

# Recognition of Betaine as an Inhibitor of Lipopolysaccharide-Induced Nitric Oxide Production in Activated Microglial Cells

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Received 26 October 2011; revised 18 January 2012; accepted 22 February 2012

## ABSTRACT

**Background:** Neuroinflammation, as a major outcome of microglia activation, is an important factor for progression of neurodegenerative disorders including Alzheimer's disease and Parkinson's disease. Microglial cells, as the first-line defense in the central nervous system, act as a source of neurotoxic factors such as nitric oxide (NO), a free radical which is involved in neuronal cell death. The aim of this study was to inhibit production of NO in activated microglial cells in order to decrease neurological damages that threaten the central nervous system. **Methods:** An *in vitro* model of a newborn rat brain cell culture was used to examine the effect of betaine on the release of NO induced by lipopolysaccharide (LPS). Briefly, primary microglial cells were stimulated by LPS and after 2 minutes, they were treated by different concentrations of betaine. The production of NO was assessed by the Griess assay while cell viability was determined by the MTT assay. **Results:** Our investigations indicated that LPS-induced NO release was attenuated by betaine, suggesting that this compound might inhibit NO release. The effects of betaine on NO production in activated microglial cells after 24 h were "dose-dependent". It means that microglial cells which were treated with higher concentrations of betaine, released lower amounts of NO. Also our observations showed that betaine compound has no toxic effect on microglial cells. **Conclusion:** Betaine has an inhibitory effect on NO release in activated microglial cells and may be an effective therapeutic component to control neurological disorders. *Iran. Biomed. J. 16 (2): 84-89, 2012*

**Keywords:** Betaine, Lipopolysaccharides (LPS), Nitric oxide (NO), Microglia

## INTRODUCTION

Amongst three types of glial cells, including microglia, oligodendrocytes and astrocytes, it seems that, microglial cells with a mesenchymal origin [1-3] are the first-line defense [4, 5] in the central nervous system. Microglia, which behave so much like macrophages, are known as "Brain macrophages" [3, 6-8] and have a pivotal role in immune surveillance and host defense [6-10]. In response to brain infection, microglial cells show rapid reaction, and the earliest phenotypic alterations from quiescent microglia to the activated form [8, 10-13], initiates an inflammatory signaling cascade. Upon activation, microglial cells prepare suitable responses that include migration to the site of injury, proliferation, phagocytosis and also the expression of surface receptors [3, 9-11]. These quick alterations and inflammatory events result in expression of several

genes encoding most innate immune proteins, such as cytokines, chemokines and enzymes including cyclooxygenase 2 and inducible nitric oxide synthase (iNOS), which are mediated by an important transcription factor: nuclear factor-kappa B (NF- $\kappa$ B) [13-16]. In this way, microglial cells orchestrate effective immune responses against foreign invaders [8, 11]. A wide array of infectious pathogens can trigger inflammatory responses by penetrating the central nervous system and activating microglial cells [8]. Lipopolysaccharides (LPS), from Gram-negative bacteria, are one of the potent stimuli which activate microglia via the Toll like receptor 4 [8]. Binding of LPS to its receptor launches intracellular signaling, which produces activation of NF- $\kappa$ B and subsequent expression of iNOS [8]. Recent reports show that microglia are the major cell types, which are responsible for initiation and progression of neurodegenerative disorders especially through the

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production of nitric oxide (NO) [11-13, 16]. NO, as a gaseous free radical, is a key molecule that plays a pivotal role in normal signal transduction and it may result in neuronal cell damage and death [17]. Numerous studies suggest that NO is capable of making alterations in the chemical biology of protein function via reaction with the cysteine residue of target proteins, which form S-nitrosothiols, a process known as S-nitrosylation [17, 18]. Accumulating evidences indicate that S-nitrosylation of specific proteins is likely to affect the degradation process, which leads to the aggregation of misfolded proteins, a significant sign of many neurodegenerative disorders [17, 18].  $\alpha$ -synuclein and synfilin 1 in patients suffering from Parkinson's disease and amyloid beta and tau in those with Alzheimer's disease are examples of misfolded, aggregated proteins [17, 18]. By using anti-inflammatory drugs, which inhibit iNOS expression, neuronal damages can be decreased via the NO reduction.

