650 nm. Forward scatter (FSC), sideward scatter (SSC), and fluorescence signals were adjusted using the negative and positive controls. SSC and the FSC were assessed using the linear mode: FL 1 and FL 3 in logarithmic mode without compensation. Erythrocytes and cell debris were excluded according to a high threshold adjusted in the FSC signals. All of the results were obtained with a constant photomultiplier gain value. Data files were stored in list mode and analyzed in dot plots using the PC-LYSYS software (Becton Dickinson). Neutrophils were included by setting a polygonal gate in FSC versus SSC. These gated cells were transferred to an FL 3/SSC dot plot for exclusion of dead neutrophils due to their high fluorescence in FL 3 as a result of the intracellular PI content. Finally, only vital neutrophils were included in an FL 1/SSC dot plot and the effects of the tested agents on the neutrophil respiratory burst were estimated at approximately 5000 vital neutrophils per sample.

NO production

The NO production was analyzed by sampling the supernatants after a 12-h incubation for the presence of nitrite, using the Griess reaction kit (Nanjing Jiancheng, Nanjing, China) according to the manufacturer protocol. Briefly, 100 μL supernatant was removed from individual wells and placed on a separate 96-well plate. To each sample, 50 μL 1% sulphanilamide in 2.5% phosphoric acid was added, followed by 50 μL 0.1% *N*-naphthyl-ethylenediamine in 2.5% phosphoric acid. The optical density of each well was determined at 540 nm. The approximate concentration of nitrite in the samples was determined from a standard curve generated using known concentrations of sodium nitrite.

iNOS activity assay

Cell lysis buffer (1 mL; Beyotime, Suzhou, China) was added to cells cultured in T25 flasks. The supernatants of the cellular lysates were used for iNOS activity assay using an NOS Assay Kit (Nanjing Jiancheng) following the manufacturer protocol.

Statistical analysis

All statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS) 19.0 software (SPSS Inc., Chicago, IL, USA). The data are reported as means ± SD from three separate experiments. Statistical significance between two groups (DEX treatment groups versus negative control group) was determined the by paired or unpaired Student *t*-test in cases of standardized expression data. Differences were considered statistically significant at $P < 0.05$.

RESULTS

DEX suppressed respiratory burst after stimulation with *E. coli*

Necrotic neutrophils were excluded due to their high intracellular PI content. The percentage of necrotic neutrophils was under 2% for all samples. The differences compared to the positive controls without drugs are shown in Figure 1.

