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The excitotoxity of NMDA receptor NR2D subtype mediates human fetal lung fibroblasts proliferation and collagen production

MingJie Wang[a], ZiQiang Luo[b], Yinyan Yue[a], YanRui Wang[a,c], Shu Wu[d], ChuanDing Cao[a], ZhengChang Liao[a], XiaoHe Yu[a], ShaoJie Yue[a,*]

[a] Department of Neonatology, Xiangya Hospital, Central South University, Changsha 410008, China
[b] Department of Physiology, Xiangya Medical College, Central South University, Changsha 410008, China
[c] Department of Neonatology, Heze City Hospital, Heze 274000, China
[d] Department of Pediatrics, Division of Neonatology, Batchelor Children's Research Institute, University of Miami Miller School of Medicine, Miami, FL, United States

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ABSTRACT

Studies have suggested that endogenous glutamate and *N*-methyl-D-aspartate (NMDA) receptor have an excitotoxity role during acute lung injury. Fibroblasts play a critical role in lung development and chronic lung disease after acute lung injury. This study aims to explore the immediate role of *NMDAR* activation in human lung fibroblasts. The expression of *NMDAR 1* subtype (*NR1*) and four individual *NMDAR 2* (*NR2*) subtypes (*NR 2 A to D*) was measured in human fetal lung fibroblasts (HFL-1 and MRC-5). Five *NMDARs* expression were all detectable in two cell lines. Although the expressions of *NMDARs* were different between MRC-5 and HFL-1, 1 mM NMDA elicited the same trend in the downregulation of *NR2A* expression, the upregulation of *NR2D*, and the increase of cells proliferation and collagen production. Glutamate stimulation after 24-h of NMDA exposure resulted in weaker and more delayed but more prolonged iCa^{2+} elevation in HFL-1 than no NMDA exposed cells. NMDA increased the level of $pERK_{1/2}$, cells proliferation and collagen production, whereas nonspecific *NMDAR* antagonist MK-801, *NR2D*-preferring receptor antagonist UBP141 and $ERK_{1/2}$ phosphorylation inhibitor U0126 suppressed it, respectively. In conclusion, we found that *NMDAR* activation, *NR2D* in particular, is involved in human fetal lung fibroblast proliferation and collagen production through a potential $ERK_{1/2}$-mediated mechanism.

1. Introduction

Glutamate is a major excitatory neurotransmitter that is abundantly present in the mammalian central nervous system (CNS). It regulates the proliferation, migration, and survival of neuronal progenitors and immature neurons during development (Zorumski and Olney, 1993). The NMDA glutamate receptor subtype is characterized by its functions in the CNS and its relationship with neuronal excitotoxicity in neurological disorders, through over-activation of *N*-methyl-D-aspartate receptors (*NMDAR*) (YM et al., 1996). Recently, some studies support the important functions of endogenous glutamate and *NMDAR* during acute lung injury and in airway inflammation (Said et al., 2000; Said et al., 2001; Said et al., 1996; Kathleen et al., 2004). Our team had revealed the abundant release of glutamate in acute lung injury induced by hyperoxia in the newborn rat (Wang et al., 2009), and bleomycin in C57BL/6 mice (Li et al., 2015). However, whether or not the glutamate participates in pulmonary fibrosis following acute lung injury through *NMDAR* remains not well established.

Our previous studies have showed that *NMDAR* over-activation mediated not only hyperoxia induced newborn rat acute (Wang et al., 2009) and chronic (Wang et al., 2016) lung injury, but also intrauterine hypoxia induced fetal lung development retardation (Liao et al., 2016). The results also showed that *NMDAR* antagonist MK-801 could inhibit rat fetal lung development in vivo and in vitro (Liao et al., 2016). Which means *NMDAR* may be a crucial factor in the regulation of lung injury repair and normal lung development.

