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Inhibition of the induction of the inducible nitric oxide synthase in murine brain microglial cells by sodium salicylate

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SUMMARY

The induction of the inducible nitric oxide synthase (iNOS) has been proposed to play a role in a variety of inflammatory diseases. Sodium salicylate (NaSal) is the most commonly used anti-inflammatory agent. We investigated whether NaSal can diminish the induction of iNOS in murine brain microglial cells. In primary cultures, interferon-γ (IFN-γ) or lipopolysaccharide (LPS) separately did not stimulate nitric oxide (NO) production, whereas IFN-γ combined with LPS synergistically induced iNOS. NaSal inhibited both the production of NO and expression of iNOS in microglial cells. Synergy between IFN-γ and LPS was mainly dependent on tumour necrosis factor-α (TNF-α) secretion as the increase of the induction of the iNOS by IFN-γ plus LPS was associated with the increase of TNF-α secretion and IFN-γ plus LPS-induced TNF-α secretion by microglial cells was decreased by the treatment with NaSal. These results suggest a possible use of NaSal in managing inflammation of the central nervous system through inhibition of the iNOS induction.

INTRODUCTION

Nitric oxide (NO), first identified as an endothelium-derived relaxation factor,[1] is now recognized to be an intra- and extracellular mediator of cell function.[2–5] NO produced by the constitutive isoform of nitric oxide synthase (NOS) is a key regulator of homeostasis, whereas the generation of NO by inducible NOS (iNOS) plays an important role in inflammation, host-defence responses, and tissue repair.[2–4] Vane and co-workers have implicated NO as an important mediator of inflammation in animal models.[6] Furthermore, because iNOS is up-regulated by lipopolysaccharide (LPS), tumour necrosis factor (TNF-α), and interferon-γ (IFN-γ), the increased synthesis of NO has been implicated in autoimmune diseases, allograft rejection, graft-versus-host disease, and systemic response to sepsis. Brain microgial cells have been shown to be inflammatory, and cytotoxic effector cells in a variety of pathological processes in the central nervous system (CNS) and express a variety of immunological characteristics, such as homeostatic control and defence of the organism against infection and injury.[7] Recent reports have shown that murine brain microglial cells can synthesize NO in response to IFN-γ and LPS.[8,9]

The anti-inflammatory properties of extracts from willow trees have been documented for almost 2000 years, and the active ingredient has been identified as salicylate.[10] Since plants, particularly fruits and vegetables, such as apples, apricots, cherries, grapes, peaches, plums, cucumbers, peppers and tomatoes, contain natural salicylates, it has been suggested that these naturally occurring salicylates contribute to the reduced risk of various human diseases associated with fruit and vegetable consumption. Recently, salicylates have been shown to exhibit additional effects. For example, administration of low-dose salicylate to physicians and patients suffering from angina pectoris significantly reduced the rate of heart attack and stroke.[10] It has been reported that salicylates inhibit neurotoxicity elicited by the excitatory amino acid glutamate in rat primary neuronal cultures and hippocampal slices.[11] Several previous studies have described the inhibition of NO production by salicylates, and a reduction in steady-state mRNA levels for iNOS occurred when salicylate was added either to rat alveolar macrophages or rat cardiac fibroblasts.[12–14] In the present study, we investigated the possibility that the anti-inflammatory agent sodium salicylate (NaSal), because of its wide spectrum of pharmacological activities and multiple sites of action, may diminish the induction of iNOS in murine brain microglial cells.

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Abbreviations: CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NaSal, sodium salicylate; N^GMMA, N^G-monomethyl-l-arginine; NO, nitric oxide; NOS, nitric oxide synthase; TNF-α, tumor necrosis factor-α.

