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NOS1 mediates AP1 nuclear translocation and inflammatory response

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ABSTRACT

A hallmark of the AP1 functioning is its nuclear translocation, which induces proinflammatory cytokine expression and hence the inflammatory response. After endotoxin shock AP1 transcription factor, which comprises Jun, ATF2, and Fos family of proteins, translocates into the nucleus and induces proinflammatory cytokine expression. In the current study, we found, NOS1 inhibition prevents nuclear translocation of the AP1 transcription factor subunits. Pharmacological inhibition of NOS1 impedes translocation of subunits into the nucleus, suppressing the transcription of inflammatory genes causing a diminished inflammatory response. In conclusion, the study shows the novel mechanism of NOS1- mediated AP1 nuclear translocation, which needs to be further explored.

1. Introduction

The inflammatory response protects the host against infectious challenges [1].Macrophages play a central role in mediating the inflammatory response by activating the cascade of transcription factors that further regulate the expression of cytokine genes leading to an outburst of inflammatory reactions [2–4]. The balance between the prolonged activation and resolution of inflammation determines the fate of inflammatory response towards tissue repair or damage [1,3].

Inflammation is marked by a robust release of potent signaling molecule nitric oxide (NO) produced by a vital enzyme of the immune cells nitric oxide synthase (NOS) to eliminate the damage caused by pathogen [5]. Early studies have identified three isoforms of NOS, neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3)[6,7].Considerable advances illustrating the involvement of NOS2 and NOS3 in inflammatory processes have established their role as an important mediator of inflammation [8–17]. However, the role of NOS1 in inflammation is not well elucidated. This study highlights the potential of NOS1 in modulating the inflammatory response in macrophages.

Coordination of the inflammatory cascade encompasses series of intracellular and extracellular receptors that interact with pathogenic stimulus and modulate the signaling pathway. Pathogen recognition receptors such as toll-like receptors (TLRs) induce the signal transduction pathway in response to bacterial infection [18–20]. Recognition of microbial product such as LPS activates TLR4thereby inducing the signaling adaptors that culminates the activation of key transcription factors such as activator protein-1 (AP1), nuclear factor kappa-light-chain-enhancer (NF-κB), signal transducers and activators of transcription factors (STAT1), as well as interferon regulatory factors (IRFs), that regulate the inflammatory gene expression and thereby impact the duration and severity of pro and anti-inflammatory response [21–23].Chronic inflammatory diseases are characterized by activation of key transcription factor NF-κB and AP1 that cause the enormous release of proinflammatory cytokines such as interleukin-12 (IL12), interleukin-23 (IL23), tumor necrosis factor-α(TNF-α) that are implicated in the augmentation of the inflammatory reaction [24–27]. This study explores the NOS1 mediated regulation of AP1 that turns on the downstream cascade to generate the appropriate inflammatory response to the pathogenic stimulus.

AP1 is composed of homo and heterodimeric complexes of Jun, Fos, and ATF2 [28]. Multiple environmental stimuli such as microbial stress activate AP1 via stress-responsive MAP kinases such as JNK [29]. Jun, the dynamic subunit of AP1, is capable of forming homo and heterodimer with Fos and ATF2, however, Fos and ATF2 do not possess the ability to homodimerize. Active AP1 dimers translocate to the nucleus and bind to immunomodulatory genes and regulate their transcription [30].Recently identified role of NOS1 in early cycles of inflammation [31] lead us to determine its involvement in the regulation of prominent mediators of inflammation including transcription factor AP1. This study explores the significance of NOS1 as a key modulator of AP1 nuclear translocation. Role of AP1 in inflammatory bone and skin disease [27,32,33], allergic asthma [34,35] and psoriasis [36–38] delineate its importance as a key transcription factor and is yet to be explored in the development of therapeutic strategies for chronic inflammatory diseases.

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2. Material and methods

2.1. Generation of BMDMs

Preparation of bone-marrow-derived macrophages (BMDM) was based on the protocol described by Baig et al. [31]. BMDMs were isolated from bone marrow of the femur of Swiss albino mice. Bones were collected in sterile Phosphate-buffered saline (PBS) and subsequently washed in 70% ethanol. They were cut from both ends followed by flushing of marrow into fresh Dulbecco's Modified Eagle Medium (DMEM). Following centrifugation, BMDMs were then cultured in DMEM (Thermo Fisher Scientific) containing 10% heat-inactivated fetal bovine serum (Life Technologies), 100 U/ml penicillin and 100 g/ml streptomycin (Life Technologies) along with 20% L929- cell conditioned media as a source of macrophage colony-stimulating factor (M-CSF). The medium was replenished on the 5th day. The non-adherent cells were purged and the macrophages (adherent cells) were collected by treatment with 1 mM EDTA in PBS for 10 min at 4 °C. The synchronized cell suspension was achieved by supplementing cells with serum-free DMEM medium overnight. Fresh DMEM media with 10% FBS was added to the differentiated macrophages prior to plating the cells in six-well plates. Cells were incubated in a humidified incubator maintained with 5% CO_2 at 37 °C.

2.2. Nitrite measurement by Greiss reagent

Detection of nitrite, a stable metabolite of nitric oxide, was done using Griess reagent (Sigma-Aldrich) as per manufacturer's instructions. BMDM were seeded in twelve well plates at a density of 10^4 cells/well. Prior to treatment, serum-free DMEM medium was added to the wells. Cells were then treated with LPS (Sigma-Aldrich, 250 ng/ml) and TRIM (Sigma-Aldrich, 100 nM) or LPS alone up to 2 h. The supernatant media were collected from each well and nitrite level was determined compared with standard nitrite concentration using Griess reagent.The absorbance of samples was measured at 570 nm in Synergy H1 Bio-Tek microplate reader and nitrite (μM) was estimated per milligram of total protein in each sample.