Besides further study which has proved the role of NO in accumulation of misfolded proteins [18], other data have implicated that high concentrations of some metabolites are also potent inducers of aggregation of abnormal proteins. Homocysteine (hcy), a derivative of methionine metabolism, is a non-essential sulfur containing amino acid, which is a risk factor for neurodegenerative disorders [19]. Evidences have shown that elevated hcy levels lead to hypomethylation of DNA and altered gene expression results in neuronal damage [19, 20]. The role of hcy in aggregation of amyloid and tau proteins, apoptosis, neuronal death and brain atrophy has been demonstrated by other researchers [19, 20].

Betaine or trimethylglycine, first founded in the juice of sugar beets, is widely available in plants, animals and other organisms [21, 22]. This molecule has two biological effects: as an osmolyte, it can protect cells, proteins and enzymes under conditions of stress, including low water content, high salinity and extreme pressure; and as an important methyl donor, betaine participates in many biological pathways [21, 22]. In methionine cycle, transition of a methyl group from betaine to hcy results in methionine formation. Therefore, betaine prevents noxious accumulation of hcy [21, 22]. An imbalance in the methionine cycle leads to elevation of serum hcy level, which is closely associated with cardiovascular disorders, brain atrophy and neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease and dementia. In the present study, the anti-inflammatory effects of betaine were investigated by examining NO levels in activated microglial cells. Our results showed some satisfactory conclusions, which could be effective for controlling neurodegenerative disorders.

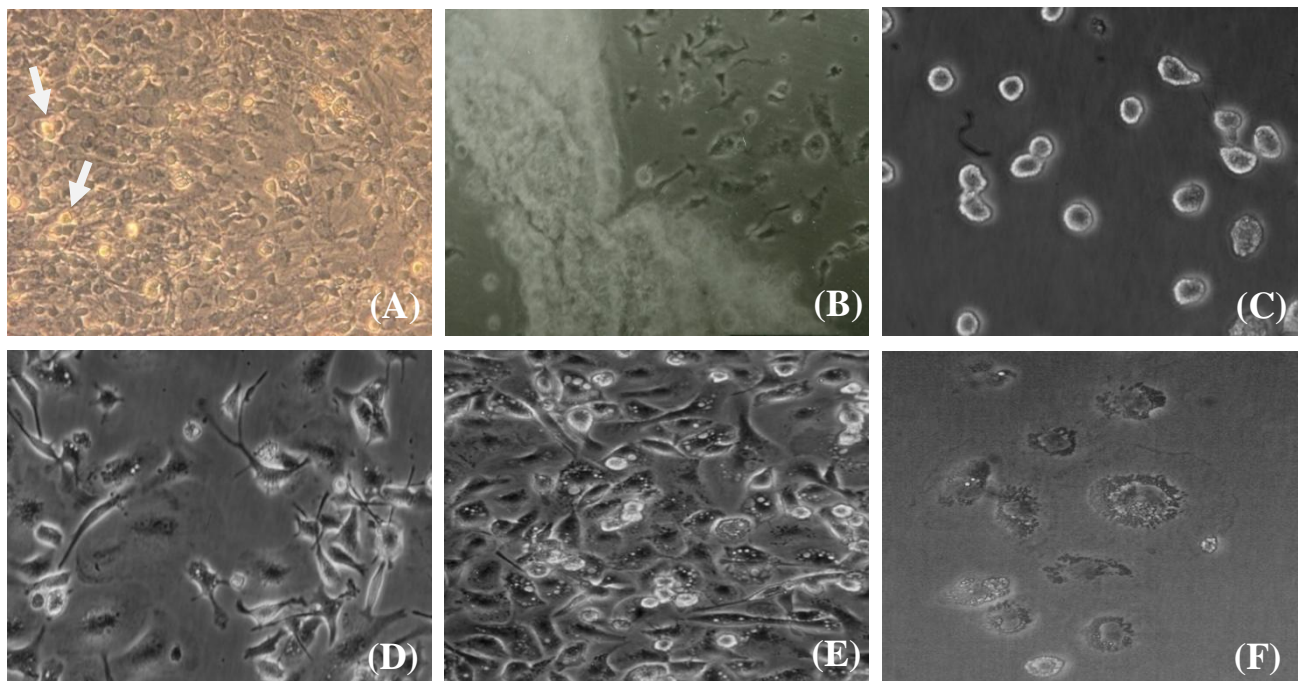
## MATERIALS AND METHODS

**Reagents.** Bacterial LPS (from *E. coli* 026:B6), Griess reagent kit (G4410), DMSO, MTT kit (M2128-500 MG) and betaine (EC No. 203-490-6) were purchased from Sigma (USA). DMEM and FBS were obtained from Gibco (USA). Antibiotics including streptomycin and penicillin were purchased from Merck (Germany).