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MATERIALS AND METHODS

Reagents and mice

Murine recombinant IFN-γ (1×10^7 U/mg), recombinant TNF-α (1×10^5 U/ml), and rabbit anti-murine TNF-α antibody were purchased from Genzyme (Munich, Germany). Phosphatase-labelled anti-rabbit IgG was purchased from Serotec (Oxford, UK). NaSal, Dulbecco's modified Eagle's medium (DMEM), LPS (phenol-extracted *Salmonella enteritidis*), L-arginine, LiCl, urea, *N*-(1-naphthyl)-ethylenediamine dihydrochloride, sodium nitrite and sulphanilamide were purchased from Sigma Chemical Co. (St. Louis, MO). Agarose, phenol, Moloney murine leukaemia virus reverse transcriptase, RNasin, DNA polymerase I, Taq polymerase and deoxynucleotide triphosphate were purchased from Life Technologies (Gaithersburg, MD). DuPont NEN (Boston, MA) was the source of [α-^{32}P]dCTP. The pBluescript II KS(−) plasmid was purchased from Stratagene Inc. (San Diego, CA). Rabbit polyclonal antisera to iNOS were obtained from Transduction Laboratories (Lexington, KY). N^G-monomethyl-L-arginine (N^GMMA) was purchased from Calbiochem (San Diego, CA). All reagents and media for tissue culture experiments were tested for their LPS content using a colorimetric *Limulus* amoebocyte lysate assay (detection limit, 10 pg/ml; Whittaker Bioproducts, Walkersville, MD). None of the reagents (including NaSal) contained endotoxins. Ninety-six-well tissue culture plates and 100-mm diameter Petri dishes were purchased from Nunc (Naperville, IL). DMEM containing L-arginine (84 mg/l), Hanks' balanced salt solution (HBSS), fetal calf serum (FCS) and other tissue culture reagents were purchased from Life Technologies. The original stock of BALB/c mice was purchased from The Dae-Han Experimental Animal Centre (Eumsung, South Korea) and the mice were maintained at the College of Pharmacy, Wonkwang University (Iksan, South Korea). To obtain microglial cells, mice were used at 2–5 days of age.

Preparation of glial cell cultures

Primary glial cell cultures were established from the brains of newborn, pathogen-free BALB/c mice as described elsewhere.[15] After removal of the meninges, brains of newborn BALB/c mice were disaggregated using nylon sieves (120-µm pore size) and seeded in 50-ml tissue culture flasks in DMEM containing 20% heat-inactivated FCS. The inoculation volume was 4 ml/flask and contained 60 mg of dissociated brain. Growth medium was replaced on the 3rd and 6th days with DMEM containing 10% FCS. On day 10, subcultures were made by treating the cells with 0·25% trypsin/0·05% ethylene diaminetetraacetic acid (EDTA) and, after washing, 1×10^6 cells/ml were plated in either a 50-ml tissue culture flask or a 24-well tissue culture plate in DMEM with 5% FCS.

Isolation of murine microglial cells

To isolate microglia from cultured glial cells, culture flasks were vigorously agitated for 1 hr on a rotary shaker (Shaker SI 100; Pharmacia Diagnostics, Upsala, Sweden) at 800 r.p.m., filtered through a 20-µm nylon mesh, plated in a fresh flask, and attached for 15 min at 37° followed by extensive washing with HBSS.[16] Adherent cells were harvested with a rubber policeman and resuspended in DMEM containing 10% heat-inactivated FCS. Dispersed cells were then plated in 24-well tissue culture plates at a concentration of 2×10^5 cells/well. The adherent cells were identified as microglial cells and astrocytes using immunofluorescent staining with monoclonal anti-Mac-1 antibody[17] and antiglial fibrillary acidic protein (GFAP) antibody.[16] These cultures are >95% Mac-1 positive and GFAP negative as determined by immunohistochemistry indicating that they are composed of microglial cells.

Measurement of nitrite and nitrate

Microglial cells (5×10^5 cells/well) were incubated with reagents as detailed in the Results. Supernatants were collected after a 20-hr incubation, and the nitrite (NO_2^-) concentration in the medium was measured by a microplate assay method as previously described.[18] Briefly, 100-µl aliquots were removed from the conditioned medium and incubated with an equal volume of Griess reagent (1% sulphanilamide/0·1% *N*-(1-naphthyl) ethylenediamine dihydrochloride/2·5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was determined in a Titertek Multiskan (Flow Laboratories, North Ryde, Australia). Nitrite was determined using sodium nitrite as a standard. In some experiments, nitrate (NO_3^-) was measured by reducing nitrate to nitrite with bacterial nitrate reductase measuring nitrite by using Griess reagent.[19]

Immunocytochemical analysis

Cells attached on coverslips were air-dried and fixed in cold acetone. The coverslips were treated with normal goat serum, and then incubated with anti-iNOS antisera (1 : 500 dilution) for 1 hr at room temperature. After three washes with phosphate-buffered saline (PBS), peroxidase-labelled secondary antibodies were sequentially applied. Peroxidase was developed with diaminobenzidine–hydrogen peroxidase solution (0·001% 3,3′-diaminbenzidine and 0·01% hydrogen peroxidase in 0·05 M Tris buffer). Finally, colour-developed coverslips were counterstained with haematoxylin and mounted.

