CHAPTER IV

RESULTS

1. Cellular activation of microglia

A rat microglial cell line HAPI was selected as an in vitro model to study the effect of iron on the expression of MMPs in microglia. This particular cell line retains phenotypic and morphological characteristics of primary microglia. These characteristics include phagocytosis, an ability upon activation to produce and secrete proinflammatory cytokines, the cell surface expression of macrophage-associated antigens, and an ability to produce nitric oxide as previously described (Cheepsunthorn *et al.*, 2001a). In this very first experiment, cellular activation of HAPI cells induced by lipopolysaccharide (LPS, 1 μg/ml) was examined by a RT-PCR assay. Markers for cellular activation included an inducible isoform of nitric oxide synthase (iNOS), interleukin (IL)-1β, and tumor necrossis factor (TNF)-α as previously reported (Giulian *et al.*, 1986b; Rothwell *et al.*, 1997; Chauvet *et al.*, 2001). Total RNA from untreated and LPS-treated HAPI cells was isolated and purified at 6 h time point and subjected to reverse transcription. The target specific primers described in the method section were used in PCR to amplify fragments of iNOS (311 bp), IL-1β (200 bp), TNF-α (659 bp), and GAPDH (300 bp). The results demonstrated that HAPI cells responded to LPS by increasing the expression of iNOS, IL-1β, and TNF-α as shown in Figure 6.

2. Cellular iron-loading in microglia

The objective of this experiment was to mimic intracellular iron loading of activated microglial cells as seen in AD. The expression of iron storage protein ferritin was selected as an indicator to examine whether an addition of iron into cell culture

medium was sufficient to induce intracellular accumulation of iron in microglia. This is because the levels of ferritin in the cytoplasm were directly regulated by the amount of intracellular iron. Ferritin expression in response to different culture conditions was determined using rabbit anti-rH ferritin antiserum as previously described (Cheepsunthorn *et al.*, 1998). The results were shown in Figure 7. In untreated control at 6 h, ferritin expression in HAPI cells was undetectable (Figure 7A; lane 1). Exposure to iron (50 μg/ml) for 6 h was sufficient to induce the expression of ferritin in HAPI cells (Figure 7A; lane 2). Treatment with LPS (1 μg/ml) was not enough to induce the expression of ferritin in HAPI cells. If the cells were supplemented with iron during the LPS treatment, ferritin expression was more robust to that seen for iron alone. The expression of an internal control beta actin was similar in all conditions (Figure 7B). These results demonstrated that the addition of iron into the microglial medium was sufficient to cause intracellular iron accumulation.

3. Iron enhances MMP-9 secretion from activated microglia.

The production of matrix-degrading enzymes known as matrix matalloproteinases is associated with microglial function as phagocyte and generally accompanied by cellular activation of microglia. Because the functional activation of microglia as reported can be modified by intracellular iron loading (Cheepsunthorn *et al.*, 2001a), this experiment was designed to examine whether iron could also affect the secretion of MMP-9, also known as gelatinase B, in microglia. As described in the materials and methods, HAPI cells were treated with serum-free medium supplemented with iron (50 μg/ml) or LPS (1 μg/ml) in the presence or absence of iron for 48 hour. The medium collected from each condition was concentrated and examined for the secretion of MMP-9 by gelatin zymography. The experiments were repeated three times and the representative zymogram was shown in Figure 8A. All samples possessed bands of gelatinolytic activity at 92 kDa corresponding to proform

of MMP-9. Treatment of HAPI cells with LPS (1 μg/ml, Lane 3) induced the secretion of MMP-9 more than that seen for untreated control or iron treated alone (lanes 1 and 2, respectively). However, the greatest expression was observed, when the cells were exposed to iron during the LPS treatment (lane 4). These results demonstrated that intracellular accumulation of iron enhanced the secretion of MMP-9 from activated microglia.

