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Sodium Benzoate, a Metabolite of Cinnamon and a Food Additive, Reduces Microglial and Astroglial Inflammatory Responses

Saurav Brahmachari, Arundhati Jana, and Kalipada Pahan

Upon activation, microglia and astrocytes produce a number of proinflammatory molecules that participate in the pathophysiology of several neurodegenerative disorders. This study explores the anti-inflammatory property of cinnamon metabolite sodium benzoate (NaB) in microglia and astrocytes. NaB, but not sodium formate, was found to inhibit LPS-induced expression of inducible NO synthase (iNOS), proinflammatory cytokines (TNF-α and IL-1β) and surface markers (CD11b, CD11c, and CD68) in mouse microglia. Similarly, NaB also inhibited fibrillar amyloid β (Aβ)-, prion peptide-, double-stranded RNA (polynosinic-polycytidylic acid)-, HIV-1 Tat-, 1-methyl-4-phenylpyridinium1-, IL-1β, and IL-12 p40-induced microglial expression of iNOS. In addition to microglia, NaB also suppressed the expression of iNOS in mouse peritoneal macrophages and primary human astrocytes. Inhibition of NF-κB activation by NaB suggests that NaB exerts its anti-inflammatory effect through the inhibition of NF-κB. Although NaB reduced the level of cholesterol in vivo in mice, reversal of the inhibitory effect of NaB on iNOS expression, and NF-κB activation by hydroxymethylglutaryl-CoA, mevalonate, and farnesyl pyrophosphate, but not cholesterol and ubiquitnone, suggests that depletion of intermediates, but not end products, of the mevalonate pathway is involved in the anti-inflammatory effect of NaB. Furthermore, we demonstrate that an inhibitor of p21ras farnesyl protein transferase suppressed the expression of iNOS, that activation of p21ras alone was sufficient to induce the expression of iNOS, and that NaB suppressed the activation of p21ras in microglia. These results highlight a novel anti-inflammatory role of NaB via modulation of the mevalonate pathway and p21ras. The Journal of Immunology, 2009, 183: 5917–5927.

Activation of glial cells (microglia and astroglia) has been implicated in the pathogenesis of a variety of neurodegenerative diseases, including Alzheimer’s disease (AD),1 Parkinson’s disease, Creutzfeld-Jacob disease, HIV-associated dementia (HAD), stroke, and multiple sclerosis (MS) (1, 2). It has been found that activated microglia and astroglia accumulate at sites of injury or plaques in neurodegenerative CNS (1–7). Although activated microglia scavenge dead cells from the CNS and secrete different neurotrophic factors for neuronal survival, it is believed that severe activation causes various autoimmune responses leading to neuronal death and brain injury (1–7). During activation, microglia and astroglia express various genes related to inflammation, such as proinflammatory cytokines, proinflammatory enzymes, and proinflammatory adhesion molecules (1–9). Therefore, characterization of signaling pathways required for the activation of glial cells is an active area of investigation since compounds capable of antagonizing such signaling steps may have therapeutic effect in neurodegenerative disorders.

Cinnamon contains three major compounds (cinnamaldehyde, cinnamyl acetate and cinnamyl alcohol), which are converted into cinnamic acid by oxidation and hydrolysis, respectively. In the liver, this cinnamic acid is β-oxidized to benzoate (10) that exists as sodium salt (sodium benzoate; NaB) or benzoyl-CoA. It has been reported that minor amount of NaB is also excreted in the urine of humans (11, 12). NaB is of medical importance because it is a component of Ucephan, a Food and Drug Administration (FDA)-approved drug used in the treatment for hepatic metabolic defects associated with hyperammonemia such as urea cycle disorder in children (13, 14). It is also widely used as a preservative in a broad range of foods and cosmetic products (15). It is nontoxic and can be administered as a solution in drinking water. It has been reported that 2% solution of NaB in drinking water is safe for lifelong treatment in mice without any noticeable side effects (16). Because Ayurvedic as well as Yunani medicines have been using cinnamon as vital medicines for inflammatory diseases like arthritis for centuries, we were prompted to test the effect of NaB on the expression of proinflammatory molecules in glial cells.

Here we provide the first evidence that NaB attenuates the expression of inducible NO synthase (iNOS) and proinflammatory cytokines in microglia, astrocytes, and macrophages. Although NaB reduced the level of cholesterol in vivo in mice, it was not the cause behind the anti-inflammatory activity of NaB. Alternatively, hydroxymethylglutaryl-CoA (HMG-CoA), mevalonate, and farnesyl pyrophosphate reversed NaB-mediated inhibition of iNOS, indicating the involvement of intermediates, but not the end product, of the mevalonate pathway in the anti-inflammatory effect of NaB.
Consistently, inhibition of the expression of iNOS and the production of NO by farnesylpyrophosphate transferase inhibitor, suppression of p21WAF1 activation by NaB and induction of iNOS by the activated p21WAF1 alone suggest that NaB exerts its anti-inflammatory effect in glial cells via modulating farnesylation and activation of p21WAF1. Our findings raise a possibility that NaB, a component of a prescribed drug for human urea cycle disorder and a widely used food preservative, may find further application in neuroinflammatory and neurodegenerative disorders.