Preparation of probe

To detect of iNOS mRNA transcripts, sense and antisense oligonucleotide primers specific for the coding regions of that gene were synthesized by using conventional technology. The following oligonucleotide primers were used: forward primer, 5′ GGCCTTGGCTCCAGCATGTAC 3′, 1856 to 1876; reverse primer, 5′ GCTGCCGCTCTCATCCAGAAC 3′, 2395 to 2415. The numbers represent the nucleotide numbers on the complementary strands of the iNOS cDNA sequence.[20] Total cellular RNA (5 µg) from IFN-γ- and LPS-stimulated macrophages of BALB/c mice was used as a template, and the single-stranded cDNA was synthesized with downstream antisense primers by reverse transcriptase. The cDNA was amplified in a 50-µl reaction mixture by using a DNA thermal cycler. For the analysis of the DNA sequence, polymerase chain reaction (PCR) products were gel purified, treated with T4 polynucleotide kinase, and then with the Klenow fragment of DNA polymerase I. The products were subcloned into the *Eco*RV site of the pBluescript II KS(−) plasmid. One microgram of plasmid DNA was radiolabelled by random priming with [α-^{32}P]dCTP. The resultant specific activity was $\approx 1 \times 10^8$ c.p.m./µg and was used at 1×10^7 c.p.m./blot.

RNA extraction and Northern blotting

Total RNA was prepared by using the modified LiCl–urea method,[21] electrophoresis in 1·2% agarose–formaldehyde gels,

and then transference to nylon membranes by capillary action in 20 × SSC (1 × SSC = 0·15 M NaCl and 0·015 M sodium citrate, pH 7·2). After prehybridization, the filters were hybridized with random [α-^{32}P]dCTP-labelled probes, having specific activity of $10^0 - 10^8$ c.p.m./μg in 10% dextran sulphate, 50% formamide, 4 × SSC, 1 × Denhardt's solution and 10 μg/ml salmon sperm DNA for 15 hr at 42°. Then the filters were washed, dried and examined by autoradiography.

Assay of TNF-α secretion

TNF-α secretion was measured by a modification of an enzyme-linked immunosorbent assay (ELISA) as described.[22] The ELISA was sensitive to TNF concentrations in medium above 40 pg/ml. The ELISA was devised by coating 96-well plates with 6·25 ng/well of murine monoclonal antibody with specificity for murine TNF-α. Before use and between subsequent steps in the assay, coated plates were washed twice with PBS containing 0·05% Tween-20 (PBS–Tween) and twice with PBS alone. All reagents used in this assay were incubated for 1 hr at room temperature with coated wells. For the standard curve, rTNF-α was added to serum previously determined to be negative for endogenous TNF-α. After exposure to medium, assay plates were sequentially exposed to rabbit anti-TNF-α, phosphatase-conjugated goat anti-rabbit IgG, and *p*-nitrophenyl phosphate. Optical density readings were made within 10 min of addition of the substrate on a Titertek Multiscan (Flow Laboratories, North Ryde, Australia) with a 405-nm filter. Appropriate specificity controls were included.

RESULTS

In order to characterize whether NaSal may affect NO_2^- accumulation, microglial cells were treated with various concentrations of NaSal and stimulated with IFN-γ and LPS. As shown in Fig. 1, control cells, incubated in the absence of IFN-γ and LPS for up to 20 hr, induced low levels of NO_2^-. In contrast, exposure of microglial cells to both IFN-γ and LPS stimuli resulted in maximal levels of NO_2^- production (65·1 ± 4·7 μM). NaSal (2–20 mM) concentration dependently inhibited the IFN-γ plus LPS-induced NO_2^- production (Fig. 1). NaSal was an equally effective inhibitor of NO_2^- accumulation if given either 4 hr before or 4 hr after the addition of IFN-γ plus LPS (data not shown). In parallel groups, N^GMMA, known to inhibit NOS, was included. IFN-γ plus LPS-induced NO_2^- production were abrogated upon addition of N^GMMA. The N^GMMA-mediated effects were not due to a toxic effect on microglial cells, as assessed by Trypan blue exclusion.