Furthermore, the effect of iron on the secretion of MMP-2 or gelatinase A was also examined using an in vitro model of fibroblasts. As described for MMP-9, the secretion of MMP-2 presented in each sample (serum-free medium with iron or LPS with or without iron) at 48 h was subjected to gelatin zymography. The experiments were repeated three times and the representative results were shown in Figure 8B. All samples possessed bands of gelatinase activity at 72 kDa corresponding to proform of MMP-2. Exposure of fibroblasts to iron had no affect on the secretion of MMP-2 (lane 1 and 2). Treatment with LPS enhanced the secretion of MMP-2 from fibroblasts as shown in lane 3. The secretion of MMP-2 was diminished in response to LPS plus iron (lane 4) compared to that seen for LPS alone. However, the secretion of MMP-2 induced by LPS plus iron was still larger than that seen for control and iron alone.

4. Iron has differential effects on the expression of MMP-10 and MMP-1 in activated microglia.

Recent data obtained from gene expression profiling experiments indicated that activated microglia expressed at least two more types of MMPs in addition to MMP-9. The first was MMP-10 or stromelysin 2 and the second was MMP-1 also known as interstitial collagenase (Cheepsunthorn *et al.*, 2001b). Therefore in this set of experiments, the effect of iron on the expression of MMP-10 and MMP-1 was examined using a RT-PCR assay. The experiments were repeated three times and the representative gels were present as shown in Figure 9. At 6 h, the expression of MMP-

10 and MMP-1 in untreated control cells was minimally detectable (lane 1; A and B). Treatment of HAPI cells with LPS increased the expression of both MMP-1 and MMP-1 (lane 2; A and B). However, LPS plus iron decreased mRNA expression of MMP-10 (lane 3A), but did not alter the expression of MMP-1 compared to that seen for LPS treatment alone (lane 3B). The expression of GAPDH was not effected by any treatment (C). These results suggested that MMP-10, but not MMP-1, can be regulated by iron at transcription level.

5. Iron enhances the secretion of MMP-1 in activated microglia.

Since iron did not appear to affect mRNA expression of MMP-1, western blot analysis was performed to examine whether iron could influence MMP-1 expression at the protein level. Equal amount of total protein from serum free media and total cell lysates of HAPI cells exposed to different conditions at 48 h were collected and separated on SDS-PAGE. The presence of MMP-1 in both fractions was detected using a mouse monoclonal anti rat MMP-1 antibody. The results were shown in Figure 10. MMP-1 was detected in all medium samples (Figure 10A), but not in any cell lysate samples (Figure 10B). LPS treatment increased the secretion of MMP-1 from HAPI cells (Figure 10A; lane 3) compared to that seen for untreated and iron treated cells (Figure 10A; lanes 1and 2). Surprisingly, iron appeared to enhance the effect of LPS on the expression of MMP-1 as shown in lane 4.

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6. Intracellular iron loading impairs phagocytic activity of activated microglia.

Results in the previous experiments have demonstrated that cellular iron loading differently influence the expression of MMPs in activated microglia. In this experiment, the effect of iron on microglial phagocytic activity was further examined. This is because histological evidences have implicated iron-rich activated microglia as the converter of diffuse plaques to dense core plaques (Grundke-Iqbal *et al.*, 1990;

Styren *et al.*, 1990; Robinson *et al.*, 1995). However, It has never been shown whether cellular iron loading will affect phagocytic activity of activated microglia. To begin the experiment, HAPI cells were exposed for 24 h to serum free medium containing iron or LPS in the presence or absence of iron. Cells in serum free medium were served as control group. Then, the media were removed before the addition of an assay mixture containing zymosan A and NBT to the cells. At 1 h later, phagocytic activities of HAPI cells in different conditions were measured as described in the materials and methods. Absorbancy at 570 nm represents phagocytic activities of the cells in each condition. The experiment was performed in duplicate and repeated three times with different cell populations. Results represented the mean ± SEM of three separate experiments as shown in figure 11. The results demonstrated that LPS increased phagocytic activity of HAPI cells (significantly in comparison with control, $p < 0.05$; LPS = 0.444 ± 0.039 and untreated control = 0.359 ± 0.056, n = 6). Interestingly, LPS plus iron decreased phagocytic activity of activated cells (significantly in comparison with LPS, $p < 0.05$; LPS plus iron = 0.341 ± 0.014). However, exposure to iron alone did not affect HAPI cell phagocytic activity ($p < 0.005$; iron = 0.338 ± 0.013).