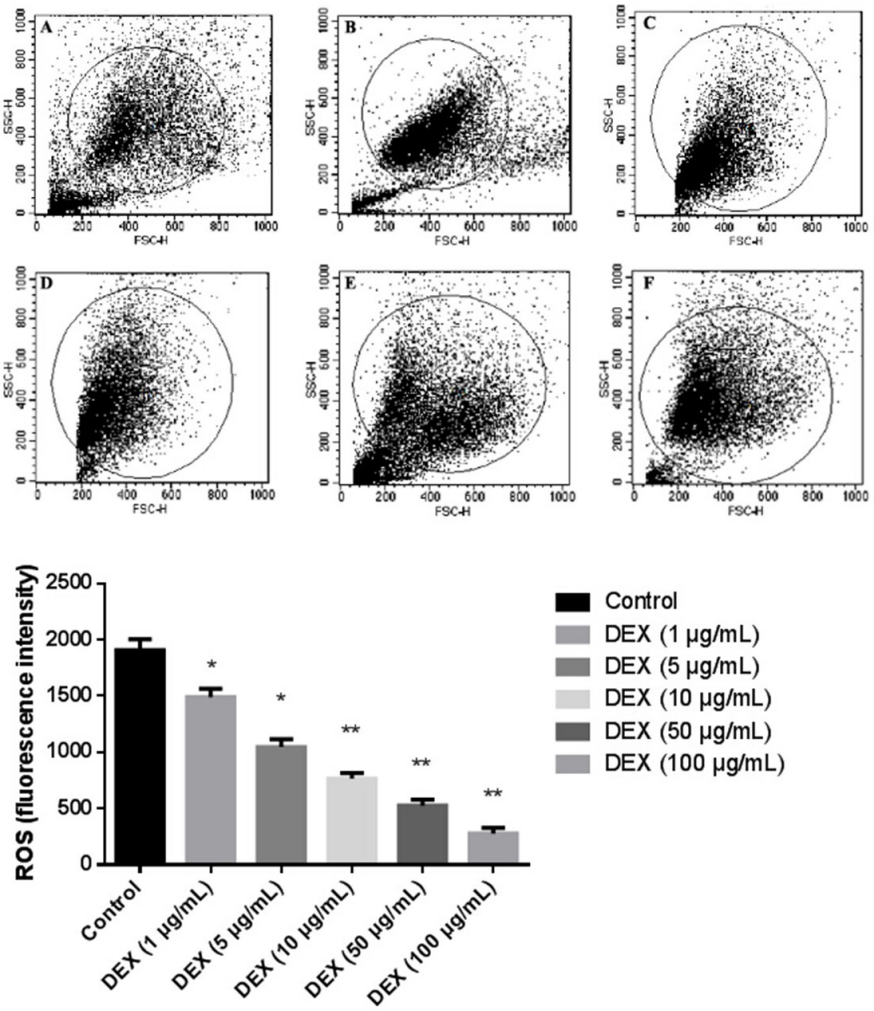

Figure 1. Adjustment of the acquisition dot plots for analysis of the neutrophil respiratory burst due to the green fluorescence of rhodamine. **A.** Positive control after *Escherichia coli* stimulation; **B.** 1 µg/mL DEX after *E. coli* stimulation; **C.** 5 µg/mL DEX after *E. coli* stimulation; **D.** 10 µg/mL DEX after *E. coli* stimulation; **E.** 50 µg/mL DEX after *E. coli* stimulation; **F.** 100 µg/mL DEX after *E. coli* stimulation. The bar graphs show, for all experimental conditions, the mean fluorescence intensity, measured by computerized image analysis, reported as means ± SD; *P < 0.05, **P < 0.01.

Inhibition of NO production

NO synthesis is catalyzed by iNOS in the presence of superoxide production. Normally, the amount of nitrite is used to represent NO production. Incubation of the neutrophils with DEX has significantly decreased NO production of the cells after stimulation with *E. coli* (Figure 2). Furthermore, the quantity of iNOS at the protein level was determined and the activity of iNOS was measured. The results show that the activity of iNOS was significantly decreased in the DEX-treated neutrophils after stimulation with *E. coli* (Figure 3).

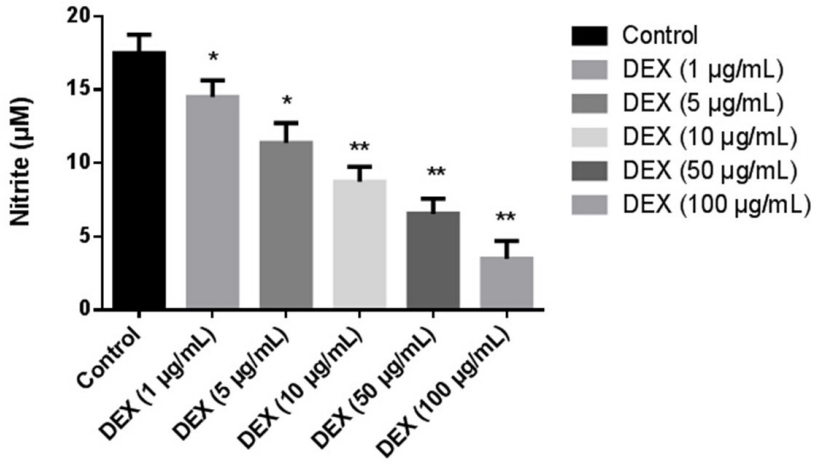

Figure 2. Nitric oxide (NO) production by macrophages incubated with 1, 5, 10, 50, 100 μg/mL DEX after *Escherichia coli* stimulation, reported as means ± SD, *P < 0.05, **P < 0.01.