Materials and Methods
Reagents
NaB, sodium formate (NaFO), LPS (Escherichia coli), polyinosinic-polycytidylic acid (polyIC), and 1-methyl-4-phenylpyridinium (MPP+) were purchased from Sigma-Aldrich. FBS, HBSS, trypsin, and DMEM-F-12 were from Mediatech. HIV-1 coat protein gp120 (expressed in Chinese hamster ovary cells; strain HIV-1 MN) was obtained from U.S. Biological. Prion peptides and human Aβ peptides 1–42 were obtained from Bachem Bioscience. Recombinant mouse IL-1β and IL-12 p40 homodimer (p40β) were obtained from R&D Systems. 125I-Labeled protein A and [α-32P]dCTP (3000 Ci/mmol) were purchased from PerkinElmer Life Sciences. Pravastatin and 125I were isolated using Ultraspec-II RNA reagent (Biotecx Laboratories) according to the manufacturer’s protocol. For Northern blot analyses, 20 μg of total RNA were electrophoresed on 1.2% agarose gel, transferred to nitrocellulose membrane, and hybridized at 68°C with 32P-labeled cDNA probe. After hybridization, filters were washed two or three times in solution I (2× SSC, 0.05% SDS) for 1 h at room temperature followed by solution II (0.1× SSC, 0.1% SDS) at 50°C for another hour. The membranes were then dried and exposed to x-ray films (Eastman Kodak). The same amount of RNA was hybridized with probe for GAPDH.

Semiquantitative RT-PCR analysis
To remove any contaminating genomic DNA, total RNA was digested with DNase. Semiquantitative RT-PCR was conducted as described earlier (27, 28) using a RT-PCR kit from Clontech. Briefly, 1 μg of total RNA was reverse transcribed using oligo(dT)12–18, as primer and Moloney murine leukemia virus reverse transcriptase (Clontech). The resulting cDNA was appropriately diluted and then cDNA amplified. Amplified products were electrophoresed on a 1.8% agarose gel and visualized by ethidium bromide staining.

iNOS, sense, 5’-CCCTCCTGAGAAGAAGCAGGACACAGC-3’; antisense, 5’-GCGCTTCGAGCCTCTGAGGTTG-3’; II-β, sense, 5’-CTCCATGACCTTTGTTACAAGG-3’; antisense, 5’-TGTCATGTAGCAGCTGTTG-3’; IL-6, sense, 5’-GACACCATTTAGGCTAGTTG-3’; antisense, 5’-ATGACGGAGATTGTTGTCG-3’; TLR4, sense, 5’-CAGAAATCTCCTGCAAGGTGT-3’; antisense, 5’-GTTAGCGAGCTTGAAGGAAACG-3’;IL-1β, sense, 5’-TGTGGATCTGACAGCTGTTG-3’; antisense, 5’-GCCACACATTGCCCGGAATTG-3’; B7-2, sense, 5’-GGGCTGTTTCCTTACAGCGC-3’; antisense, 5’-CTCTGTCGACGTGACATCTC-3’; CD68, sense, 5’-CAGATCAACAAATGATGGTTATGGG-3’; antisense, 5’-CATCATGTCCTTCTGACTGCCGTTG-3’; HMC-II, sense, 5’-AGGCTTTGAGTGAGGTTAAGA-3’; antisense, 5’-GGTGGAGCAGAAAGCTGTCG-3’; IL-12 p40, sense, 5’-AAATCTCCTGCTGACATCT-3’; antisense, 5’-CCCTGACATTGACCCGATG-3’; GAPDH, sense, 5’-GGTGAAATGAGGACCTGT-3’; antisense, 5’-TGGTCCACCCCTTCAAGTG-3’.

Real-time PCR analysis
It was performed using the ABI-Prism7700 sequence detection system (Applied Biosystems) as described earlier (27, 28). The mRNA expressions of respective genes were normalized to the level of GAPDH mRNA. Data were processed by the ABI Sequence Detection System 1.6 software and analyzed by ANOVA.

Assay of iNOS promoter-driven reporter activity
Cells plated at 50–60% confluence in six-well plates were cotransfected with 1 μg of phINOS(7,2)Luc and 50 ng of pRL-TK (a plasmid encoding Renilla luciferase), used as transfection efficiency control; Promega) by LipofectAMINE Plus (Invitrogen) as described in many studies (20, 26). Twenty-four hours after transfection, cells were treated with different stimuli for 6 h. Firefly and Renilla luciferase activities were obtained by analyzing total cell extract according to standard instructions provided in the Dual Luciferase Kit (Promega) in a TD-20/20 Luminometer (Turner Designs). Relative luciferase activity of cell extracts was typically represented as the ratio of firefly luciferase value to the R. luciferase value × 10–3.

Preparation of nuclear extracts and EMSA
Nuclear extracts were prepared from microglial cells as described previously (17, 19, 20). Nuclear extracts were used for EMSA using 32P-end-labeled double-stranded (NF-κB) or single-stranded (NF-κB) oligonucleotide, as described in many studies (20, 26). Briefly, 10 μg of nuclear extract was electrophoresed on a 12% polyacrylamide gel and transferred onto a nitrocellulose membrane, and the EMSA band was visualized by immunoblotting with Abs against human iNOS.

Analysis of transcriptional activities of NF-κB
Cells plated at 70–80% confluence in 12-well plates were cotransfected with 0.25 μg of either PBIX-Luc (an NF-κB-dependent reporter construct) and 12.5 ng of pRL-TK using LipofectAMINE Plus (22, 23). After 24 h of transfection, cells were treated with different stimuli for 6 h. Firefly and R. luciferase activities were obtained as described above.

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INHIBITION OF INFLAMMATION BY CINNAMON METABOLITE
Assay of cytokines by ELISA

Microglial cells preincubated with NaB for 6 h were stimulated with LPS. After 24 h of stimulation, supernatants were collected to assay TNF-α and IL-1β by high-sensitivity ELISA kits (BD Biosciences).