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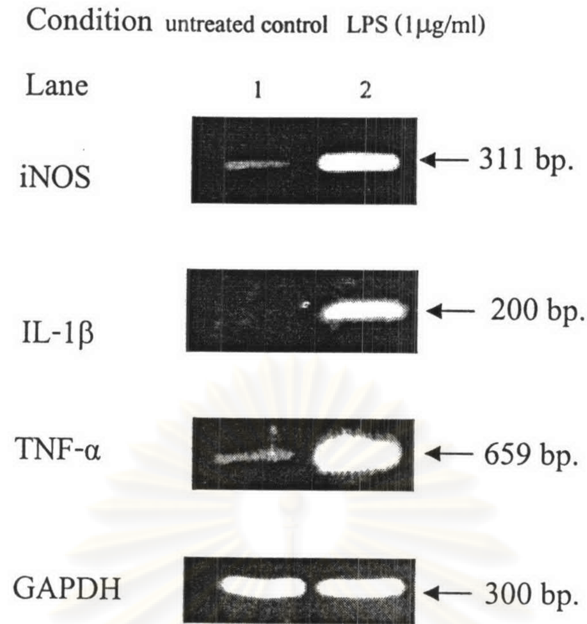

Figure 6 LPS induces gene expressions of iNOS, IL-1β and TNF-α in rat microglial cells. HAPI cells were grown in 5% FBS supplemented DMEM. When cells reached 80% confluence, the serum concentration was decreased to 0% and grown for 24 h before LPS treatment. To begin the experiment, the medium was removed and fresh serum free DMEM supplemented with LPS (1 μg/ml) was added to the cells. Cells exposed to the medium without LPS were used as control. Total RNA from cells in each condition was isolated at 6 h. A volume of 2 μg total RNA from each sample was subjected to RT-PCR assay in the presence of primers specific for either iNOS, IL-1β, TNF-α or GAPDH. The PCR protocols were described in materials and methods. The resulting amplification products were analyzed on 1.5 % agarose gel. The experiments were repeated three times. The representative gels were shown. Bands in lane 1 indicated basal expression of iNOS, IL-1β, and TNF-α in untreated control cells. Bands in lane 2 shown an increase in expression of iNOS, IL-1β, and TNF-α induced by LPS treatment. The expression of GAPDH in each sample was used as an internal control.

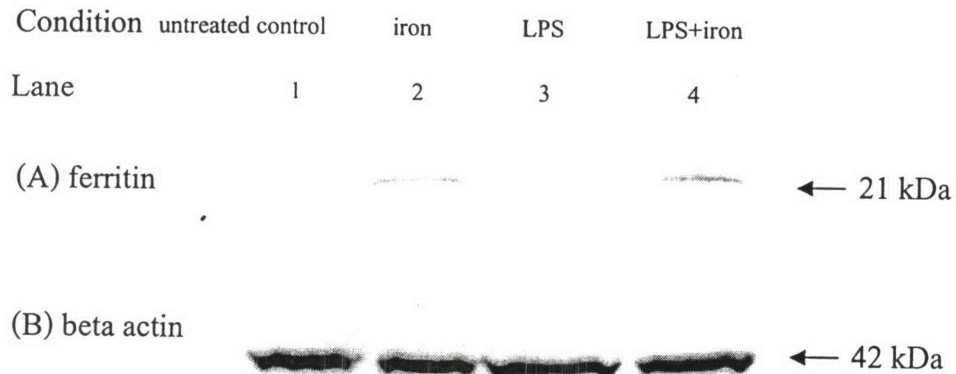

Figure 7 Exposure of microglia to iron increases the expression of intracellular iron storage protein ferritin. An immunoblot analysis of ferritin was included in these studies to demonstrate that the addition of iron into the medium was sufficient to induce a change in expression of an intracellular iron storage protein. Equal amounts (40 μg protein/lane) of total cell lysates isolated from HAPI cells at 6 h exposure time was separated on 10% bis-tris gel in the presence of 2-mercaptoethanol. Protein samples were transferred to PVDF membranes, blocked in 5% non fat dry milk for 1 h at room temperature, washed with TBS, and incubated overnight at 4°c in primary antibodies either rabbit anti rH-ferritin or mouse anti human beta actin. Subsequently, the membranes were washed with TBS and incubated in appropriate secondary antibodies conjugated with HRP for 1 h at room temperature. Bound antibodies were visualized with DAB substrate for the detection of HRP. The results demonstrated that (A) exposure of iron at a final concentration of 50 μg/ml for 6 h induced a robust expression of ferritin in HAPI cells (lane 2). The greater induction was seen with combined treatment of iron and LPS (lane 4). (B) Beta actin in each sample was used as an internal control.

