

## Inhibitory Effects of Long-Term Administration of Ferulic Acid on Microglial Activation Induced by Intracerebroventricular Injection of $\beta$ -Amyloid Peptide (1–42) in Mice

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**Flavonoids and monophenolic compounds have been well described in recent years as antioxidants and scavengers of reactive oxygen and nitrogen species. In the present study, we aimed to characterize the effects of long-term administration of ferulic acid on the centrally administered  $\beta$ -amyloid peptide ( $A\beta$ )<sup>1–42</sup>-induced activation of microglial cells in mice.  $A\beta$ <sup>1–42</sup> increased the immunoreactivity of OX-42, a microglial marker, and interferon- $\gamma$  in the hippocampus at 8 h after the intracerebroventricular injection. The effects were suppressed by long-term (4-week) pretreatment with ferulic acid. This inhibition of microglial cell activation may underlie the beneficial effects of long-term administration of ferulic acid on  $A\beta$ <sup>1–42</sup>-induced toxicity *in vivo*.**

**Key words**  $\beta$ -amyloid peptide ( $A\beta$ ); microglia; endothelial nitric oxide synthase; ferulic acid; Alzheimer's disease

Accumulating evidence indicates that glial cells, *i.e.*, astrocytes and microglia, are actively involved in the pathogenesis of Alzheimer's disease.<sup>1,2)</sup> Thus one promising target of preventive or therapeutic intervention in Alzheimer's disease is suppression of the proinflammatory activity of activated glial cells in the brains of Alzheimer's disease patients.<sup>3,4)</sup>

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is a phenolic compound present in a variety of plants. We recently reported that long-term administration of ferulic acid for 4 weeks protects mice against learning and memory deficits induced by centrally administered  $\beta$ -amyloid ( $A\beta$ )<sup>1–42</sup>.<sup>5)</sup> In that report, we found that the  $A\beta$ <sup>1–42</sup>-induced increases in immunoreactivities of glial fibrillary acidic protein (GFAP), the astrocyte marker, and interleukin-1 $\beta$  in the hippocampus are also suppressed by pretreatment with ferulic acid. In the present study, we further characterize the effects of long-term administration of ferulic acid on the activation of microglial cells induced by centrally administered  $A\beta$ <sup>1–42</sup> in mice.

### MATERIALS AND METHODS

**Materials** Male ICR mice weighing 18–26 g at the beginning of experiments (Myung-Jin, Inc., Seoul, Korea) were used in all experiments. The mice were housed 5 per cage in a room maintained at 22±1 °C with an alternating 12-h light–dark cycle. Food was available *ad libitum*.  $A\beta$ <sup>1–42</sup> (American Peptide Company, U.S.A.) and  $A\beta$ <sup>42–1</sup> (Bachem, Switzerland) were prepared as stock solutions at a concentration of 37  $\mu$ g/ $\mu$ l in sterile 0.1 M phosphate-buffered saline (PBS) (pH 7.4) and aliquots were stored at –20 °C until use. Ferulic acid (Sigma) was dissolved each day in tap water at a concentration of 0.006% (w/v). Assay with HPLC with electrochemical detection showed 11% loss of ferulic acid (0.006% w/v) in tap water in 24 h at room temperature.

**Experimental Design** Mice were allowed free access to normal drinking water or ferulic acid solution for 4 weeks. Subsequently,  $A\beta$ <sup>1–42</sup> was administered *via* intracerebroventricular injection, and at various time points after the  $A\beta$ <sup>1–42</sup> injection the brains were analyzed immunohistochemically.

Control animals were injected with  $A\beta$ <sup>42–1</sup>.

**Intracerebroventricular Injection of  $A\beta$ <sup>1–42</sup>** The administration of  $A\beta$ <sup>1–42</sup> was performed according to the procedure established by Laursen and Belknap.<sup>6)</sup> Briefly, each mouse was injected at the bregma with a 50- $\mu$ l Hamilton microsyringe fitted with a 26-gauge needle that was inserted to a depth of 2.4 mm. The injection volume was 5  $\mu$ l.