Immunofluorescence analysis

It was performed as described earlier (19, 28). Briefly, coverslips containing 100–200 cells/mm² were fixed with 4% paraformaldehyde followed by treatment with cold ethanol and two rinses in PBS. Samples were blocked with 3% BSA in PBS-Tween 20 (PBST) for 30 min and incubated in PBST containing 1% BSA and goat anti-CD11b (1/50), rabbit anti-iNOS (1/200), or goat anti-GFAP (1/50). After three washes in PBST (15 min each), slides were further incubated with Cy2 (Jackson ImmunoResearch Laboratories). For negative controls, a set of culture slides was incubated under similar conditions without the primary Abs. The samples were mounted and observed under a Bio-Rad MRC1024ES confocal laser-scanning microscope.

Assay of cholesterol in serum

Total cholesterol was quantified in serum by using an Amplex Red Cholesterol Assay kit from Invitrogen. Briefly, cholesterol was oxidized by cholesterol oxidase to yield H₂O₂, which then reacted with 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red). In the presence of HRF, this Amplex Red-H₂O₂ complex produced highly fluorescent resoruvin, which was detected by fluorometry.

Statistics

Statistical comparisons were made using one-way ANOVA followed by Student’s t test.

Results

NaB attenuates the expression of iNOS and proinflammatory cytokines in LPS-stimulated mouse microglia

Activated microglia are known to produce excessive amount of NO having the potential of damaging neurons and oligodendrocytes. Because cinnamon has been used as a natural supplement for normal human health for centuries, we investigated the effect of NaB, a major metabolite of cinnamon, on the expression of iNOS in microglia. LPS is a prototype inducer of various proinflammatory molecules in different cell types including mouse microglia. Therefore, BV-2 microglial cells preincubated with different doses of NaB and sodium formate (NaFO), the negative control for NaB, for 6 h were stimulated with LPS under serum-free condition. Although at lower concentrations (50 and 100 μM), NaB was not effective in inhibiting the production of NO (data not shown), at higher concentrations, NaB markedly suppressed LPS-induced production of NO in microglial cells as evident from estimation of total NO (nitrite and nitrate; Fig. 1A). Significant inhibition of LPS-induced NO production was observed at 500 μM NaB, and maximum inhibition was noted at 1 mM or higher concentration (Fig. 1A). Alternatively, NaFO had no effect on LPS-induced production of NO (Fig. 1A). However, a 1- or 2-h preincubation of microglial cells with NaB was not enough for this molecule to exhibit its inhibitory effect on LPS-induced NO production (data not shown). To understand the mechanism further, we investigated the effect of NaB on the mRNA expression of iNOS in microglial cells. It is clearly evident from RT-PCR in Fig. 1B and real-time PCR in Fig. 1C that NaB, but not NaFO, inhibited LPS-induced mRNA expression of iNOS in BV-2 microglial cells. It is well known that LPS requires TLR4 to transduce its signal and subsequently to induce iNOS. Therefore, there is a possibility that NaB might inhibit the expression of TLR4, and as a result decreased expression of iNOS is observed. To examine this possibility, we investigated the effect of NaB on the expression of TLR4. The RT-PCR results in Fig. 1D show that NaB had no effect on the expression of TLR4 in LPS-treated microglial cells, clearly suggesting that NaB-mediated inhibition of iNOS is not due to any inhibition of expression of TLR4.

Next, to investigate whether NaB suppresses the induction of NO production in primary cells, we used peritoneal macrophages and primary microglia. As evident from semiquantitative RT-PCR in Fig. 1E and quantitative PCR in Fig. 1F, NaB, but not NaFO, markedly suppressed the expression of iNOS mRNA in LPS-stimulated peritoneal macrophages. Similar to BV-2 microglial cells, NaB dose-dependently suppressed LPS-induced production of NO in primary mouse microglia (Fig. 1G). On the other hand, NaFO had no effect on LPS-induced production of nitrite (Fig. 1G). MTT results show that neither NaB nor NaFO was toxic to microglia at any of the concentrations tested (Fig. 1H), suggesting that the inhibitory effect of NaB on microglial expression of iNOS was not due to any change in cell viability.

In addition to producing NO, activated microglia also secrete various proinflammatory cytokines. Therefore, we examined...
whether NaB was capable of suppressing the expression of proinflammatory cytokines in BV-2 microglial cells. Similar to the inhibition of iNOS, NaB dose-dependently inhibited the production of TNF-α and IL-1β protein (Fig. 2A) and the expression of TNF-α and IL-1β mRNA (Fig. 2, B and C). On the other hand, NaFO had no effect on the expression of these proinflammatory cytokines.

**NaB inhibits Aβ-, prion peptide (PrP)-, dsRNA (poly(IC))--, HIV-1 gp120-, MPP+-, IL-1β-, and IL-12 p402-induced expression of iNOS in microglial cells**