**Cell culture.** Primary mixed glial cultures were obtained from the cerebral cortices of 1-4-day-old newborn Wistar rats (Fig. 1A). According to Giulian and Baker's method [23], after removing meninges and blood vessels in Hank's buffer, the brain tissues were isolated and dissociated mechanically into small pieces in DMEM. Four brains were transferred to T-25 tissue culture flasks containing DMEM supplemented with 10% heat-inactivated FBS (0.5 hemisphere/flask) and grown at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. After four days, the media and tissues were removed from the flasks and the fresh media were replaced. All cells except microglia were removed after 10 days of incubation by mild trypsinization (Fig. 1B and 1C). After 24 h (Fig. 1D), the microglial cells were detached from the flasks with a cell scraper and were stained with trypan blue and counted by a hemocytometer. The cells were seeded onto 96-well plates at a density of  $1 \times 10^4$  cells per well and allowed to attach to the plate in 10% FBS by incubation at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 24 h. The cells were subsequently stimulated by LPS (1  $\mu$ g/ml) and treated with different concentrations of betaine (50-1000  $\mu$ M) (Fig. 1).

**Nitrite quantification.** The concentrations of NO were estimated by measuring the amounts of nitrite secreted by microglial cells into the culture medium, using a colorimetric reaction with the Griess reagent. The culture supernatants were collected 24 and 48 h after LPS stimulation and drug treatment, and then centrifuged at 226.6  $\times$ g for 10 minutes and mixed with an equal amount of the Griess reagent in a 96-well microtiter plate. The light absorbance of the mixture was read at 540 nm using a microplate reader (Fig. 2).

**Cell viability assay.** For the cell viability assay, cultures were incubated with modified MTT solution at 37°C for 4 h. The MTT solution was then removed and formation of formazan crystals which dissolved in DMSO, showed the metabolic activity of the cells. Absorption was determined at 580 nm using a microplate reader (Figs. 3 and 4).



**Fig. 1.** Purification and activation of microglia cells. Microglial cells in a mixed glial and neuron culture after 2 weeks (arrows). Mixed cell population containing neurons and other glial cell types (A). Purification of microglial cells by trypsin (B). Microglial cells after trypsinization (C). After exposure to trypsin, phenotypic changes of microglial cells from ramified shape to reactive form will occur. Microglial cells 24 h after trypsinization (D). After 24 h, microglial cells regain their ramified phenotype. Untreated microglial cells (E) in comparison with LPS-treated microglial cells (F) (Phase contrast [200×]). Untreated microglial cells (E) are ramified in comparison with LPS-treated microglial cells (F), which have altered and activated form.

**Statistical analysis.** Data were statistically analyzed as factorial experiments in a completely randomized design with at least three separate experiments carried out in triplicate. The Walter-Duncan k-ratio was then used to determine the significant difference among the means at  $P < 0.05$  using SPSS v16.

## RESULTS

**Microscopic investigations of microglial cells after treatment with lipopolysaccharide and betaine.** In this research, 1  $\mu\text{g/ml}$  of LPS was used to activate microglial cells and different concentrations of betaine (50-1,000  $\mu\text{M}$ ) were prepared for the subsequent treatments. Morphological alterations of microglial cells after treatment with LPS are evident when compared with the untreated negative controls ([DMEM + FCS] and DMEM) (Fig. 1E and 1F).