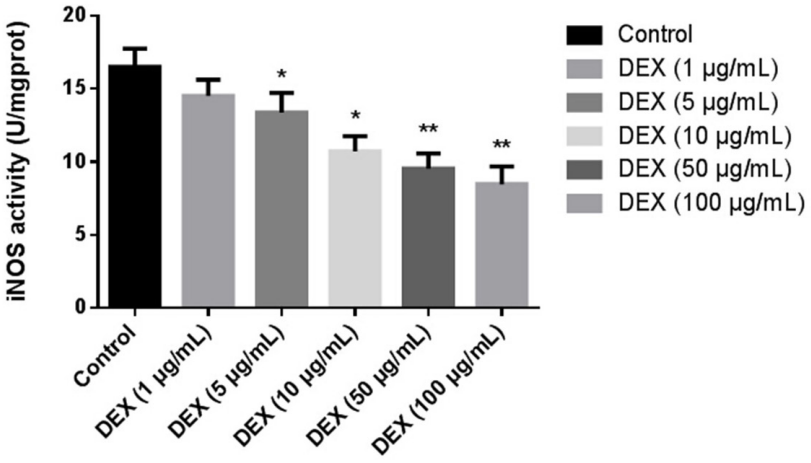

Figure 3. iNOS activity of macrophages incubated with 1, 5, 10, 50, 100 μg/mL DEX after *Escherichia coli* stimulation, reported as means ± SD, *P < 0.05, **P < 0.01.

DISCUSSION

Drugs commonly used in anesthesia for the treatment of sedation, analgesia, and anesthesia are α_2-agonists (Bergendahl et al., 2004; Constant et al., 2004). Özcengiz et al. (2011) showed that DEX administered orally could prevent post-sevolurane agitation in children. Yuen et al. (2008) reported that 1 μg/kg intranasal DEX produced significantly higher sedation in children aged 2-12 years compared with oral midazolam. The authors emphasized that DEX and midazolam created similar premedication conditions and that both were acceptable (Yuen et al., 2008). In another study, Yuen et al. (2010) found that sedation began an average of 25 min after intranasal administration of DEX and that the mean duration of sedation was 85 min. Sakurai et al. (2010) reported that 3-4 μg/kg DEX administered to children 1 h before surgery was reliable and effective.

In a previous study, respiratory burst was stimulated by phagocytosis of *E. coli* or by priming with tumor necrosis factor (TNF)-α followed by receptor activation with the bacterial peptide FMLP. Phagocytosis raised the intracellular Ca^{2+} content, leading to activation of protein kinase C followed by NADPH oxidase activation (Babior, 1992). Priming with TNF-α was suggested to upregulate neutrophil FMLP receptor expression (Elbim et al., 1993). Priming is defined as the exposure of PMNs to a triggering agent so that there is a markedly increased response to a second stimulus. TNF-α priming is mediated by the 55-kD TNF-α receptor on the surface of neutrophils (Menegazzi et al., 1994). FMLP itself binds to a formyl-peptide receptor, which leads to changes in intracellular calcium concentration followed by oxidase activation (Babior, 1992).

In contrast to the NADPH oxidase, the activity of iNOS is mainly regulated at the transcriptional level (Fang, 2004). Upon the stimulation of microbial pathogens, neutrophils are typically induced to produce ROS immediately. However, RNS production requires *de novo* protein synthesis (Fang, 2004). ROS and RNS can interact with numerous cellular compounds, including thiols, metals, protein tyrosines, nucleotide bases, and lipids. In the presence of oxygen, $NO\cdot$ can converted to $NO_2\cdot$ and $ONOO^-$, which can result in oxidative modifications and resemble those that are mediated by ROS alone (Spek et al., 2001). Under certain conditions, the combined antimicrobial activities of ROS and RNS are greater than that of either pathway alone (Pacelli et al., 1995). The synergy of ROS and RNS in the neutrophils stimulated with DEX is not known and needs to be further addressed.

Numerous factors are involved in the regulation of RNS and ROS synthesis, such as proinflammatory cytokines (IFNs, IL-1β and TNF-α). In our results, DEX was able to reduce the synthesis of ROS and RNS in the neutrophils derived from blood, indicating that the inhibition of ROS and RNS by the neutrophils was associated with lower expression of some proinflammatory cytokines. However, the mechanisms underlying the ROS-RNS inhibition and the lower expression of proinflammatory cytokines by DEX are enigmatic and need to be further characterized.

Conflicts of interest

The authors declare no conflict of interest.

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