Activated microglia are considered to play an important role in various pathological conditions associated with viral encephalopathy, AD, MS, Parkinson’s disease, HAD, Creutzfeldt-Jakob disease, etc. (1). Because NaB inhibited LPS-induced expression of proinflammatory molecules in microglia, we were prompted to investigate whether NaB was also capable of negating the expression of iNOS in microglial cells stimulated with etiological reagents of various neurological disorders. BV-2 microglial cells were challenged with fibrillar Aβ peptides (etiologic reagent for AD), fibrillar PrP peptides (etiologic reagent for prion diseases), dsRNA in the form of poly(IC) (one of the etiological reagents for viral encephalopathy), HIV-1 gp120 (one of the etiological reagents for HAD), IL-12 p402 (one of the etiological reagent for MS), and MPP+ (Parkinsonian toxin). It is clearly evident from Fig. 3 that Aβ peptides (Fig. 3A), gp120 (Fig. 3B), MPP+ (Fig. 3C), PrP (Fig. 3D), poly(IC) (Fig. 3E), IL-12 p402 (Fig. 3F), and IL-1β (Fig. 3G) induced the expression of iNOS mRNA in microglial cells. However, NaB knocked down Aβ-, gp120-, MPP+-, PrP-, poly(IC)-, p402-, and IL-1β-induced microglial expression of iNOS (Fig. 3). On the other hand, NaFO had no such inhibitory effect (Fig. 3) suggesting the specificity of the effect. We further examined the effect of NaB on the expression of TLR3, the prototype receptor of poly(IC). It is clearly evident from the Fig. 3 that NaB had no effect on the mRNA level of TLR3, indicating that inhibition of iNOS by NaB in poly(IC)-stimulated cells is not due to any suppression of TLR3.

**NaB inhibits the expression of microglial surface markers, MHC class II, and costimulatory molecules in LPS- or MPP+-stimulated mouse microglial cells**

During severe activation, microglia not only secrete various neurotoxic molecules but also express different proteins and surface markers. Among different surface markers, CD11b is the most potential one with immense biological significance (29–31). It is reported that in various neuroinflammatory diseases, the increased CD11b expression corresponds to the severity of microglial activation (1). We examined whether NaB could abrogate increased expression of various surface molecules in microglia. As expected, LPS increased the expression of CD11b, CD11c, and CD68 in BV-2 microglial cells (Fig. 4A). We also used MPP+ (Parkinsonian neurotoxin) to stimulate CD11b in microglia. Double-label immunofluorescence analysis of CD11b and iNOS expression in mouse primary microglia shows that MPP+ stimulation increased the expression of CD11b and iNOS and that NaB attenuated LPS-mediated CD11b and iNOS expression (Fig. 4B). We further examined the surface expression of CD11b and CD68 by FACS. It is evident from Fig. 4 that NaB, but not NaFO, markedly inhibited the surface expression of CD11b (Fig. 4C) and CD68 (Fig. 4D) in LPS-stimulated microglial cells. Taken together, these studies suggest that NaB is capable of suppressing the activation of microglia.
Activated microglia also express MHC class II and costimulatory molecules like B7-1 and B7-2, which are critical for Ag presentation. As evident from Fig. 4A, LPS increased the expression of class-II MHC and the costimulatory molecules B7-1 and B7-2 (Fig. 4A). However, NaB, but not NaFO, markedly suppressed the expression of these activation markers and costimulatory molecules in microglial cells (Fig. 4A).

**NaB inhibits the activation of NF-κB in mouse BV-2 microglial cells**

LPS, proinflammatory cytokines (TNF-α and IL-1β) and other stimuli (HIV-1 gp120, IL-12 p40, Aβ, PrP, etc.) are known to induce iNOS expression via activation of NF-κB (19–24, 26, 27). Because NaB attenuated the expression of iNOS in mouse microglia, we examined the effect of NaB on the activation of NF-κB. Activation of NF-κB was monitored by both DNA binding and transcriptional activity of NF-κB. As expected, treatment of BV-2 microglial cells with LPS resulted in the induction of DNA-binding activity of NF-κB (Fig. 5A). However, NaB inhibited LPS-induced DNA-binding activity of NF-κB in microglial cells (Fig. 5A). We then tested the effect of NaB on LPS-induced transcriptional activity of NF-κB. Consistent to the effect of NaB on the DNA binding activity of NF-κB, NaB also suppressed NF-κB-dependent transcription of luciferase in a dose-dependent manner in LPS-stimulated cells (Fig. 5B). NaB had no effect on the LPS-induced transcriptional activity of NF-κB in microglial cells (Fig. 5B). We next examined the effect of NaB on other proinflammatory stimuli-induced transcriptional activity of NF-κB. The wild-type IκB kinase-γ (IKK-γ) NEMO binding domain peptide (wtNBD), a specific inhibitor of induced NF-κB activation, was used as a positive control. As expected, NaB markedly suppressed NF-κB-dependent transcription of luciferase in microglial cells stimulated by Aβ, IL-1β, gp120, PrP, and IL-12 p40 (Fig. 5, C–G). These results suggest that NaB attenuates the expression of iNOS by suppressing the activation of NF-κB.

**NaB suppresses the expression of iNOS and GFAP in primary human astrocytes**

Next we examined whether NaB could suppress the expression of iNOS in human brain cells. Astroglia are the major glial cells in the CNS, and astroglial activation also plays a role in various neurodegenerative disorders. Therefore, we investigated the effect of NaB on the expression of iNOS in primary human astroglia. Earlier, we found that IL-1β is the only cytokine that induces iNOS in primary astroglia (20). Consistently, IL-1β induced the production of nitrite (Fig. 6A) and the expression of iNOS protein (Fig. 6B) in primary astroglia isolated from human fetal brains. NaB markedly inhibited IL-1β-induced production of NO (Fig. 6A) and the expression of iNOS protein (Fig. 6B) in human fetal astroglia.