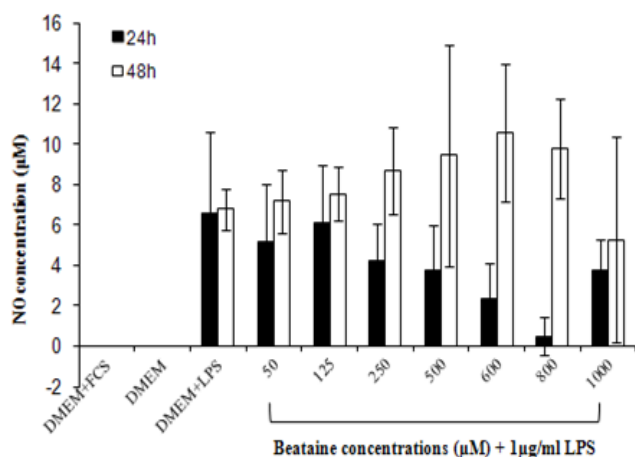
**The effect of betaine on lipopolysaccharide-induced NO production.** The investigations revealed that betaine could decrease LPS-induced NO release in the brain. Results showed that betaine decreases the amount of NO production in a dose-dependent manner, 24 h after treatment. In fact, with an increase in betaine concentration, NO levels decreased (Fig. 2). Moreover, it seems that the effective concentration of betaine that

controls the release of NO is up to 800  $\mu\text{M}$ . In addition, there is a significant difference between the amounts of NO release in positive control (DMEM + LPS) and treated cells with LPS and higher concentrations of betaine (500-800  $\mu\text{M}$ ). After 48 h, there was no significant reduction of NO in LPS- and betaine-treated cells (50-1000  $\mu\text{M}$ ) when compared with positive control (DMEM + LPS). This result may indicate that during 24 h, betaine was consumed by microglial cells.

**The effect of betaine on viability of cells.** To investigate the cytotoxic effect of betaine, viability of cells were assessed by MTT (Fig. 3). Formation of formazan crystals in treated microglial cells with different concentrations of betaine reveals that this compound has no toxic effect on microglial cells (Fig. 4).

## DISCUSSION

Inflammation is a natural reaction of the organism to injury and invading pathogens [24]. Within the brain, this reaction is initiated by microglial cells, which serve as the resident mononuclear phagocytes of the brain [24]. High sensitivity of these cells to environmental threats triggers a rapid transformation of these cells from resting and non-motile forms to altered and



**Fig. 2.** The effect of betaine on nitric oxide (NO) production in lipopolysaccharide (LPS)-activated microglial cells. First, microglial cells were activated by LPS (1 µg/ml), and then treated by different concentrations of betaine. Nitrite concentration (µM) in culture media was assessed by Griess reaction. In treated microglial cells, the culture supernatants were collected 24 and 48 h after LPS-stimulation and drug treatment, and centrifuged at 226.6 ×g for 10 minutes and mixed with an equal amount of the Griess reagent in a 96-well microtiter plate. The light absorbance of the mixture was read at 540 nm. The effects of betaine on NO production in activated microglial cells after 24 h are "dose-dependent", with an increase in betaine concentration, NO levels decreased. (DMEM + FCS) and DMEM are negative controls, while (DMEM + LPS) is positive control. It seems that after 48 h, betaine had no effect on reduction of NO release in LPS and betaine-treated cells (50-1,000 µM), probably because of consumption of betaine by microglial cells in 24 h. Values with  $P < 0.05$  are statistically significant.

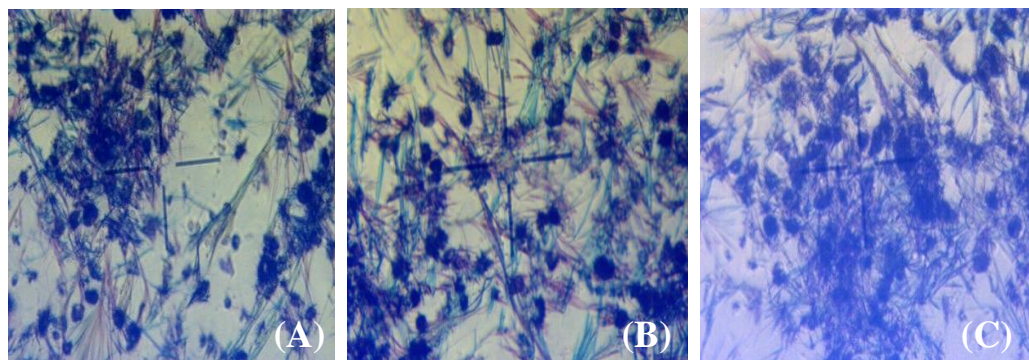
activated phenotypes [25, 26]. Activated microglial cells are capable of producing and releasing a plethora of secretory products ranging from cytokines and chemokines to toxic defense metabolites, such as NO, in response to foreign invaders.