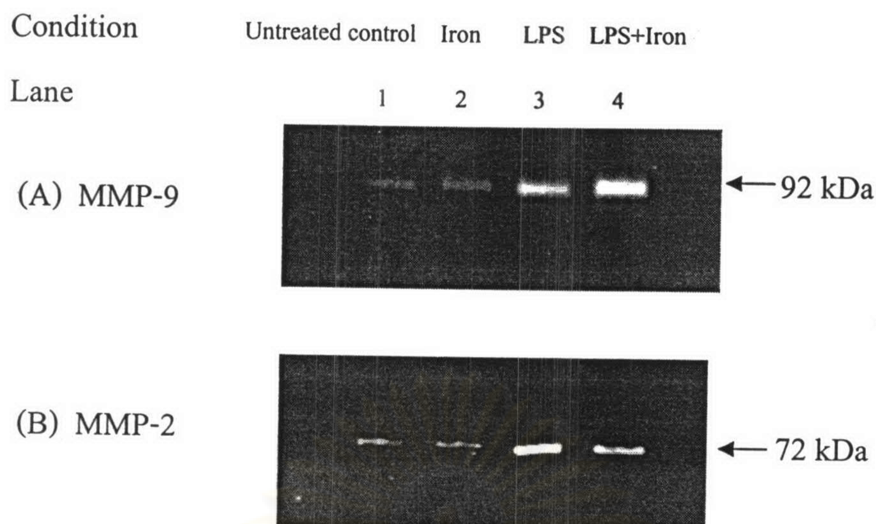

Figure 8 Treatment with iron increases gelatinase activity of MMP-9 in activated microglia, whereas it decreases gelatinase activity of MMP-2 in fibroblasts. To analyze the amount of MMP-9 secreted by HAPI cells and MMP-2 by fibroblasts, total protein from each serum free conditioned medium (300 μg for MMP-9 and 120 μg for MMP-2) at 48 h were separated on 10% polyacrylamide gel containing 1 mg/ml of gelatin substrate. The experiments were repeated three times. The representative zymography was shown. The results of this analysis demonstrated that LPS increased the secretion of MMP-9 secreted by HAPI cells (lane 3A). However, in the presence of LPS and iron, the secretion of MMP-9 was greater as shown in lane 4A. (B) Stimulation of fibroblasts with LPS increased the secretion of MMP-2 (lane 3B). In contrast to the effect of iron on MMP-9, treatment of fibroblasts with LPS in the presence of iron decreased the secretion of MMP-2 (lane 4B). As it was indicated by a decrease in size and intensity of band in lane 4 compared to lane 3.