To determine if the inhibition of NO_2^- production by NaSal was due to a decrease in steady-state protein levels of iNOS, immunocytochemical analysis was performed. Figure 2 shows the results of immunocytochemical analysis using iNOS antibody in microglial cells. Expression of iNOS protein was induced by IFN-γ plus LPS (Fig. 2a), while pretreatment of murine microglial cells with NaSal did not increase iNOS protein (Fig. 2b). We also determined whether decreased iNOS protein expression by NaSal correlates with iNOS mRNA levels. Total RNA was isolated from cultured murine microglial cells, separated by denaturing agarose gel electrophoresis, and analysed by Northern blotting with radiolabelled cDNA that encoded the iNOS gene (Fig. 3). In control samples, no iNOS

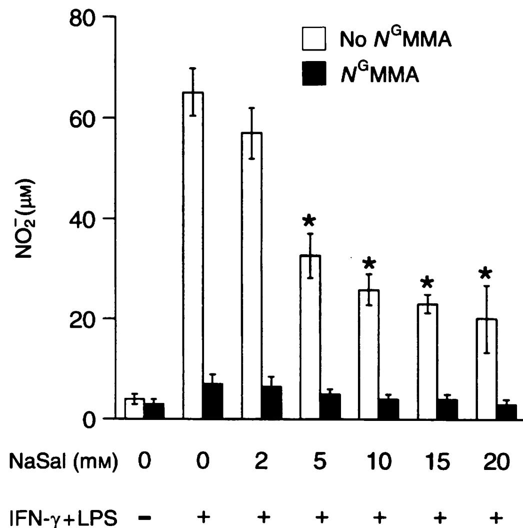

Figure 1. Effect of NaSal on IFN-γ plus LPS-induced NO synthesis in brain microglial cells. Microglial cells (5 × 10^5 cells/ml) were dispensed on four-well plates, and treated with the indicated concentrations of NaSal. After 2 hr cells were activated with IFN-γ (30 U/ml) plus LPS (1 μg/ml), in the absence or presence of N^GMMA (1 mM) for 1 hr. Cells were then washed with fresh medium, and further cultured for 20 hr. Cell-free supernatants were then assayed for NO production by evaluation of NO_2^- levels, as detailed in the Materials and Methods. Data shown are the mean ± SD of three independent experiments. * $P < 0·05$; NaSal + IFN-γ + LPS-treated versus IFN-γ + LPS-treated cells.

mRNA was evident. There was a clear induction in IFN-γ plus LPS-treated cells, which was about threefold reduced by the addition of NaSal. The β-actin transcript shows the even loading of the lanes with total RNA. On the basis of the Northern blotting data, expressed iNOS mRNA levels were substantially associated with iNOS protein.

We finally investigated whether the inhibitory effect of NaSal on NO synthesis was dependent on IFN-γ plus LPS-induced TNF-α secretion in microglial cells. As Fig. 4 shows, NaSal in combination with IFN-γ plus LPS concentration dependently decreased TNF-α secretion in microglial cells. NaSal significantly inhibited IFN-γ plus LPS-induced TNF-α secretion at concentrations of 5–20 mM. In addition, as Fig. 5 shows, IFN-γ plus LPS-induced NO production was progressively inhibited by the use of anti-TNF-α antibody, indicating that TNF-α induction is crucial for induction of iNOS in brain microglial cells.