To understand the effect of NaB on the transcription of the iNOS gene, primary human astrocytes were transfected with phiNOS(7,2)Luc, a construct containing the human iNOS promoter fused to the luciferase gene, and activation of this promoter was measured after stimulating the cells with IL-1β in the presence or absence of NaB. As we found earlier (20, 23), IL-1β induced iNOS promoter-driven luciferase activity by ~4-fold (Fig. 6C). Consistent with the effect of NaB on the expression of iNOS, NaB itself had no effect on iNOS promoter-driven luciferase activity but it significantly inhibited iNOS promoter-driven luciferase activity in IL-1β-stimulated cells (Fig. 6C), suggesting that NaB inhibits IL-1β-induced production of NO and the expression of iNOS by inhibiting the activation of iNOS promoter.
Increased expression of glial fibrillary acidic protein (GFAP) represents astroglial activation and gliosis during neurodegeneration. Because NaB decreased the expression of iNOS in human astroglia, we investigated whether this drug was capable of inhibiting the increased expression of GFAP in primary human astroglia. As expected, IL-1β markedly increased the mRNA expression of GFAP in astroglia (Fig. 6E). However, NaB, but not NaFO, suppressed IL-1β-induced astroglial mRNA expression of GFAP (Fig. 6E). Immunofluorescence analysis in Fig. 6D also indicates that IL-1β stimulation increased the level of GFAP and iNOS compared with control and that NaB markedly attenuated IL-1β-mediated up-regulation of GFAP and iNOS in human astrocytes. Western blot analysis in Fig. 6F further substantiates our findings and also indicates that NaB has no additive effect when used in combination with wtNBD, suggesting that NaB-mediated inhibition of proinflammatory markers is solely due to the inhibition of NF-κB.

Intermediates of the mevalonate pathway reverses the inhibitory effect of NaB on the expression of iNOS and the activation of NF-κB in mouse BV-2 microglial cells

The requirement of at least 6 h of preincubation of cells with NaB to see its anti-inflammatory effect suggests that metabolite(s) sensitive to NaB may be involved in the process. Earlier, Pahan et al.
concentration (25–250/\text{H}9262\text{NF-}
expression of iNOS and activation of NF-
ferase with the IC\text{50 of }75 \text{nM}. In whole cells, however, a higher
(Fig. 9\text{B}), NO production (Fig. 8\text{A}), and the activation of NF-
action in the activation of NF-
in microglial cells. Alternatively, cholesterol (the end product of
(32) have demonstrated that intermediates of the mevalonate path-
yway play a role in the expression of iNOS and proinflammatory
cytokines in glial cells. The end product of the mevalonate path-
way is cholesterol; therefore, we investigated whether NaB had
any effect on the level of cholesterol in vivo in mice. After 7 days
of treatment, NaB reduced the level of cholesterol in serum of
by ~28%, and this reduction was comparable (~30%) with that by
the so-called cholesterol-lowering drug pravastatin (Fig. 7). Alter-
atively, NaFO had no effect on serum level of cholesterol (Fig. 7),
indicating the specificity of the effect. These results are important
because they suggest that NaB may be used to lower cholesterol in
patients with hypercholesterolemia.

Next we examined the role of different members of the meval-
one pathway in the anti-inflammatory effect of NaB, HMG-CoA, mevalo-
one, and farnesyl pyrophosphate abrogated the inhibitory
effect of NaB on the expression of iNOS mRNA (Fig. 8\text{A}), the
production of NO (Fig. 8\text{B}), and the activation of NF-
(Fig. 8\text{C}) in microglial cells. Alternatively, cholesterol (the end product of
the mevalonate pathway) and coenzyme Q (an unrelated lipid mol-
ceule) had no effect on NaB-mediated inhibition of iNOS mRNA
(Fig. 8\text{A}), NO production (Fig. 8\text{B}), and the activation of NF-
(Fig. 8\text{C}). These results suggest that depletion of intermediary
products rather than end products of the mevalonate pathway is
responsible for the observed anti-inflammatory effect of NaB.

An inhibitor of p21\text{ras} farnesyl protein transferase (FPT
inhibitor II) suppresses LPS-induced expression of iNOS mRNA
and activation of NF-\kappa B in mouse \textit{BV-2} microglial cells
Inhibition of LPS-induced expression of iNOS and activation of
NF-\kappa B by NaB and its reversal by farnesyl pyrophosphate (FPP)
and other upstream members of the mevalonate pathway (HMG-
CoA and mevalonate), but not by the end product of the same
pathway, suggest a possible involvement of the farnesylation re-
action in the activation of NF-\kappa B and the expression of iNOS.
Because farnesylation is a necessary step for the activation of
p21\text{ras}, we examined the effect of FPT inhibitor II on LPS-induced
expression of iNOS and activation of NF-\kappa B in microglial cells.
FPT inhibitor II selectively inhibits p21\text{ras} farnesyl protein transfer-
ase with the IC\text{50 of }75 \text{nM}. In whole cells, however, a higher
concentration (25–250 \text{mM}) of FPT inhibitor II is required to in-
hbit farnesylation of p21\text{ras} by 90% (33). Therefore, BV-2 cells
were pretreated with 100, 200, and 300 \text{mM} FPT inhibitor II for 2 h
followed by stimulation with LPS. It is clearly evident from Fig. 9
that FPT inhibitor II dose-dependently suppressed LPS-induced
expression of iNOS mRNA (Fig. 9\text{A}) and activation of NF-\kappa B
(Fig. 9\text{B}) in microglial cells. These results suggest that p21\text{ras}
farnesylation plays an important role in the activation of NF-\kappa B and
the expression of iNOS in LPS-stimulated microglia.

