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Original article

Patterns of microglial innate immune responses elicited by amyloid $\beta_{1–42}$ and lipopolysaccharide: the similarities of the differences

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Background: As part of their innate immune response to changes in the central nervous system environment, normally quiescent microglia become activated and increase expression of pattern recognition receptors, scavenger receptors, and production of inflammatory cytokines, proteinases, reactive oxygen species (ROS), and free radicals. These molecules have been implicated in the pathogenesis and progression of several neurodegenerative disorders including Alzheimer disease (AD).
Objective: We compared patterns of microglial innate immune responses elicited by nonfibrillar amyloid β peptide ($nfA\beta_{1–42}$) to those elicited by lipopolysaccharide (LPS).
Methods: Murine BV-2 microglial cells were exposed to either $nfA\beta_{1–42}$ or LPS for 12 h. Then, total RNA from each condition was isolated and expression levels of Toll-like receptor (TLR)-4, scavenger receptor class A (SR-MARCO) and class B (SR-BI), CD36, and matrix metalloproteinase (MMP)-9 were determined by reverse transcription–quantitative real-time polymerase chain reaction. The amount of hydrogen peroxide (H_2O_2) and nitric oxide (NO) in the cell-free supernatant at 24 h were determined using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) and Griess reagent, respectively.
Results: $nfA\beta_{1–42}$ and LPS significantly increased expression of TLR-4, SR-MARCO, CD36, and MMP-9 and production of H_2O_2 and NO in BV-2 microglial cells compared with that of unstimulated cells. However, expression of SR-BI was significantly induced only when the cells were exposed to $nfA\beta_{1–42}$.
Conclusion: These findings indicate that pattern of microglial innate immune responses elicited by $nfA\beta_{1–42}$ overlap with that elicited by LPS and suggest a specific role of microglial SR-BI expression in AD pathogenesis.

Keywords: Alzheimer disease, innate immunity, matrix metalloproteinase, microglia, scavenger receptors, toll-like receptor

The deposit of amyloid beta (Aβ) peptides in the neuropil is the most distinctive and widespread pathology in Alzheimer disease (AD) [1]. The peptides of varying length, ranging from 39–43 amino acids, are derived from combined and sequential cleavage of the amyloid precursor protein by enzymes known as β- and γ-secretases. $A\beta_{1–42}$ is a highly aggregable variant of the peptides and its toxicity is relevant to the pathological processes in AD that include synaptic dysfunction, glial cell activation, inflammation, and neuronal loss [2-5]. It has been shown that Aβ interacts with a diverse classes of proteins including those expressed by neurons, such as the α7 nicotinic acetylcholine receptor [6] and the 75 kDa neurotrophin receptor ($p75^{NTR}$) [7], and those on microglial cells, such as scavenger receptors (SR) [8-11].

Microglia are innate immune cells comprising approximately 10%–20% of glial cells in the brain [12]. They are on the front line of defense against invading pathogens and resemble tissue macrophages. They have diverse functions being either protective or destructive. Normally quiescent microglia rapidly become activated in response to brain insult. Activated microglia provide multiple neurotrophic factors to support neuronal survival and functions, such as nerve growth factor [13], brain-derived neurotrophic factor [14], and insulin-like growth factor [15]. The capability of microglia to migrate and perform phagocytosis is crucial for the clearance of cellular debris and extracellular protein aggregates that interfere with the neuroregenerative processes [16, 17]. However, it has been shown that activated microglia are an important

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source of neurotoxic molecules that include proinflammatory cytokines (e.g. interleukin (IL)-1, IL-6, and tumor necrosis factor-α) [18], glutamate [19], matrix metalloproteinase (MMP)-9 [20], reactive oxygen species, free radicals [21], and neurotoxin [22]. Therefore, the cellular mechanisms involved in the activation of microglia have been the focus of various research that aims to understand better the pathogenesis and progression of several neurodegenerative disorders including AD.

