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The alpha2-adrenoreceptor agonist dexmedetomidine protects against lipopolysaccharide-induced apoptosis via inhibition of gap junctions in lung fibroblasts

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ABSTRACT

The α2-adrenoceptor inducer dexmedetomidine protects against acute lung injury (ALI), but the mechanism of this effect is largely unknown. The present study investigated the effect of dexmedetomidine on apoptosis induced by lipopolysaccharide (LPS) and the relationship between this effect and gap junction intercellular communication in human lung fibroblast cell line. Flow cytometry was used to detect apoptosis induced by LPS. Parachute dye coupling assay was used to measure gap junction function, and western blot analysis was used to determine the expression levels of connexin43 (Cx43). The results revealed that exposure of human lung fibroblast cell line to LPS for 24 h increased the apoptosis, and pretreatment of dexmedetomidine and 18α-GA significantly reduced LPS-induced apoptosis. Dexmedetomidine exposure for 1 h inhibited gap junction function mainly via a decrease in Cx43 protein levels in human lung fibroblast cell line. These results demonstrated that the inhibition of gap junction intercellular communication by dexmedetomidine affected the LPS-induced apoptosis through inhibition of gap junction function by reducing Cx43 protein levels. The present study provides evidence of a novel mechanism underlying the effects of analgesics in counteracting ALI.

1. Introduction

Gap junctions are intercellular membrane channels that directly mediate transfer of cytoplasmic signaling molecules between adjacent cells. Gap junction channels are composed of two hemichannals, each of which contains six connexin (Cx) monomers for docking to its counterpart in the coupled cell membrane and form a gap junction channel [1]. Connexin43 (Cx43) is abundant in lung fibroblast cells, which suggests a substantial amount of Cx43-mediated gap junctional coupling [2]. Gap junction intercellular communication is crucial in the development of acute lung injury (ALI), and it has been reported that inhibition of gap junction intercellular communication decreased the onset of ALI [3–7]. Yao et al. reported that Cx43 expression was upregulated in mouse lungs during ovalbumin-induced asthma [8]. In addition, in Cx43-KO mice, ALI was alleviated due to blockage of Ca^{2+}-mediated release of inflammatory factors [6]. Therefore, inhibition of gap junction function might alleviate ALI.

Dexmedetomidine is a highly selective alpha-2 adrenoceptor inducer with sedative, analgesic, and anesthetic effects. It has been widely used in anesthesia and intensive care. Recent studies focused on its protective effects on multiple organs including heart [9], nerve [10], kidney [11] and liver [12]. Documents indicated that dexmedetomidine has a protective effect against renal hypoxia/reoxygenation injury in mice [13]. Evidence has proved that gap junction may promote apoptosis via the transfer of pro-apoptotic signals between cells [14,15]. Chenfang et al. reported that blockade of Cx32-composed gap junction in renal tubular epithelial cells reduced the spread of apoptosis. In addition, dexmedetomidine is reported to protect against ALI [16], and the apoptosis of lung cells is an established attribute of ALI [17]. However, there remains a lack of evidence of the effects of dexmedetomidine on the regulation of gap junctions composed of Cx43, and on LPS-induced apoptosis of lung.

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In the present study, human lung fibroblast cell line (HLF) was selected to investigate whether the effects of dexmedetomidine on LPS-induced apoptosis are mediated by alterations in gap junction intercellular communication. The results of the present study may help elucidate a novel mechanism underlying the effects of analgesics in counteracting ALI.

2. Materials and methods

2.1. Materials

Lipopolysaccharides (LPS), 18α-glycyrrhetinic acid (18α-GA), anti-Cx43, anti-β-tublin mouse lgG and secondary antibodies for western blot analysis were from Sigma-Aldrich (ST. Louis, MO. USA). Primary antibody for cleaved caspase-3 was obtained from Cell Signal Technology (Danvers, MA, USA). Dexmedetomidine (Dex) was purchase from Sun Yat-Sen Memorial Hospital (Guangzhou, China). Calcein-acetoxymethyl ester (calcein-AM) and cell culture reagents were from Invitrogen (Carlsbad, CA, USA). All other reagents were purchased from Sigma-Aldrich, unless stated otherwise.

2.2. Methods

2.2.1. Cell lines and cell culture

The human lung fibroblast cell line (HLF) was obtained from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in an atmosphere of 5% CO_2 in air.

2.2.2. Flow cytometry apoptosis detection assay

HLF cells were exposed to LPS (10 μg/ml) for 24 h in the dark. Cells were treated with dexmedetomidine (0–1 μM) or 18α-GA (50 μM) for 1 h before incubation with LPS and remained during LPS treatment. Afterward, the cells were washed in PBS and then trypsinized and harvested. Cells were re-suspended in binding buffer and dual staining with Annexin V-FITC and propidium iodide (PI) using the Annexin V-FITC apoptosis detection kit (Dojindo, Shanghai, China) according to the manufacturer's protocol. Apoptosis rate was analyzed by BD Accuri C6 Plus Flow Cytometer and data was analyzed using the FlowJo software.