To exclude any possibility that FPTI-II inhibits iNOS indepen-
dent of NF-\kappa B, we examined the effect of wtNBD and FPT inhib-
itor II, alone or together, on the mRNA expression of iNOS. It is
evident from Fig. 9\text{C} that FPT inhibitor II did not show any addi-
tive inhibitory effect on the expression of iNOS in wtNBD-
treated cells, suggesting that inhibition of iNOS by FPT inhibitor
II is purely due to the suppression of NF-\kappa B activation. Fur-
thermore, inability of FPP to reverse the inhibitory effect of wtNBD on
iNOS as evident from Fig. 9\text{D} clearly indicates that farnesylation of
p21\text{ras} is an upstream event of NF-kB activation and thus
whether NF-\kappa B is directly inhibited, inhibition of iNOS is not
reversed by FPP. The semiquantitative RT-PCR studies in Fig. 9\text{E}
again shows that NaB has no additive inhibitory effect on iNOS
over wtNBD, further substantiating the fact that NaB-mediated
inhibition of iNOS in microglial cells solely involves suppression
of NF-kB activation. To further confirm, we investigated the effect

![FIGURE 7. Effect of NaB on serum level of cholesterol in male C57BL/6 mice. Mice (6–8 wk old) were treated with NaB (100 mg/kg body weight), NaFO (100 mg/kg body weight), and pravastatin (1 mg/kg body weight) separately via gavage for 7 days followed by quantification of cholesterol in serum using a simple fluorometric method. Results represent means ± SD of five mice per group (n = 5).](http://www.jimmunol.org/)

![FIGURE 8. Intermediates of the mevalonate pathway negate the inhib-
itory effect of NaB on the expression of iNOS and the activation of NF-kB in
mouse \textit{BV-2} microglial cells. Cells were treated with NaB in the pres-
ence or absence of different concentrations of HMG-CoA, mevalonate,
FPP, cholesterol, and ubiquinone for 6 h followed by stimulation with LPS.
A. After 5 h of stimulation, the expression of iNOS mRNA was monitored
by RT-PCR. B; After 24 h of stimulation, the level of nitrite was measured
in supernatants using Griess reagent. Results are means ± SD of three
independent experiments. a, p < 0.001 vs LPS; b, p < 0.001 vs LPS+NaB.
C. Cells were cotransfected with 0.25 \text{pg} of PBIX-Luc and 12.5 \text{ng} of
pRl-TK. Twenty-four hours after transfection, cells were incubated with
NaB in the presence or absence of HMG-CoA, mevalonate, FPP, choles-
terol, and ubiquinone. After 6 h of incubation, cells were stimulated with
LPS for 6 h followed by assay of firefly (\textit{ff}-Luc) and \textit{Renilla} (\textit{r}-Luc) lu-
ciferase activities. Results are means ± SD of three different experiments.
a, p < 0.001 vs LPS; b, p < 0.001 vs LPS plus NaB.)
STATs, which is independent of NF-κB/H11006/H11021/H9262 FPT inhibitor II (200 μM). The results represent three independent experiments.

Because p21ras activation of NF-κB is sufficient for the expression of iNOS, we examined the whether activation of p21ras alone was sufficient for the expression of iNOS. It is clear from Fig. 11A that the expression of p21ras alone markedly induced the production of NO in microglial cells whereas the expression of empty vector was unable to induce NO production. Inhibition of RasV12-induced production of NO by arginase, an enzyme that degrades the substrate (L-arginine) of iNOS, indicates that the induction of NO production in RasV12-transfected cells is dependent on iNOS-mediated arginine metabolism (data not shown). To understand the mechanism of induction of NO production, we analyzed the status of iNOS protein and mRNA in RasV12-transfected cells. Western blot analysis with Abs against murine macrophage iNOS and Northern blot analysis for iNOS mRNA clearly showed that RasV12 induced the expression of iNOS protein (Fig. 11B) and mRNA (Fig. 11C), suggesting that signal(s) provided by the activation of p21ras alone is sufficient to induce the expression of iNOS.

Because the activation of NF-κB is important for the induction of iNOS (19–23, 26–28), to understand the basis of iNOS expression by RasV12, we also investigated whether Ras alone was sufficient for the activation of NF-κB. Activation of NF-κB was monitored by transcriptional activity of NF-κB using the expression of luciferase from a reporter construct, PBIIX-Luc. Cells were co-transfected with PBIIX-Luc and RasV12 followed by incubation in serum-free medium. As evident from Fig. 11D, RasV12 markedly induced NF-κB-dependent transcription of luciferase, whereas the empty vector was unable to induce the transcriptional activity of NF-κB.

FIGURE 9. Effect of FPT inhibitor II (FPTI II) on the expression of iNOS and the activation of NF-κB in mouse BV-2 microglial cells. A, Cells treated with different concentrations of FPT inhibitor II for 2 h were stimulated by LPS. After 5 h of stimulation, the expression of iNOS mRNA was monitored by RT-PCR. B, Cells were cotransfected with 0.25 μg of PBIIX-Luc and 12.5 ng of pRL-TK. Twenty-four hours after transfection, cells were incubated with different concentrations of FPT inhibitor II for 2 h followed by stimulation with LPS. After 6 h of stimulation, activities of firefly (p-Luc) and Renilla (r-Luc) luciferase were monitored. Results are mean ± S.D. of three different experiments. a, p < 0.001 vs LPS; b, p < 0.05 vs LPS. C–E, Cells treated with appropriate concentrations of FPT inhibitor II (200 μM) for 2 h (C), FPP (200 μM; D), or NaB (1 mM; E) alone for 6 h or along with wtNBD (10 μM; 1 h before stimulation) were stimulated by LPS. After 5 h of stimulation, the expression of iNOS mRNA was monitored by RT-PCR. F, Cells treated with different concentrations of NaB for 6 h were stimulated by IFN-γ (12.5 U/ml). After 5 h of stimulation, the expression of iNOS mRNA was monitored by RT-PCR. The results represent three independent experiments.