Studies in vivo and in vitro regarding the activation of microglia have been accomplished using lipopolysaccharide (LPS) derived from the outer membrane component of gram negative bacteria [23-26]. Exposure to LPS activates the MyD88-dependent and independent signaling pathways downstream of TLR-4 leading to the activation of the nuclear factor NF-κB and subsequent production of major inflammatory cytokines and reactive oxygen species (ROS) [27]. Furthermore, expression of several cell surface receptors as part of microglial innate immune responses to LPS including SR and β1 integrin have been shown to be crucial for microglial interaction with fibrillar/aggregated Aβ [8, 28, 29]. A host of molecules derived from LPS-activated microglia have also been described in AD [30], supporting a pivotal role of microglial innate immunity in AD pathogenesis. To provide a better understanding regarding the role of nonfibrillar (nf) $A\beta_{1-42}$ in microglial innate immune responses, BV-2 cells [31], a widely used alternative model system for primary microglial cultures, were exposed to $nfA\beta_{1-42}$. Then, the microglial expression patterns of Toll-like receptor (TLR)-4, scavenger receptor class A macrophage receptor with collagenous structure (SR-MARCO), scavenger receptor class B (SR-BI), CD36, and MMP-9 were determined by reverse transcription–quantitative real-time polymerase chain reaction (RT-qPCR) and compared with those elicited by LPS. The ability of $nfA\beta_{1-42}$ compared with that of LPS to stimulate BV-2 cells to produce hydrogen peroxide (H_2O_2) and nitric oxide (NO) was also examined. Results demonstrated that $nfA\beta_{1-42}$ elicited a target gene expression pattern similar to that of that elicited by LPS. One exception to this was that of expression of SR-BI, which was specifically induced by $nfA\beta_{1-42}$.

Materials and methods

Reagents

LPS was obtained from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin were from HyClone (South Logan, UT, USA). Fetal bovine serum (FBS) was from Gibco (Paisley, UK). $A\beta_{1-42}$ (human), TRIzol reagent, SuperScript VILO cDNA Synthesis Kit, Griess reagent, and 10-acetyl-3,7-dihydroxyphenoxazine (Amplex red) were purchased from Invitrogen (Carlsbad, CA, USA). Power SYBR Green PCR Master Mix was purchased from Applied Biosystems (Warrington, UK).

Microglial cell cultures and treatments

The murine BV-2 microglial cell line was kindly provided by Dr. James R Cornor (The Pennsylvania State University, College of Medicine, Hershey, PA). BV-2 cells were grown and maintained in DMEM supplemented with 5% FBS, penicillin 100 U/mL and streptomycin 100 μg/mL under standard conditions at 37°C. Cultures were exposed to either $nfA\beta_{1-42}$ (10 μM) or LPS (1 μg/ml) for different times as indicated in each experiment. A stock of $nfA\beta_{1-42}$ was freshly prepared in PBS according to the manufacturer's protocol and used immediately before a β-sheet conformation. A 10 μM concentration of $A\beta_{1-42}$ was used in this study, because this concentration was reported to stimulate phagocytosis and expression of inflammatory cytokines in BV-2 microglial cells [32]. Unstimulated cultures served as controls.

RNA isolation and RT-qPCR assay

BV-2 cells were exposed to either $nfA\beta_{1-42}$ or LPS for 12 h. Total RNA from each condition was isolated using TRIzol reagent, according to the manufacturer's protocol. Total RNA from each sample was subjected to reverse-transcriptase to cDNA using a SuperScript VILO cDNA Synthesis Kit. qPCR was performed using Power SYBR Green PCR Master Mix containing the following primers: TLR-4 [33], SR-MARCO, CD36, SR-BI, MMP-9, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [34]. Relative quantification of gene expression was determined using a comparative threshold (CT) method. The difference in mRNA expression levels was calculated following normalization to GAPDH. The ratios obtained after normalization are expressed as fold change compared with that of the control.

Hydrogen peroxide assay

Levels of H_2O_2 in BV-2 culture medium (1×10^5 cells/well, 24-well plate) were determined using

10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) in combination with horseradish peroxidase, according to the manufacturer's instructions. In the presence of peroxidase, the Amplex Red reagent reacts with H_2O_2 to produce resorufin, a red fluorescent compound [35]. To begin the experiment, BV-2 cells (1×10^4 cells/well, 96-well plates) were exposed to either nf$A\beta_{1-42}$ or LPS for 24 h. Then, the cell-free supernatants were collected and 100 μM Amplex Red reagent and 0.2 U/ml HRP were added. The reactions were incubated at room temperature for 30 min and the fluorescence intensity was measured at excitation/emission wavelengths of 545/590 nm on a Synergy HT multimode microplate reader (BioTek Instruments, Winooski, VT, USA). The concentration of H_2O_2 in each sample was calculated using a H_2O_2 standard curve.

