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Research paper

Profiling of cytosolic and mitochondrial H_2O_2 production using the H_2O_2-sensitive protein HyPer in LPS-induced microglia cells

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HIGHLIGHTS

- Subcellular H_2O_2 formation was confirmed using the HyPer in BV-2 microglia.
- Cytosolic H_2O_2 started increasing constantly from early time in response to LPS.
- Mitochondrial H_2O_2 rapidly increased at later time by LPS treatment.
- MAPK, such as JNK and p38 affected cytosolic H_2O_2, but not mitochondrial H_2O_2.

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ABSTRACT

Dysregulation of the production of pro-inflammatory mediators in microglia exacerbates the pathologic process of neurodegenerative disease. ROS actively affect microglia activation by regulating transcription factors that control the expression of pro-inflammatory genes. However, accurate information regarding the function of ROS in different subcellular organelles has not yet been established. Here, we analyzed the pattern of cytosolic and mitochondrial H_2O_2 formation in LPS-activated BV-2 microglia using the H_2O_2-sensitive protein HyPer targeted to specific subcellular compartments. Our results show that from an early time, cytosolic H_2O_2 started increasing constantly, whereas mitochondrial H_2O_2 rapidly increased later. In addition, we found that MAPK affected cytosolic H_2O_2, but not mitochondrial H_2O_2. Consequently, our study provides the basic information about subcellular H_2O_2 generation in activated microglia, and a useful tool for investigating molecular targets that can modulate neuroinflammatory responses.

1. Introduction

Microglia are immune cells resident in the brain that perform essential roles in neuronal maintenance by promoting neurogenesis, debris cleaning, and neuron defense against infection or injury. However, consistently activated microglia can exert neurotoxic effects because of the excessive production of cytotoxic mediators such as nitric oxide and pro-inflammatory cytokines; preventing the production of such pro-inflammatory mediators attenuates neuronal damage. Therefore, microglia activation is considered an early indicator of various neurodegenerative diseases [3,21].

Reactive oxygen species (ROS), including superoxide (O_2^-) and the resultant hydrogen peroxide (H_2O_2), are neurotoxic when excessively produced [7]. Microglial ROS are mainly produced from the action of NADPH oxidase (NOX) or in mitochondria, and can regulate the immune response by acting as secondary messengers capable of modifying the expression of pro-inflammatory genes [4,6]. However, the molecular mechanisms of ROS generation in individual subcellular organelles during microglia activation have not been elucidated.

Mitogen-activated protein kinases (MAPK), such as ERK, JNK, and p38, play pivotal roles in the production of pro-inflammatory

Abbreviations: DPI, diphenyleneiodonium; ERK, extracellular signal-regulated kinases; JNK, c-Jun N-terminal kinases; LPS, lipopolysaccharide; NAC, N-acetyl-L-cysteine; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; p38 MAPK, p38 mitogen-activated protein kinases.

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mediators in activated microglia [1]. MAPK activity is regulated by ROS [20], and ROS generation is affected by the MAPK pathway in activated microglia and macrophages [11,12]. However, more studies are required to clearly establish the effect of the MAPK pathway on subcellular ROS generation in activated microglia.

HyPer, a highly specific genetically encoded fluorescence probe for detecting H_2O_2, has been used as a tool to target specific organelles such as peroxisomes, the endoplasmic reticulum, and mitochondria, in living cells [2,10,17].

Here, we explored the pattern of cytosolic and mitochondrial H_2O_2 in LPS-induced activated microglia using lentivirus-mediated HyPer expressing BV-2 cells. We found that cytosolic H_2O_2 started increasing constantly soon after LPS treatment, whereas mitochondrial H_2O_2 increased at a later time point. Furthermore, we determined the effect of MAPK on the increasing levels of cytosolic and mitochondrial H_2O_2.

2. Materials and methods

2.1. Cloning of HyPer and preparation of stable cell lines

The HyPer-cyto gene was obtained from pHyPer-cyto (Evrogen, Russia, Moscow), and the HyPer-mito gene was generated by fusing the mitochondrial targeting sequence with the HyPer-cyto gene. The HyPer-cyto and HyPer-mito genes were inserted into the pLenti6.3/V5-DEST (Invitrogen). BV-2 murine microglial cells were transduced with HyPer-cyto and HyPer-mito lentiviruses. Lentivirus production was performed as previously described [15]. BV-2 cells were transduced with HyPer-cyto and HyPer-mito lentiviruses. Lentivirus-infected BV-2 cells were selected using 4 μg/mL blasticidin (Invitrogen)

2.2. Cell culture and treatment

BV-2 cells were cultured in DMEM (Welgene, Daegu, Korea) with 10% FBS (Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (Welgene) at 37 °C in a humidified 5% CO_2 incubator. Non-transduced, and HyPer-cyto- and HyPer-mito-expressing BV-2 cells were pre-treated with NAC, mito-TEMPO, DPI (Sigma-Aldrich, St. Louis, MO, USA), SP600125 (Abcam, Cambridge, MA, USA), PD98059, and SB203580 (Invitrogen, Carlsbad, CA, USA) for 1 h, followed by stimulation with LPS from *E. coli.* serotype O26:B6 (Sigma-Aldrich). Concentration of LPS and all reagents were selected based on our past studies [14,18,23]. HyPer-cyto- and HyPer-mito-expressing BV-2 cells were treated with H_2O_2 (100 μM; Sigma-Aldrich) for 30 min.