DISCUSSION

In this study, we demonstrated that NaSal inhibited IFN-γ plus LPS-induced NO synthesis and TNF-α secretion in the murine brain microglial cells. Additionally, NaSal had the ability to diminish the induction of iNOS protein and mRNA. Microglial cells have been shown to generate NO upon activation with IFN-γ plus LPS.[9,23] The data presented here indicate that microglial cells produce significant levels of NO following treatment with IFN-γ plus LPS which act synergistically on the microglial cells. Moreover, NO production is totally abrogated by N^GMMA, indicating that it is likely to depend upon a NOS. It is clear from these experiments that

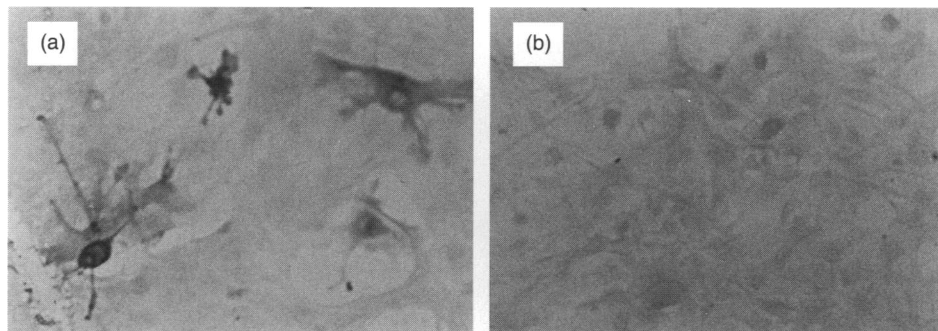

Figure 2. Effect of NaSal on expression of IFN-γ plus LPS-induced iNOS protein in brain microglial cells. Microglial cells were attached on coverslips, and treated with the NaSal (10 mM). After 2 hr cells were activated with IFN-γ (30 U/ml) plus LPS (1 μg/ml) for 1 hr. Cells were then washed with fresh medium, further cultured for 20 hr and then analysed for the iNOS protein expression by immunocytochemical analysis, as detailed in the Materials and Methods. (a) IFN-γ plus LPS, (b) NaSal and IFN-γ plus LPS.

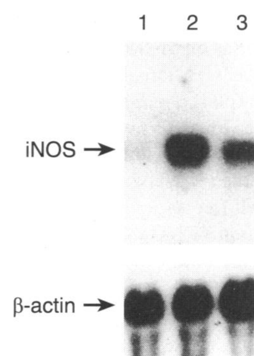

Figure 3. Effect of NaSal on expression of IFN-γ plus LPS-induced iNOS mRNA in brain microglial cells. Microglial cells (2×10^7 cells/ml) were dispensed on 100-mm diameter Petri dishes, and treated with the NaSal (10 mM). After 2 hr cells were activated with IFN-γ (30 U/ml) plus LPS (1 μg/ml) for 1 hr. Cells were then analysed for the iNOS mRNA expression by Northern blotting. Total cellular RNA was hybridized either to [α-^{32}P]dCTP-radiolabelled iNOS cDNA probe or to [α-^{32}P]dCTP-radiolabelled β-actin cDNA probe and exposed to X-ray film for 18 hr. Lane 1, medium alone (control); lane 2, IFN-γ plus LPS treatment; lane 3, NaSal and IFN-γ plus LPS.

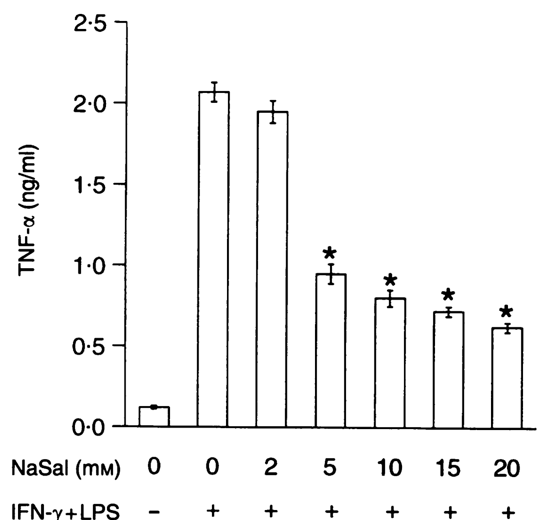

Figure 4. Effect of NaSal on IFN-γ plus LPS-induced TNF-α secretion in brain microglial cells. Microglial cells (5×10^5 cells/ml) were dispensed on four-well plates, and treated with the NaSal (10 mM). After 2 hr, cells were activated with IFN-γ (30 U/ml) plus LPS (1 μg/ml) for 1 hr. Cells were then washed with fresh medium, and further cultured for 20 hr. Cell-free supernatants were then assayed for TNF-α secretion, as detailed in the Materials and Methods. Data shown are the mean ± SD of three independent experiments. * $P < 0.05$; NaSal + IFN-γ + LPS-treated versus IFN-γ + LPS-treated cells.