NaB suppresses the activation of p21ras in mouse BV-2 microglial cells

Because p21ras farnesylation is involved in the activation of NF-κB and the expression of iNOS and NaB-mediated inhibition of these events were reversed by FPP, we examined the whether NaB suppressed the activation of p21ras. Earlier, we have seen activation of p21ras in human astrogloma cells within 2–4 min of LPS stimulation (34). Therefore, at different times (2, 3, and 5 min) of stimulation by LPS, microglial cells were analyzed for the activation of p21ras. Although we did not see p21ras activation at 2 min of LPS stimulation, marked activation was observed at 3 and 5 min of stimulation (Fig. 10A). Therefore, we examined the effect of NaB on the activation of p21ras at 3 min of LPS stimulation. It is clearly evident from Fig. 10B that NaB, but not NaFO, markedly inhibited LPS-induced activation of p21ras in microglial cells. These results suggest that NaB attenuates the expression of proinflammatory molecules in glial cells probably by suppressing the activation of p21ras. 

Activation of p21ras alone is sufficient to induce the expression of iNOS and the activation of NF-κB in mice BV-2 microglial cells

Our results that FPT inhibitor II inhibited microglial expression of iNOS and that NaB suppressed microglial activation of p21ras and the expression of iNOS prompted us to examine whether activation of p21ras alone was sufficient for the expression of iNOS in microglial cells. Activation of p21ras in BV-2 glial cells was achieved by the expression of RasV12, a constitutively active mutant of p21ras. It is clear from Fig. 11A that the expression of RasV12 alone markedly induced the production of NO in microglial cells whereas the expression of empty vector was unable to induce NO production. Inhibition of RasV12-induced production of NO by arginase, an enzyme that degrades the substrate (L-arginine) of iNOS, and N-methyl-L-arginine acetate, a competitive inhibitor of NOS activity, suggests that the induction of NO production in RasV12-transfected cells is dependent on NOS-mediated arginine metabolism (data not shown). To understand the mechanism of induction of NO production, we analyzed the status of iNOS protein and mRNA in RasV12-transfected cells. Western blot analysis with Abs against murine macrophage iNOS and Northern blot analysis for iNOS mRNA clearly showed that RasV12 induced the expression of iNOS protein (Fig. 11B) and mRNA (Fig. 11C), suggesting that signal(s) provided by the activation of p21ras alone is sufficient to induce the expression of iNOS.
Discussion

Although microglial activation has an important repairing function through scavenging of unwanted bodies in the CNS and activation of astrocytes may have important beneficial effects in the recovery of injured CNS by actively monitoring and controlling the extracellular water, pH, and ion homeostasis, once microglia and astroglia are activated in the neurodegenerating microenvironment, activation always goes beyond control, and eventually detrimental effects of glial activation override its beneficial effects. Activated glia produce NO, a number of proinflammatory cytokines, reactive oxygen species, etc., in excessive amounts for a prolonged time period that ultimately damage neurons and oligodendrocytes. Therefore, understanding mechanisms that regulate microglial and astroglial activation is an important area of investigation.

Cinnamon, the brown bark of the cinnamon tree, is a commonly used spice and flavoring material for desert, candies, chocolate, etc. It has a long history as a medicine as well. Medieval physicians used cinnamon in medicines to treat a variety of disorders including arthritis, coughing, hoarseness, and sore throats. In addition to containing manganese, dietary fiber, iron, and calcium, cinnamon contains three major compounds, cinnamaldehyde, cinnamyl acetate, and cinnamyl alcohol. After intake, these three active compounds are converted into cinnamic acid by oxidation and hydrolysis, respectively. Then cinnamic acid is β-oxidized to benzoate in the liver. This benzoate exists as sodium salt (NaB) or benzoyl-CoA. This NaB is a widely used food preservative due to its antimicrobial properties. Earlier, we have demonstrated that NaB modifies T cells at multiple steps and protects experimental allergic encephalomyelitis, an animal model of MS (35). Recently, one study by Cao et al. (36) demonstrates that cinnamon polyphenol extract increases the expression of tristetraprolin, an anti-inflammatory molecule, more rapidly than those of proinflammatory cytokines in macrophages. Several lines of evidence presented in this study clearly support the conclusion that the cinnamon metabolite NaB attenuates the activation of mouse microglia and human astroglia. Our conclusion is based on the following observations. First, LPS, a prototype inducer of inflammation in many cell types including microglia, induced the expression of iNOS and proinflammatory cytokines and up-regulated the expression of various surface markers in mouse microglia. However, NaB attenuated LPS-induced expression of proinflammatory molecules without altering cell survival suggesting that this attenuation is not due to any cell death. This inhibition was also specific as NaFO, a compound structurally similar to NaB but without having the benzoate moiety, had no effect. Second, we extended the study beyond LPS and examined whether NaB was capable of suppressing microglial expression of iNOS induced by other neurotoxins and etiological reagents of various neurodegenerative disorders. It is important that NaB, but not NaFO, attenuated microglial expression of iNOS mRNA induced by various neurotoxins such as Aβ (related to AD), poly(IC) (related to viral neuropathy), HIV-1 gp120 (related to HAD), MPP+ (related to Parkinson’s disease), PrP (related to prion disorders), IL-1β (related to neuroinflammation), and IL-12 p40, (related to MS). Third, IL-1β is critical for inducing iNOS and activating primary human astrocytes (20). NaB also suppressed the production of NO, the expression of iNOS, the activation human iNOS promoter, and the up-regulation of GFAP in human astrocytes. Because these proinflammatory molecules have been implicated in the pathogenesis of demyelinating and neurodegenerative diseases, our results provide a potentially important mechanism whereby cinnamon metabolite NaB may ameliorate neural injury.