Fibroblasts play a critical role in the neonatal lung development (Kauffman et al., 1974) and the lung injury repair following acute lung injury (Pardo and Selman, 2002; Jordana et al., 1994). The principal role of lung fibroblasts is to maintain the integrity of the alveolar structure via the synthesis, secretion, maintenance, degradation, and remodeling of the extracellular matrix (ECM) (McGowan and Torday, 1997; Baum et al., 1978). However, lung fibroblasts could also play an important role in exacerbating the fibrotic process in chronic lung disease in premature infants (Alejandre-Alcázar et al., 2007; Chen et al., 2007) and pulmonary fibrosis (Pardo and Selman, 2002; Jordana et al.,

* Corresponding author at: Department of Neonatology, Xiangya Hospital, Central South University, Xiangya Road 87, Changsha 410008, China.
E-mail address: shaojieyue@csu.edu.cn (S. Yue).

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1994) by producing collagens and ECM proteins.

In 1980, Gray et al. reported that 30 mM of L-glutamate elicited toxicity in Huntington's disease fibroblasts (Gray et al., 1980). Glutamate induces cellular degeneration in Huntington's disease and normal skin fibroblast (Archer and Mancall, 1983; May and Gray, 1985; Stahl et al., 1984). In the past few years, *NMDARs* have been detected in fibroblasts. *NMDARs* modulated the expression of MMP-2 in human fibroblast-like synoviocytes (Flood et al., 2007), and regulated cells differentiation in human periodontal ligament fibroblasts (JH et al., 2009). Which indicated that the lung fibroblasts may stimulated through *NMDAR* activation by excessive release of glutamate following acute lung injury (Wang et al., 2009; Li et al., 2015).

Seven NMDA receptor subtypes have been identified; the *NR1* subtype, four different *NR2* subtypes (*NR 2A–D*), and two *NR3* subtypes (*NR 3A*, *B*). NMDA receptors are assembled from two *NR1* subtypes and two *NR2* subtypes and are activated by simultaneous binding of glycine and glutamate to *NR1* and *NR2* subtypes, respectively (Lynch and Guttmann, 2002; Traynelis et al., 2010a). The four *NR2* subtypes play different roles during neuronal development and in the adult central nervous system, and endow *NMDARs* with different biophysical and pharmacological properties (Traynelis et al., 2010b; Luk and Sadikot, 2004). The affinity for glutamate differs among the isoforms such that *NR2A* has the lowest affinity, *NR2D* the highest, and *NR2B* and *NR2C* have intermediate affinities (Monaghan and Jane, 2009). Generally, high binding affinity indicates a low dissociation rate of glutamate from the receptor and more prolonged *NMDAR* opening following glutamate binding. Studies previously demonstrated that lung *NR2D* was strongly expressed after NMDA (Said et al., 1996), hyperoxia (Li et al., 2015) and intrauterine hypoxia (Liao et al., 2016) induced lung injury. For the predominant expression of *NR2D* in the peripheral, gas-exchange regions of the lung (Kathleen et al., 2004) and the alveolar macrophage (Kathleen et al., 2004; Shang et al., 2010), *NR2D* may be the receptor subtype most closely associated with lung injury and development.

In the case of hyperoxia-induced chronic lung disease (CLD) in newborn rats, our previous study showed the *NMDAR* activation mediated lung fibroblast proliferation and differentiation, in parallel with the upregulation of four *NR2* genes, especially *NR2A* and *NR2D* (Wang et al., 2016). For the different biophysical properties between *NR2A* and *NR2D*, it is still not clear which *NR2* receptor activation has the greatest involvement in the activation of lung fibroblast. Additionally, aside from glutamate stimulation, there are many other factors that participate in lung fibroblast proliferation and collagen production. To rule out the effect of oxygen radicals and various cytokines and further understand the immediate action of *NMDAR* activation in lung fibroblasts, the effects of *NMDAR*-specific agonist on lung fibroblasts must be investigated.

Early structure activity studies established that an ideal structure for activating *NMDARs* is represented by glutamate (Watkins, 1981). However, NMDA shows a low affinity for plasma membrane transporters and no activity at other glutamate receptors, thus appear more potent than glutamate in some physiological assays (Furukawa et al., 2005; Platenik et al., 2000). We used NMDA as an agonist rather than glutamate in this study. The activation of *NMDARs* in neurons drives the phosphorylation of the extracellular signal-regulated kinase ($ERK_{1/2}$)-signaling cascade in the nucleus, resulting in the activation of genes that promote survival (Navon et al., 2012). We hypothesize that *NMDAR* activation regulates collagen production and the proliferation of human fetal lung fibroblasts through $ERK_{1/2}$-signaling.