2.2.3. Parachute dye-coupling assay

A dye-coupling assay was used to examine gap junction intercellular communication as previously described [18,19]. Briefly, cells were frown to confluence, and the donor cells were labeled with 5 μM calcein-AM for 30 min at 37 °C, and then trypsinized and seeded onto the receiver cells at a 1:150 donor:receiver ratio. The cells were allowed to attach to the monolayer of the receiver cells to form gap junctions for 4 h at 37 °C, and were then monitored under a fluorescence microscope (Olympus DP73, Tokyo, Japan). For each experimental condition, the average number of receiver cells around every donor cell was recorded as an index of gap junction intercellular communication.

2.2.4. Western blot analysis

The cells were washed with cold PBS and harvested in lysis buffer (Bio-Rad, Hercules, CA), sonicated and centrifuged at 14,167 g for 30 min at 4 °C. Proteins were quantified using Pierce ™ BCA Protein Assay Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Subsequently, 25 μg of protein from each sample was added into SDS-PAGE, and then transferred onto a polyvinylidene fluoride membrane. Membranes were blocked with 5% milk for 1 h at room temperature and incubated with the specific primary antibodies overnight at 4 °C. Monoclonal antibodies for Cx43 (C8093; diluted 1:2000), cleaved caspase-3 (9664S; diluted 1:1000) and β-tublin (T4026; diluted 1:10,000) were used. Immuno-positive bands were visualized using the Immobilon ECL Western Blotting Detection Kit (Millipore, Billerica, MA, USA).

2.2.5. Statistical analysis

The data were statistically analyzed using one-way ANOVA and unpaired Student's *t*-test. The results were presented as the mean ± standard error using Prism software (Graphpad, Inc.). *P* value < 0.05 was considered to be significant.

3. Results

3.1. Apoptosis induced by LPS is gap junction-dependent

To examine whether gap junction contributed to LPS-induced apoptosis, HLF cells were pretreated with 18α-GA (50 μM), followed by LPS (10 μg/ml). First, we confirmed the effectiveness of 18α-GA on gap junction function as revealed by parachute dye-coupling assay in HLF cells. As shown in Fig. 1A, treatment with 50 μM 18α-GA for 1 h led to marked inhibition of the spread of dye between the donor cells and receiver cells in HLF cells, and the inhibition rate was approximately 50%.

We then evaluated the roles of gap junctions in LPS-induced apoptosis. As shown in Fig. 1B, 10 μg/ml LPS increased the apoptosis rate of the cell population. 18α-GA itself did not affect apoptosis (data not shown). Pretreatment of HLF cells with 18α-GA decreased the apoptotic rate to approximately 60% compared to LPS treated cells, which indicated that gap junction obviously increased LPS-induced apoptosis in HLF cells.

3.2. Effect of dexmedetomidine on apoptosis induced by LPS

HLF cells were exposed to clinically relevant concentrations (0.1 nM, 1 nM) of dexmedetomidine for 1 h immediately followed by LPS. Dexmedetomidine itself did not affect apoptosis at different concentrations (data not shown). As shown in Fig. 2A, 0.1 nM and 1 nM dexmedetomidine significantly decreased apoptotic rate for 40%–50% compared to dexmedetomidine-untreated cells. The fact that LPS-induced apoptosis depends on gap junction intercellular communication and that dexmedetomidine decreased LPS-induced apoptosis which mimicked gap junction inhibitor 18α-GA suggested that amelioration of LPS-induced apoptosis by dexmedetomidine may be partly mediated by gap junction intercellular communication.

Then we analyzed the expression of cleavage caspase-3 in LPS-treated HLF cells in the presence or absence of dexmedetomidine (1 nM) or 18α-GA (50 μM) to see whether the protection against apoptosis mediated by gap junction involved the inhibition of caspases. Western blot analysis showed that dexmedetomidine (1 nM) and 18α-GA (50 μM) downregulated the expression of cleaved caspase-3 compared to cells treated with LPS alone (Fig. 2B), which suggested that the cleavage of caspase-3 was involved in the anti-apoptosis effect by dexmedetomidine and 18α-GA.

3.3. Influence of dexmedetomidine on gap junction function and connexin 43 protein expression

The fact that dexmedetomidine decreased LPS-induced apoptosis in HLF cells expressing Cx43 suggested that the inhibition was mediated by gap junctions. To validate this possibility, we examined the effect of dexmedetomidine on dye coupling between HLF cells. As shown in Fig. 3A, treatment of dexmedetomidine (0.1 nM, 1 nM) for 1 h obviously inhibited dye spread of HLF cells

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