**Immunocytochemistry** At various time points after injection of  $A\beta$ <sup>1–42</sup>, mice were transcardially perfused and postfixed for 4 h in 4% paraformaldehyde. Brains were cryoprotected in 30% sucrose, sectioned coronally (45  $\mu$ m) on a freezing microtome, and collected in a cryoprotectant for storage at –20 °C. Floating sections of brains were processed as described previously.<sup>5)</sup> After 3 10-min rinses in PBS, sections were placed in the cryoprotectant, preincubated for 30 min in 0.1 M PBS with 1% bovine serum albumin and 0.2% Triton X-100, and incubated overnight with the following primary antisera: anti-rat interferon (IFN)- $\gamma$  (1:500, R&D); and anti-rat OX-42 (1:500, Harlan Sera-Labs). On the following day, the sections were incubated for 1 h in biotinylated rabbit and goat secondary antibody obtained from Vector Laboratories. After incubation with the Vector Elite ABC kit, antigens were detected with 3,3'-diaminobenzidine tetrahydrochloride (DAB) as the chromogen.

### RESULTS

**Inhibition of  $A\beta$ <sup>1–42</sup>-Induced Increase in OX-42 Immunoreactivity by Ferulic Acid** To examine whether central  $A\beta$ <sup>1–42</sup> injection induces microglial activation, OX-42 immunoreactivity, a marker of activated microglia, was examined. OX-42 immunoreactivity was markedly enhanced 8 h after injection of  $A\beta$ <sup>1–42</sup> (Fig. 1b), and returned to basal levels at 1 d (data not shown). In animals pretreated with ferulic acid for 4 weeks, the  $A\beta$ <sup>1–42</sup>-induced increases in hippocampal OX-42 immunoreactivity were effectively blocked (Fig. 1c).

**Inhibition of  $A\beta$ <sup>1–42</sup>-Induced Increase in IFN- $\gamma$  Immunoreactivity by Ferulic Acid** Interestingly, the time

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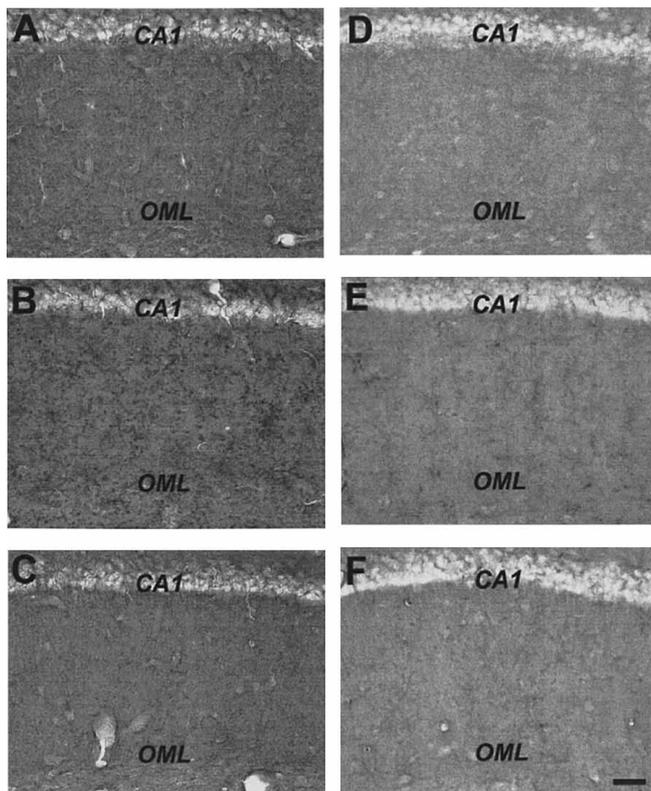


Fig. 1. Blockade of the  $A\beta^{1-42}$ -Induced Increase in OX-42 and Interferon ( $IFN$ )- $\gamma$  Immunoreactivity by Ferulic Acid