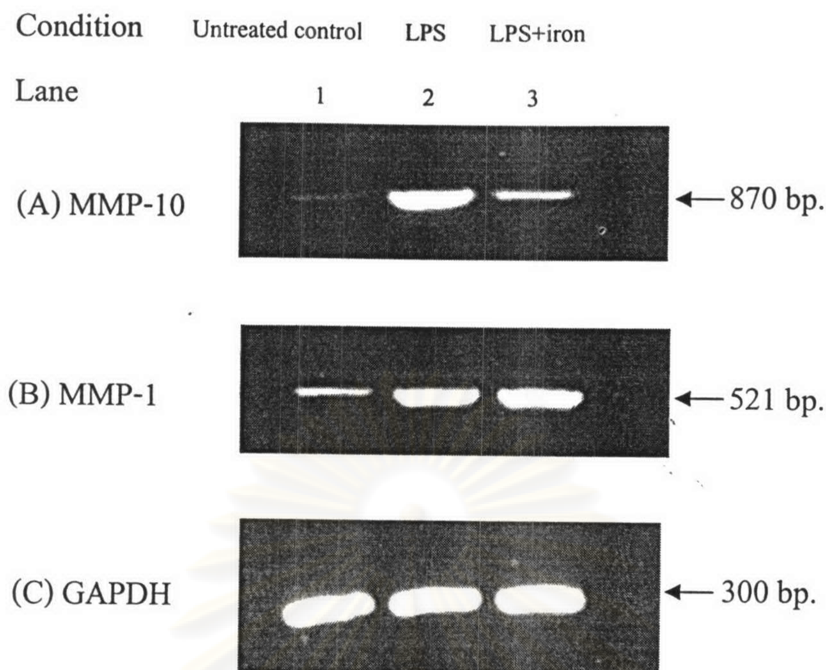

Figure 9 Iron loading in activated microglia decreased mRNA expression of MMP-10, whereas it did not change the expression of MMP-1. HAPI cells were grown in serum free medium (served as untreated control) or serum free medium containing LPS or LPS plus iron, as described in materials and methods. At 6 h, the medium was removed. Total RNAs from both untreated and treated cells were isolated and analyzed by RT-PCR using the primer pairs specific for either MMP-10, MMP-1 or GAPDH. The PCR products were separated on 1.5 % agarose gel. The experiments were repeated three times and the representative gels were shown. The results demonstrated that the expressions of MMP-10 and MMP-1 in untreated HAPI cells were minimal (lane 1; A and B). Exposure to LPS upregulated gene expression of MMP-10 as well as MMP-1 (lane 2; A and B). The effect of LPS on MMP-10 expression was diminished in the presence of iron (lane 3A). However, the presence of iron had no effect on LPS-induced MMP-1 expression (lane 3B). (C) The expression of GAPDH, a housekeeping gene, was served as an internal control.