The signaling events required for the transcription of iNOS and proinflammatory cytokines are becoming clear. Although many transcription factors such as NF-κB, C/EBPβ, AP-1, STAT, IRF-1, etc., play a role in the expression of various proinflammatory molecules, activation of NF-κB seems essential for the transcription of most of the proinflammatory molecules (19–23, 26–28, 37, 38). Therefore, for a drug to exhibit an anti-inflammatory effect, it is almost mandatory to attenuate the activation of NF-κB. Although we did not see complete abrogation of NF-κB activation, NaB significantly decreased the activation of NF-κB in microglia. However, it was unknown by which mechanisms NaB suppressed the activation of NF-κB. p21

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, a membrane-associated small guanine nucleotide-binding protein, plays a central role in transmitting extracellular signals within the cell and in controlling cellular proliferation and differentiation (39, 40). Here we present evidence that NaB suppressed the activation of p21

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and thereby inhibited the activation of NF-κB and the expression iNOS in microglia. Our conclusion is based on the following observations. First, NaB reduced the level of cholesterol in vivo in mice at a level comparable with pravastatin suggesting that this drug may be used to lower cholesterol in patients with hypercholesterolemia. However, HMG-CoA, mevalonate and farnesyl pyrophosphate, but not cholesterol, reversed the inhibitory effect of NaB on the expression of iNOS and the activation of NF-κB, suggesting that depletion of...
intermediates, but not end products, of the mevalonate pathway is involved in the anti-inflammatory effect of NaB. Second, FPT inhib- itor II, capable of inhibiting farnesylation of p21\textsuperscript{ras}, inhibited the activation of NF-κB and the expression of iNOS. Third, NaB, but not NaFO, inhibited the activation of p21\textsuperscript{ras} in LPS-stimulated microglial cells. Fourth, activation of p21\textsuperscript{ras} alone was sufficient for the activation of NF-κB and the expression of iNOS in microglial cells underlying the importance p21\textsuperscript{ras} activation in the activation of microglia.

The Ras proto-oncogene proteins, a family of GTP-binding pro- teins, function by binding to the cytoplasmic surface of the plasma membrane. This membrane localization of p21\textsuperscript{ras} involves prenyl- lation of cysteine in a CAAX motif present at the C terminus, proteolytic removal of AAX tripeptide, and then carboxymethylation of the C-terminal cysteine (39). The activation of p21\textsuperscript{ras} by receptor tyrosine kinase occurs through conversion of the GDP-bound inactive form to the GTP-bound active form by Sos and Grb2 and then transduction of signal to downstream effector mole- cules (40). The GTP-bound form is converted to the inactive form by the intrinsic GTPase activity, which is accelerated by GTPase- activating proteins (41). NaB preferentially attenuates farnesylation of p21\textsuperscript{ras} and thereby inhibits the signal transmission to the downstream signaling molecules (42, 43). One such downstream candidate is Raf-1 (serine-threonine kinase). The p21\textsuperscript{ras} interacts directly with Raf-1 and is believed to function by positioning Raf-1 at the plasma membrane in the vicinity of its activator, and tyrosine phosphorylation of Raf-1 seems to be essential for p21\textsuperscript{ras}-induced activation of Raf-1 (42, 43). Raf-1, in turn, phosphorylates and activates MEKs and ERKs (members of the MAPK cascade). Therefore, the observed inhibition of NF-κB activation and induc- tion of iNOS by NaB may be due to decrease and/or lack of signal transmission from receptor tyrosine kinase to Raf/MAPK cascade via p21\textsuperscript{ras}.

There are several advantages of NaB over other proposed an- tineurodegenerative therapies. First, NaB is fairly nontoxic. Cin- namon has been widely used as flavoring material and spice throughout the world for centuries. Cinnamon is metabolized to NaB. NaB is excreted through the urine, if in excess. NaB is an FDA-approved drug against urea cycle disorders in children. Sec- ond, NaB can be taken orally, the least painful route. Recently, we have demonstrated that NaB treatment of mice with relapsing-remitting experimental allergic encephalomyelitis, an animal model of MS, via drinking water suppressed the disease process of ex- perimental allergic encephalomyelitis (35). Third, NaB is very economical compared with other existing antineurodegenerative therapies. Fourth, although here we have not tested the premise, NaB as a lipophilic molecule is most likely able to diffuse through the blood-brain barrier. For example, glycine toxicity is a problem in urea cycle disorders. After treatment of patients with urea cycle disorders, NaB combines with glycine to produce hippurate, a compound that is readily excreted in the urine. Simultaneous se- rum and CSF sampling in those patients showed comparable levels of NaB and hippurate in the CSF (13–15), suggesting that NaB is capable of crossing the blood-brain barrier.

In summary, we have demonstrated that NaB (a metabolite of cinnamon, commonly used food additive and a FDA-approved drug for urea cycle disorders) inhibited glial activation of NF-κB and expression of iNOS and cytokines by modulating the mevalonate pathway and Ras activation. Because NaB suppressed the mevalonate pathway, it was also able to lower cholesterol in vivo in mice at a level comparable with that of pravastatin, a cholester- ol-lowering drug. These results highlight undiscovered proper- ties of NaB and indicate that this drug may be used for therapeutic intervention in neurodegenerative disorders as primary or adjunct therapy.

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Disclosures
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References

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