Mice were allowed free access to normal drinking water or water containing ferulic acid (0.006%) for 4 weeks prior to  $A\beta^{1-42}$  injection. OX-42 and  $IFN$ - $\gamma$  immunoreactivities in the hippocampus were examined 8 h after injection of 410 pmol of  $A\beta^{1-42}$  (A, D) or  $A\beta^{1-42}$  (B, C, E, F). Mice injected with  $A\beta^{1-42}$  410 pmol served as controls (A, B). A, B, and C are OX-42 immunoreactivity in the hippocampus in control,  $A\beta^{1-42}$  and FA/ $A\beta^{1-42}$  respectively. D, E, and F are  $IFN$ - $\gamma$  immunoreactivity in the hippocampus in control,  $A\beta^{1-42}$  and FA/ $A\beta^{1-42}$ , respectively. Original magnification  $\times 20$ . Scale bar, 25  $\mu$ m.  $n=6$  (6 of 9).

course of  $IFN$ - $\gamma$  immunoreactivity in the hippocampus was the same as OX-42 immunoreactivity.  $IFN$ - $\gamma$ -immunoreactive cells appeared to be microglial cells. In animals pretreated with ferulic acid for 4 weeks, the  $A\beta^{1-42}$ -induced increases in hippocampal  $IFN$ - $\gamma$  immunoreactivity were effectively blocked (Fig. 1f).

## DISCUSSION

Previously, we reported that a single intracerebroventricular injection of a picomolar dose (410 pmol) of  $A\beta^{1-42}$  in mice effectively activates astrocytes, as evidenced by increased immunoreactivities of GFAP and  $IL-1\beta$ , which were blocked by long-term pretreatment with ferulic acid.<sup>5</sup> In the present study, we extended our previous findings by showing that centrally injected  $A\beta^{1-42}$  induces activation of microglia, as evidenced by the increased immunoreactivity of OX-42, which was also effectively inhibited by ferulic acid.

We found that activation of microglia is more rapid compared with that of astrocytes, *i.e.*, we observed that OX-42 immunoreactivity is increased at 8 h after central injection of  $A\beta^{1-42}$ , which returned to the basal state at 24 h, while GFAP and eNOS immunoreactivities peaked 3 or 5 d after central  $A\beta^{1-42}$  injection.<sup>5</sup> Thus it appears likely that the mi-

croglia is activated at 8 h after  $A\beta^{1-42}$  injection, which returns to the basal state 24 h after injection, while astrocyte activation is slower, which peaks post- $A\beta^{1-42}$  injection day 3 to 5. In addition, activated microglia exhibits increased  $IFN$ - $\gamma$  immunoreactivity. The time course of  $IFN$ - $\gamma$  immunostaining is the same as that of OX-42.  $IFN$ - $\gamma$  could act as an inflammatory amplifier aggravating the neurodegenerative process by priming microglia or monocytes/macrophages for the secretion of proinflammatory cytokines.<sup>7</sup> Further,  $IFN$ - $\gamma$  was reported to show a synergistic effect on microglia activation in response to  $A\beta$ .<sup>8</sup> Although the mechanism of astrocyte activation is unclear at present, it can be speculated that microglia activation induces subsequent astrocyte activation, because the time course of astrocyte activation follows microglia activation. The primary site of action of ferulic acid could be microglia. Thus it may be suggested that long-term administration of ferulic acid could be microglia. Thus it may be suggested that long-term administration of ferulic acid induces microglia to suppress the  $A\beta^{1-42}$  stimulus.

Activation of glial cells including increased eNOS expression by preconditioning stimulus was postulated to be responsible for the induction of tolerance to neuronal damage.<sup>5,9,10</sup> In our laboratory, we noted a transient and slight increase in  $IL-1\beta$ <sup>5</sup> and eNOS (unpublished data) immunoreactivity in astrocytes on day 14 after treatment with ferulic acid, which returned to basal levels on day 28. Thus it can be speculated that activation and subsequent recovery of glial cells by ferulic acid might somehow induce resistance to the subsequent  $A\beta^{1-42}$  insult to the brain. Further study is needed to explore the exact mechanisms of ferulic acid in neuroprotection against  $A\beta^{1-42}$ .

In conclusion, long-term administration of ferulic acid prevented the  $A\beta^{1-42}$ -induced activation of microglia. This inhibition of microglial cell activation may underlie the beneficial effect of long-term administration of ferulic acid against  $A\beta^{1-42}$ -induced toxicity *in vivo*.

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