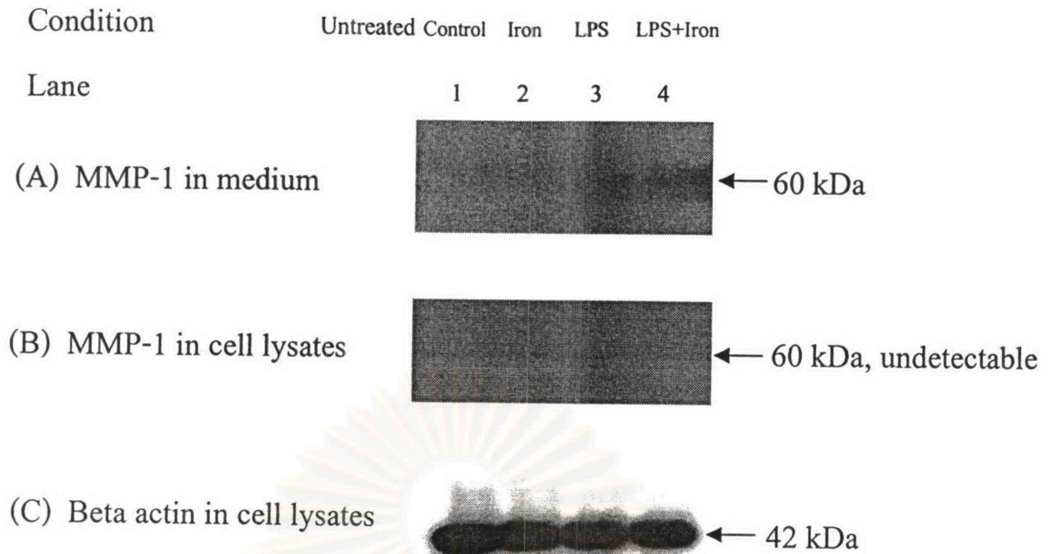

Figure 10 Iron loading in activated microglia enhances the secretion of MMP-1 into the medium. Equal amount (60 µg/lane) of total protein from conditioned media or cell lysates from HAPI cells was analyzed by western blotting for MMP-1. The samples were boiled for 10 min in the presence of 2-mercaptoethanol and separated on 10% bis-tris gel. Protein samples were transferred to PVDF membranes, blocked in 5% non fat dry milk for 1 h at room temperature, washed with TBS, and incubated overnight at 4°c in a mouse monoclonal anti rat MMP-1. Subsequently, the membranes were washed with TBS and incubated in the secondary antibody goat anti mouse HRP conjugate for 1 h at room temperature. Bound antibodies were visualized using chemiluminescent substrate for HRP. The representative blots were shown. In lane 3A, LPS increased MMP-1 secretion. In lane 4A, iron further enhanced the secretion of MMP-1 induced by LPS. (B) The expression of MMP-1 in HAPI cell lysates was undetecable. (C) Beta actin presented in each cell lysate sample was used as an internal control for (B).

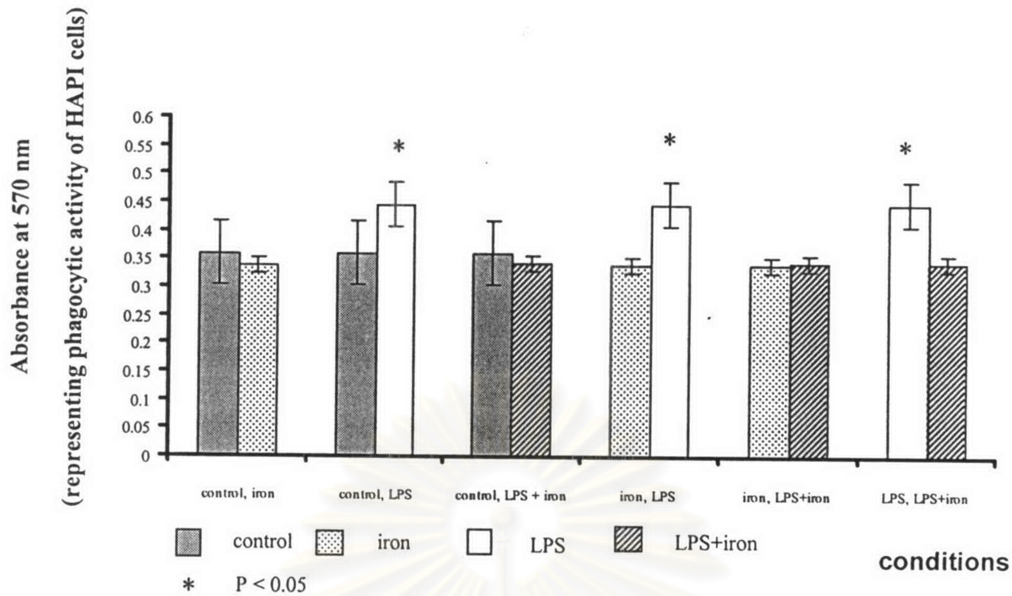

Figure 11 Intracellular iron loading decreases phagocytic activity of activated microglia. In this experiment, HAPI cells were plated at $5x10^4$ cells/well in 96 well plates. To begin the experiment, the medium was removed and fresh serum-free DMEM containing either 1 µg/ml LPS, 50 µg/ml iron, or LPS plus iron was added to the cells. Untreated cells in serum-free DMEM were served as control. After 24 h treatment, the medium was removed. The assay mixture composed of NBT and zymosan A was added into each well and incubated at $37^{O}c$ in 5% CO_2 for 1 h. The reaction was stopped by incubated at $-20^{O}c$ for 5 min. Each well was then washed with absolute methanol three times and left air dry. 120 µl of 2 M KOH and 140 µl of DMSO were added into each well, mixed vigorously, and the plates were read at 570 nm. The absorbency values (mean ± SEM) represent phagocytic activity of HAPI cells. The results demonstrated that LPS significantly increased phagocytic activity of HAPI cells in comparison with control ($p < 0.05$; LPS = 0.444 ± 0.039 and untreated control = 0.359 ± 0.056, n = 6). Interestingly, LPS plus iron significantly decreased phagocytic activity of activated cells in comparison with LPS ($p < 0.05$; LPS plus iron = 0.341 ± 0.014, n = 6). However, exposure to iron alone did not affect HAPI cell phagocytic activity ($p < 0.005$; iron = 0.338 ± 0.013, n = 6).