2.3. Nitrite measurement by DAF-FM staining

Nitrite measurement in a cell-based assay was performed by using DAF-FM (Molecular Probes) to stain cells that produce nitrite. For the assay, BMDM were seeded on coverslips in a six-well plate in DMEM medium. Stimulation was done with LPS (250 ng/ml) and TRIM (100 nM) or LPS alone up to 1 h. Post-stimulation, media was removed and cells were washed with 1X PBS. Cells were then incubated with diluted DAF-FM diacetate for 20 min at 37 °C. Excess dye was removed by washing cells with 1X PBS. 4% formaldehyde was used for fixation for 1 h at room temperature. Fixed cells were mounted on to slides in an inverted position with DAPI containing mounting media (Thermo Fisher Scientific).Visualization of nitrite in cells was done by confocal imaging usingOlympus confocal laser scanning microscope.

2.4. Quantitative real-time PCR

Total RNA was extracted from BMDM using TRIzol reagent (Takara) as per manufacturer's instructions. cDNA was synthesized using cDNA synthesis kit (Biorad) followed by qPCR using SYBR Green PCR Master Mix (Applied Biosystems). Ct values of target gene compared to that of the housekeeping gene (GAPDH) were used to quantify gene expression in each sample. Primers used to amplify genes are listed in table 1.

2.5. Immunoblotting

For immunoblotting, cells were washed in PBS and lysed in RIPA buffer (Life Technologies), containing protease and phosphatase inhibitor tablet (Invitrogen). Protein was resolved on 10% SDS-PAGE, blotted onto nitrocellulose membrane and probed with antibodies for *phospho*-NOS1, NOS2, Actin and HDAC1. Nuclear and cytoplasmic extracts from cells were prepared using nuclear/cytosol fractionation kit (Biovision) following manufacturer's instruction and subjected to immunoblot analysis for detection with Fos, Jun, and ATF2 antibodies. All antibodies were obtained from Santa Cruz.

2.6. Flow cytometry

Flow cytometry analysis was performed on BMDM seeded in six-well plates. Post LPS and TRIM treatment, cells were collected by centrifugation and resuspended in 1X PBS. Fixation of cells was done in 4% formaldehyde for 10 min at 37 °C followed by snap chill on ice for 1 min. Cells were then permeabilized by adding 90% methanol on ice. Post permeabilization, cells were repeatedly washed in incubation buffer (0.5 g bovine serum albumin in 100 ml 1X PBS). 100 μl of NOS2 primary antibody (Santa Cruz Biotechnology; 200μg/ml) at a concentration of 1μg/1 × 10^6 cells was added to the cell pellet for 1 h at room temperature. Unbound primary antibody was removed by washing in incubation buffer. The cell pellet was resuspended in FITC conjugated secondary antibody for NOS1 (Donkey anti-mouse-FITC; Santa Cruz Biotechnology) for 30 min at room temperature. Cells were then washed with incubation buffer. After final washing, cells were resuspended in 1X PBS and analyzed on a flow cytometer (BD LSR Fortessa). NOS2 protein expression in LPS and TRIM treated macrophages was analyzed by the intensity of FITC signal from a cellular population of each sample.

3. Results

3.1. NOS1 deprived macrophages show diminished proinflammatory cytokine response to LPS

Inflammatory signaling in macrophages is a tightly regulated event that prompts the production of proinflammatory cytokines upon endotoxin challenge [4,39,40]. Engagement of Toll-like receptor-4 (TLR-4) with endotoxin (or LPS) is associated with activation of key transcription factors such as NFkB and AP1 [23,41]. The downstream cooperative effect of the active NF-κB and AP1 pathway [26,42], propagate the pathogenic signals into the cellular response by rapid production of proinflammatory cytokines including IL12, IL23, TNF-α, and IFN-γ [42–45].

In this study, we examined the LPSinduced proinflammatory cytokine gene regulation and their varied expression upon NOS1 inhibition by pharmacological inhibitor TRIM (1-(2-Trifluoromethylphenyl) imidazole) in bone-marrow-derived macrophages. Proinflammatory cytokines IL12 and IL23 were highly expressed with LPS challenge in a time-dependent manner (Fig. 1A, B). LPS stimulation up to 2h significantly increased IL12 and IL23 gene expression, which however decreased in presence of TRIM. Similar to observation with IL12 and IL23, TNF-α gene expression also markedly upregulated upon LPS induction with a steady increase up to 2h. Strikingly, TNF-α gene exhibited diminished expression with the inhibition of NOS1 in macrophages by TRIM (Fig. 1C). Furthermore, expression of IFN-γ which is a critical mediator of endotoxin-induced immune response is regulated by NF-κB and AP1 in a concomitant manner, was determined in LPS stimulated macrophages. Results demonstrated a rapid increase in IFN-γ expression within 1h of LPS stimulation with a constant increase up to 2h (Fig. 1D). Subsequently, NOS1 suppression by TRIM notably declined IFN-γ expression suggesting a NOS1 dependent upregulation of proinflammatory cytokine that further orchestrates robust inflammatory signals.

In addition to the production of cytokine genes and their negative regulation by NOS1 inhibition, the study also determined the expression of inflammatory chemokine CXCL8 (interleukin-8) in