Nitric oxide assay

The levels of nitrite (NO_2), an end product of NO metabolism, were determined by using Griess reagent. In this reaction, sulfanilic acid is converted to a diazonium salt by reacting with nitrite in acid solution. The diazonium salt is then coupled to *N*-(1-naphthyl) ethylenediamine forming an azo dye, which can be detected by measuring absorbance at 540 nm. Briefly, BV-2 cells (1×10^5 cells/well, 24-well plate) were exposed to either nf$A\beta_{1-42}$ or LPS for 24 h. Then, the cell-free supernatants were collected to measure the absorbance values at 540 nm on a Synergy HT multimode microplate reader (BioTek instruments). The NO_2 concentrations were calculated using a sodium nitrite standard curve.

Statistical analysis

All data were expressed as the mean ± SEM from at least three independent experiments performed in triplicate. Statistical comparisons between groups were determined using a paired samples *t* test. Multiple comparisons of data were evaluated using a one-way ANOVA followed by LSD post hoc testing. $P < 0.05$ was considered statistically significant.

Results

The effects of nf$A\beta_{1-42}$ and LPS on expression of TLR-4, SR-MARCO, SR-BI, CD36, and MMP-9

In this set of experiments, the ability of nf$A\beta_{1-42}$ to elicit expression of genes associated with microglial innate immune responses was examined. BV-2 microglial cells were exposed to 10 μM nf$A\beta_{1-42}$ or 1 μg/ml LPS for 12 h. Relative mRNA expression levels of TLR-4, SR-MARCO, SR-B1, CD36, and MMP-9 were quantified by RT-qPCR. Results demonstrated that nf$A\beta_{1-42}$ and LPS elicited similar, but not identical, expression patterns of TLR-4, SR-MARCO, CD36, and MMP-9 in BV-2 cells. However, it was found that expression of SR-B1 was significantly increased by nf$A\beta_{1-42}$ and suppressed by LPS (**Figure 1**).

The effects of nf$A\beta_{1-42}$ and LPS on the production of H_2O_2 and NO

H_2O_2 and NO produced by cells of the immune system play an important role in host defense against infectious pathogens. These reactive intermediates have also been implicated in microglia-mediated neurotoxicity [23, 24]. Therefore, to examine the effects of nf$A\beta_{1-42}$ on microglial production of H_2O_2 and NO compared with that of LPS, BV-2 cells were exposed to nf$A\beta_{1-42}$ or LPS for 24 h and the amount of H_2O_2 and NO_2 in the cell-free supernatant was determined using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) and Griess reagent, respectively. Results demonstrated that unstimulated BV-2 cells produced a detectable level of H_2O_2. nf$A\beta_{1-42}$, like LPS, stimulated BV-2 cells to produce a significant level of H_2O_2, compared with that produced by unstimulated cells. (**Figure 2A**). nf$A\beta_{1-42}$ also stimulated BV-2 cells to produce a very low level of NO compared with the production stimulated by LPS. In the absence of stimulus, BV-2 cells produced undetectable levels of NO (**Figure 2B**).

Discussion

Positron emission tomography (PET) studies using [^{11}C]APK11195 and [^{11}C]DAA1106, which are selective ligands for the peripheral benzodiazepine binding site, as a marker for microglial activation, have revealed that microglial activation is an early event in AD [36, 37]. This provides evidence for the role of innate immunity in the pathological processes of the disease. In this study, we examined patterns of microglial innate immune responses elicited by nf$A\beta_{1-42}$, a critical trigger for AD progression. We found a significantly increased expression of TLR4, SR-MARCO, CD36, and MMP-9 in BV-2 microglia, compared with that in unstimulated cells. This pattern of expression was similar to that of LPS-stimulated cells, suggesting a "nonspecific" pattern of microglial innate immune responses to nf$A\beta_{1-42}$. This view has been supported by the fact that TLR4, SR-MARCO,