It has been shown that excessive NO and hcy are

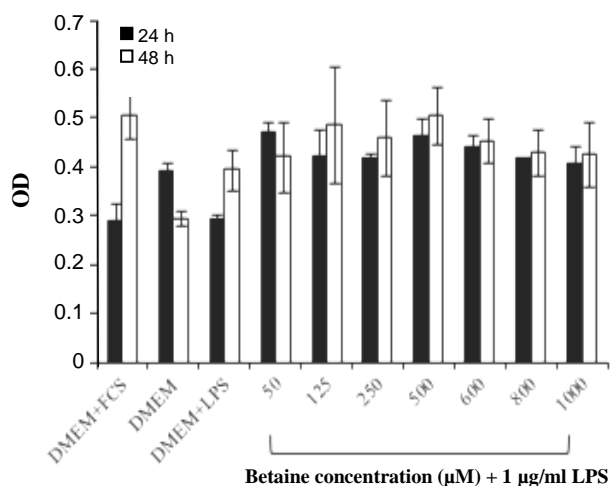
two significant reasons for neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease, and betaine is capable of decreasing both of these factors in order to control neurological damages [17-20, 27]. The result of this study showed that betaine is a strong inhibitor of NO production in LPS-stimulated microglial cells. We observed that betaine decreased the amount of NO release in a dose-dependent manner 24 h after treatment. In fact, treated cells with higher concentrations of betaine, released lesser amounts of NO. Our hypothesis is that betaine might inhibit iNOS indirectly via suppressing NF-κB. Moreover, GO and colleagues [22, 28], in their investigations of aged rat kidney cells, elucidated the anti-inflammatory effects of betaine on NF-κB activity and TNF-α expression.

Since NF-κB is a critical transcription factor involved in many inflammatory disorders, they also reported the suppressive effects of betaine on NF-κB activation via NF-κB-inducing kinase/IκB kinase and mitogen-activated protein kinase, which present betaine as a beneficial agent for the suppression of age-related inflammation [22, 28]. Other finding has demonstrated that betaine as a methyl group donor can decrease S-adenosyl homocysteine and hcy concentrations in human blood [21]. Furthermore, observations have shown that betaine may affect Aβ protein expression in the murine microglia cell line BV-2, by blocking the hypomethylation of the presenilin1 gene promoter [29].

Recent studies have identified betaine as a protective osmolyte in the brain, which is transported via an integral membrane transporter known as the betaine/gamma aminobutyric acid transporter 1 (BGT-1) [30, 31]. This transporter (BGT-1) is capable of utilizing both betaine and gamma aminobutyric acid as substrates [30]. Other investigation has implicated the effect of hyperosmotic conditions on BGT-1 expression in astrocytes, thus emphasizing the key role of betaine with regard to osmoregulation in the brain [32].



**Fig. 3.** The comparison between cell viability assay in treated microglial cells and untreated control. Microglial cells after treatment with lipopolysaccharide (LPS) and 100 µM betaine (A). Untreated microglial cells in DMEM and FCS (B). Microglial cells after treatment with LPS and 1,000 µM betaine (C). First, cultures were incubated into modified MTT solution at 37°C for 4 h. The MTT solution was then removed and formation of formazan crystals which dissolved in DMSO, showed the metabolic activity of the cells. Absorption was determined at 580 nm.



**Fig. 4.** The effect of betaine on viability of cells. First, cultures were incubated into MTT solution at 37°C for 4 h. The MTT solution was then removed and formation of formazan crystals which dissolved in DMSO, showed the metabolic activity of the cells. Absorption was determined at 580 nm. Formation of formazan crystals in treated microglial cells with lipopolysaccharide (LPS) and different concentrations of betaine (50-1,000 µM) in comparison with controls (DMEM + FCS), DMEM and DMEM + LPS show that betaine has no toxic effect on microglial cells. Values with  $P < 0.05$  are statistically significant.

Based on the findings of this study, it is rational to propose that betaine would be a useful element for reducing NO-dependent inflammation in the brain and may be an effective therapeutic component to control many neurodegenerative disorders.

### ACKNOWLEDGMENTS

We are grateful to Dr. Shariati for her kind assistance in manuscript preparation. This work was supported by the National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran.

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