NaSal does not inhibit iNOS mRNA expression completely at the concentration selected (Fig. 3). However, partial inhibition of iNOS may be sufficient to inhibit an inflammatory response. This assumption is supported by a study in animal models, where partial inhibition of NOS by NOS inhibitors in rats with adjuvant-induced arthritis was sufficient to reduce paw swelling without significantly affecting the elevated excretion of NO_2^- in the urine.[24] Thus, NaSal that incompletely inhibits iNOS expression may still be a good candidate for pharmaceutical intervention to modulate iNOS. The inducibility of iNOS has been formally shown to be dependent on two transcription factors: IFN regulatory factor-1 for IFN inducibility and NF-κB for LPS inducibility.[25,26] It has recently been demonstrated that salicylate-like drugs, but not indomethacin or acetaminophen, inhibit the activation of NF-κB by LPS.[27] Additionally, two NF-κB consensus sequences have been demonstrated in the murine iNOS promoter.[28,29] These studies suggest that NaSal and aspirin may be inhibiting iNOS induction via NF-κB-dependent mechanisms. If indeed the transcription factor NF-κB is being inhibited, then the reduction in NO_2^- accumulation could be due to a transcriptional inhibition. We confirmed that the inhibition of NO_2^- accumulation is correlated with a reduction of iNOS expression. Previously we and others reported that IFN-γ plus LPS stimulated the cells to secrete TNF-α.[9,30,31] The addition of NaSal inhibited IFN-γ plus LPS-induced TNF-α secretion (Fig. 4). TNF-α, originally defined by its anti-tumoral activity *in vitro* and *in vivo*, is now recognized as a cytokine with multiple biological functions.[32,33] The broad range of *in vivo* activities of TNF-α is apparently based on its ability to affect the growth, differentiation and function of virtually every cell type investigated. The inhibitory effect of NaSal on TNF-α secretion *in vivo* and the relative importance of microglial cells in the CNS as a source of TNF-α during inflammatory and immune responses are important areas for future studies. Even

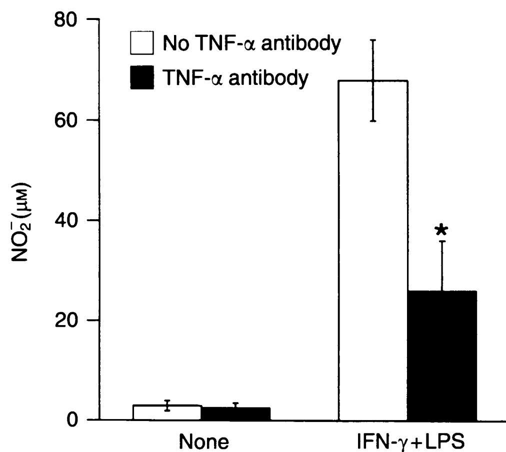

Figure 5. Effect of anti-TNF-α antibody on IFN-γ plus LPS-induced NO synthesis in brain microglial cells. Microglial cells (5×10^5 cells/ml) were dispensed on four-well plates, and treated with IFN-γ (30 U/ml) plus LPS (1 μg/ml), in the absence or presence of anti-TNF-α anibody (dilution, 1:50). After 24 hr of culture, NO production was assayed by evaluation of NO_2^- levels, as detailed in the Materials and Methods. Data shown are the mean ± SD of four independent experiments. * $P<0{\cdot}05$; anti-TNF-α antibody + IFN-γ + LPS-treated versus IFN-γ + LPS-treated cells.

though the brain has been considered to be an immunologically privileged organ, recent reports indicated that certain brain cells are involved in immunological processes. It has been reported that NO in the CNS can act as a defence mechanism against microbes, parasites, or tumour cells.[15–17,30,34] However, the exact mechanism of inhibition of NO production by NaSal in the CNS, especially in microglial cells, is unknown. In summary, we have provided evidence for inhibition of NO production and TNF-α secretion in murine microglial cells by NaSal. The ability of the anti-inflammatory NaSal to curtail NOS may provide a rationale for therapeutic options, especially in light of the ability of this drug to inhibit NOS induction.

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