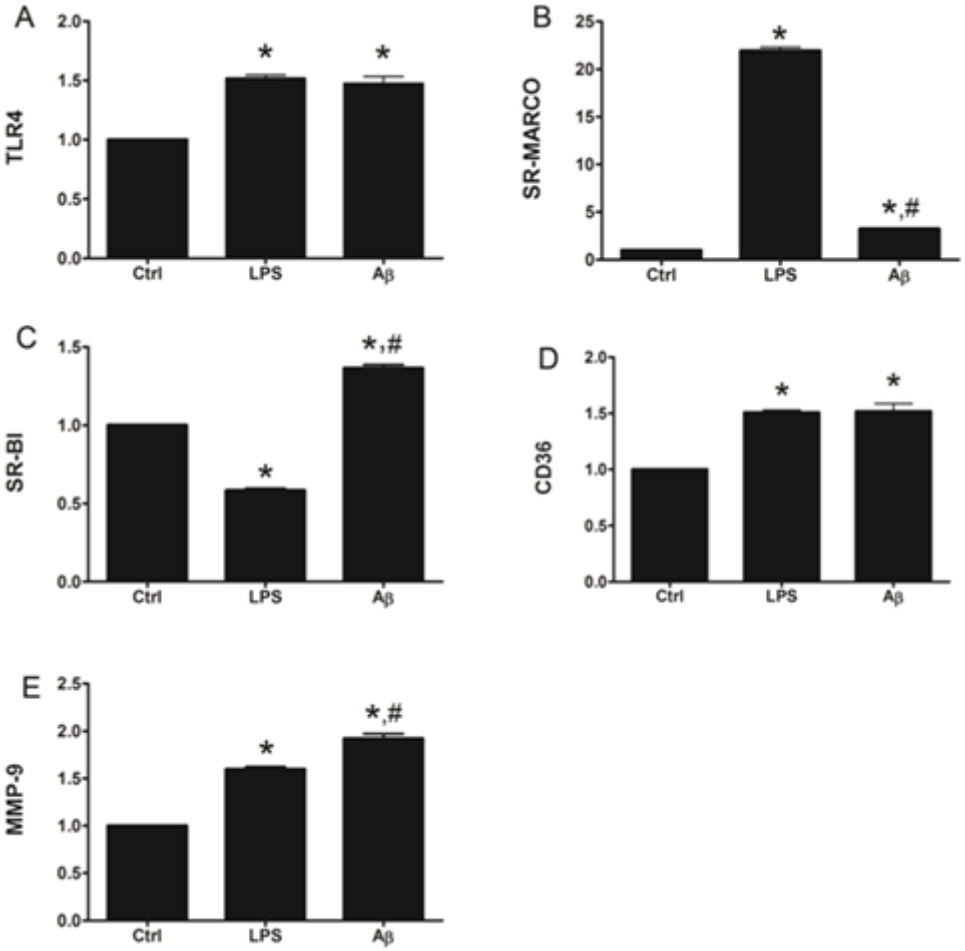

Figure 1. Expression of genes associated with microglial innate immune responses to $A\beta_{1-42}$ and lipopolysaccharide (LPS). BV-2 cells were exposed to nonfibrillar $A\beta_{1-42}$ (10 μM) or LPS (1 μg/ml) for 12 h. Relative levels of expression of mRNA for Toll-like receptor (TLR)-4 (**A**), scavenger receptor class A (SR-MARCO) (**B**) and class B (SR-BI) (**C**), CD36 (**D**), and matrix metalloproteinase (MMP)-9 (**E**) were quantified by reverse transcription–quantitative real-time polymerase chain reaction. Normalized comparative threshold levels are presented as mean ± SEM of three independent experiments performed in triplicate; $^{*}P < 0.05$ compared with unstimulated control; $^{\#}P < 0.05$ compared with LPS.