2.3. Imaging of organelle-specific H_2O_2 formation using HyPer

BV-2 cells expressing HyPer-cyto and HyPer-mito were seeded on poly-D-lysine-coated glass. BV-2 cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) and stained with Mito-ID (Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's protocol. Cell images were acquired with an LSM-710 confocal microscope (Carl Zeiss, Oberkochen, Germany).

2.4. Measurement of ROS

The levels of cytosolic and mitochondrial H_2O_2 were measured in HyPer-cyto and HyPer-mito-expressing BV-2 cells by flow cytometry. The levels of intracellular ROS were assessed after trypsinized BV-2 cells were incubated with 5 μM H_2DCFDA (Invitrogen) at 37 °C for 30 min, and then were analyzed by flow cytometry.

2.5. Statistical analysis

Statistical analysis was conducted using the GraphPad Prism software (La Jolla, CA, USA). Data are shown as the mean values ± SD from at least three independent experiments ($n \geq 3$). For group comparisons, one-way analysis of variance followed by Dunnett's multiple comparison tests were performed. A p value less than 0.05 was considered to denote statistical significance and is indicated on the graphs by an asterisk. Similarly, p values less than 0.01 and 0.001 are indicated on the graphs by two and three asterisks, respectively.

3. Results

3.1. Establishment of expression of cytosolic and mitochondrial targeted H_2O_2-sensitive protein HyPer in BV-2 microglia

To measure H_2O_2 generation during oxidative conditions in microglia, we generated a BV-2 microglia that expressed specifically targeted HyPer to the cytosol or mitochondria (HyPer-cyto and HyPer-mito, respectively) using lentivirus. We tested cytosolic and mitochondrial HyPer expression in BV-2 cells using Mito-ID, a mitochondria-selective staining dye. As shown in Fig. 1A, the HyPer signal (green) was exclusively colocalized with the Mito-ID signal (red) in HyPer-mito expressing BV-2 cells but not in HyPer-cyto expressing BV-2 cells. The expression of HyPer-cyto and HyPer-mito was also assessed by flow cytometry. HyPer signals were increased in both HyPer-cyto and HyPer-mito expressing BV-2 cells compared to mock-expressing BV-2 cells (Fig. 1B). We next investigated the sensitivity of HyPer-cyto and HyPer-mito to H_2O_2 (100 μM) in BV-2 cells by confocal microscopy and flow cytometry. Indeed, the fluorescence intensity of HyPer-cyto and HyPer-mito increased by H_2O_2 (Fig. 2). These results indicate that we could successfully achieve the specific expression of cytosolic and mitochondrial HyPer in BV-2 microglia.

3.2. Changes in HyPer fluorescence intensity in response to LPS

To obtain information for cytosolic and mitochondrial H_2O_2 production in LPS-induced microglial activation, we exposed LPS to HyPer-cyto and HyPer-mito-expressing BV-2 cells. The increase in the fluorescence intensity of both HyPer forms in response to LPS treatment for 18 h was also confirmed by confocal microscopy (Fig. 3A). We then analyzed the changes in HyPer fluorescence intensity by LPS in a time-dependent manner using flow cytometry. Our results showed that after 3 h of LPS exposure, HyPer-cyto started increasing constantly, reaching its highest level at 18 h, whereas HyPer-mito started increasing only after 12 h of LPS exposure (Fig. 3B). We also assessed the effect of the intracellular antioxidant NAC, the mitochondrial superoxide scavenger mito-TEMPO, and the NOX inhibitor DPI on the LPS-induced changes in HyPer fluorescence intensity. All three antioxidants decreased the levels of cytosolic and mitochondrial H_2O_2 (Fig. 3C). These findings demonstrate that the levels of cytosolic and mitochondrial H_2O_2 increased in microglia activated by LPS.

3.3. Effect of MAPK signaling on cytosolic and mitochondrial HyPer fluorescence intensity

We examined the effect of the MAPK pathway on the LPS-induced changes in cytosolic and mitochondrial H_2O_2 production. Before analyzing the H_2O_2 changes, we investigated the effect of MAPK signaling on intracellular ROS generation in LPS-activated BV-2 cells using H_2DCFDA. JNK and p38 inhibitors, but not an ERK inhibitor, prevented intracellular ROS upregulation (Fig. 4A). Consistently with the H_2DCFDA results, the LPS-induced increase in HyPer-cyto fluorescence intensity was suppressed by the JNK and