2. Materials and methods

2.1. Cell culture and treatment

Human fetal lung fibroblasts (HFL-1 and MRC-5; lung diploid, human cell line, passages 7–12) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were placed in a 5% CO_2-humidified incubator at 37 °C in α minimum essential medium (α-MEM) (HyClone, USA) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco, USA). To eliminate the effect of exogenous glutamate, glutamate-free α-MEM medium (HyClone, USA) was used for the treatments described below.

To select the optimal concentration of NMDA agonist inducing HFL-1 and MRC-5 proliferation and collagen production, cells were treated with various concentrations of NMDA (0.1, 1, or 10 mM) or the same volume of phosphate buffered saline (PBS) for 24 h. To study the effects of *NMDAR* and *NR2D* activation on HFL-1, cells were stimulated for 24 h or 72 h (for flow cytometric analysis of cell cycle phase distribution only) with one of the following: 1 mM NMDA (NMDAR agonist); 0.5 mM MK-801 (nonspecific *NMDAR* antagonist); 1 mM NMDA + 0.5 mM MK-801; 10 μM UBP141 (*NR2D*-preferring receptor antagonist); 1 mM NMDA + 10 μM UBP141; 20 μM U0126 ($ERK_{1/2}$ phosphorylation inhibitor); 1 mM NMDA + 20 μM U0126; or the same volume of PBS as a control. MK-801, UBP141 and U0126 were administered 30 min before NMDA treatment.

2.2. Western blotting analysis

Proteins were extracted from HFL-1 cells into a radio immunoprecipitation assay (RIPA) lysis buffer containing a protease inhibitor cocktail (Roche Diagnostics). The supernatant was collected, and the protein concentration was quantified using a bicinchoninic acid (BCA) assay (Beyotime, China). Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with monoclonal anti-phospho-$ERK_{1/2}$ (*p*$ERK_{1/2}$) and anti-$ERK_{1/2}$ antibodies (1:1000 dilutions; Cell Signaling Technology, USA) at 4 °C overnight. Thereafter, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:5000; Rockland Immunochemicals Inc. USA) for 60 min at 24 °C. The reactions were visualized using an enhanced chemiluminescence kit (Biorbyt, China) and detected on a Kodak XB-1 photographic film. The intensities of protein bands were quantified using Quantity One Imaging Analysis Program (Bio-Rad, USA). Activation of *p*ERK was measured as the ratio of *p*ERK to ERK.

2.3. Total RNA sample preparation and cDNA synthesis

Total RNA was isolated from HFL-1 cells using RNeasy Mini Kit (Qiagen, USA). Reverse transcription was performed with Super Script II Reverse Transcriptase (Invitrogen, USA) and random primers (Takara Bio, Japan), according to the manufacturer's instructions (Applied Biosystems, USA). Reverse transcription was performed using 1 μg total RNA and oligo (dT)12–18 primers (Invitrogen, Grand Island, NY) in a 20-μL reaction, according to the manufacturer's protocol (PE Applied Biosystems, USA).

2.4. Semi-quantitative reverse transcription-PCR (RT-PCR)

The following primers were designed using Primer Express software v3.0 (Thermo Scientific, USA). Procollagen I (116 bp) f: 5′-CCTCAAGGGCTCCAACGAG-3′, r:5′-TCAATCACTGTCTTGCCCCA-3′; procollagen III (485 bp) f: 5′-GCGGAG-TAGCAGTAGGAGGAC-3′, r:5′-GTCATTACCCCGAGCACCTGC-3′; *β*-actin (224 bp) f: 5′-TTCCAGCCTTCCTTCCTGGG-3′, 5′-TTGCGCTCAGGAGGAGCAAT-3′. The PCR cycling conditions were as follows: reaction mixtures were incubated for 3 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 45 s at 50–64° and 1 min at 72 °C, and then a final extension for 10 min at 72 °C. Reaction products were visualized using agarose gel electrophoresis. Semi-quantitative RT-PCR was performed with the same samples under the same cycling conditions, except that the number of cycles was serially reduced, to ensure that the amplification remained within a linear range. Relative gene expression was determined by the ratio of procollagen I or III to β-actin signal.