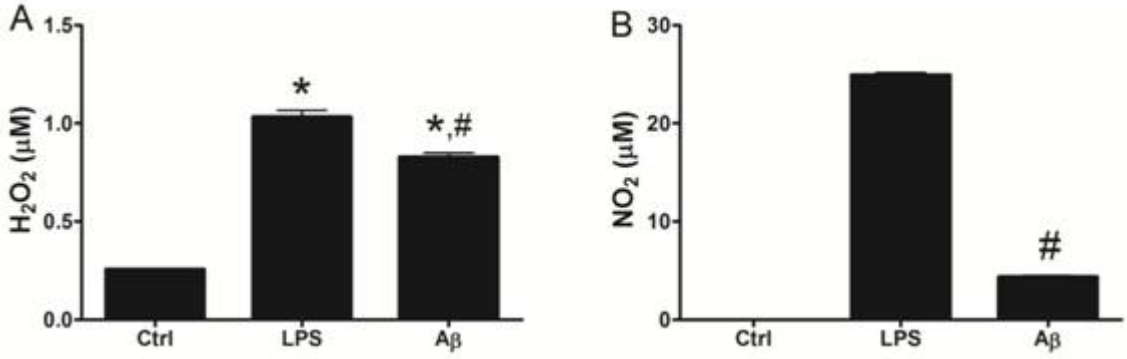

Figure 2. Microglial production of H_2O_2 and NO in response to $A\beta_{1-42}$ and lipopolysaccharide (LPS). BV-2 cells were exposed to nonfibrillar $A\beta_{1-42}$ (10 □M) or LPS (1 μg/ml) for 24 h. Then, levels of H_2O_2 (**A**) and NO (**B**) in the cell-free supernatants were determined using 10-acetyl-3,7-dihydroxyphenozasine (Amplex red) and Griess reagent, respectively. All data are presented as mean ± SEM of three independent experiments performed in triplicate; $^{*}P < 0.05$ compared with unstimulated control; $^{\#}P < 0.05$ compared with LPS.

and CD36 are multiligand receptors. In addition to bacterial LPS, TLR-4 has been shown to interact with gangliosides on neuronal cell membranes [38] and, in conjunction with CD14 and TLR-2, and fibrillar (f) A [39]. Known ligands for SR-MARCO include LPS [40], and fAβ and nfAβ [41]. CD36 recognizes diacylglycerides [42], LPS [43], and fAβ [11]. Thus, it is hypothesized that early expression of these receptors primed by $nfA\beta_{1-42}$ is essential for microglial interaction with fAβ in AD plaques and subsequent production of proinflammatory cytokines and neurotoxins. This is consistent with many other reports regarding proinflammatory and neurotoxic properties of fAβ [11, 30, 44]. Furthermore, increased expression of MMP-9 by $nfA\beta_{1-42}$, as observed herein, may amplify microglial toxicity through its direct neurotoxic effect and ability to facilitate maturation of pro-IL-1β [45, 46].

The appearance of microglial clusters within the plaques [47, 48] readily highlights a possible mechanism of microglial clearance of Aβ deposits. We showed that exposure of BV-2 microglia to $nfA\beta_{1-42}$ specifically induced expression of SR-BI, suggesting a potential role of SR-BI in the microglial–Aβ interaction. This finding is in agreement with previous reports showing that AD microglia express SR-BI [9, 10]. Similarly, perivascular macrophages, associated with fAβ deposits in the brains of a mouse model of AD, also express SR-BI. Downregulation of SR-BI compromises cellular responses of these cells to fAβ [49]. Furthermore, it has been shown by a genetic manipulation approach that SR-BI expression is essential for microglial adhesion to and uptake of fAβ [50]. Additionally, a study by Koenigsknecht and Landreth has demonstrated that induction of microglial phagocytosis of fAβ requires a receptor complex consisting of CD36, $\alpha_6\beta_1$ integrin, and integrin-associated protein CD47 [29]. Moreover, the role of TLR-4 in microglial clearance of Aβ has also been suggested [51]. Thus, microglial clearance of Aβ deposits appears to involve several classes of innate immune receptors.

Here, we reported that BV-2 microglia produced H_2O_2 and NO in response to $nfA\beta_{1-42}$. These findings are consistent with constitutive expression of TLR4, SR-BI, and CD36 in these cells, as shown in **Figure 1** and with many reports showing that stimulation of these receptors on microglia by either LPS or fAβ facilitates ROS and free radical production [24, 39, 50, 52]. Here, we report that microglia produced H_2O_2 and NO in response to $nfA\beta_{1-42}$. A study by Le and colleagues has also demonstrated that H_2O_2 and NO are potentially toxic molecules mediating microglial toxicity in an in vitro model of dopaminergic cell injury [24], suggesting a similar mechanism of microglia-mediated toxicity by $nfA\beta_{1-42}$ in AD.

In summary, this study demonstrates a "nonspecific" pattern of microglial innate immune responses elicited by $nfA\beta_{1-42}$ and this pattern overlaps with that of LPS. We also report that expression of SR-BI is a specific microglial response to $nfA\beta_{1-42}$. Taken together, early activation of microglial innate immune receptors by $nfA\beta_{1-42}$ could enhance microglial clearance of fAβ deposits. Nevertheless, collateral damage to neuronal tissue as a result of this process is unavoidable.

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