CHAPTER IV

RESULTS

1. Estrogen treatment reduces MMP-9 secretion from iron-loaded activated microglia.

To demonstrate by means of gel zymography that iron loading could enhance inflammatory effector function of LPS-activated microglia by increasing the secretion of MMP-9. As shown in figure 1 (lane 1-3), two clear bands were appeared against the dark background of coomassie blue stained gel. A band at 92 κDa represents proform of MMP-9 and 88 κDa band represents its active form. Treatment with E_2 1nM induced the production of MMP-9 from non-iron loaded microglia in (lane1, 4) but 10 nM E_2 had diminished the production of MMP-9 from the cell (lane 1, 5). As shown in lane 2, 6, treatment with E_2 1nM was not affect the production of MMP-9 from LPS-activated microglia but E_2 10nM decreased MMP-9 secretion. However, the production of MMP-9, both forms, from iron-loaded activated microglia was diminished in a dose-dependent manner, when these cells were treated with E_2 (lane 8, 9). Our results suggest that treatment with E_2 could interfere with cellular iron metabolism of activated microglia and further suggest that the activation of microglia is linked to cellular iron metabolism.

2. Estrogen treatment attenuated LPS-induced NO production in iron-loaded and non-iron loaded microglia.

To further characterize the effect of E_2 on microglia-mediated cell secretory, the production of NO was examined by means of Greiss reaction, as shown in Figure 2. Treatment with E_2 diminished the production of NO from microglia in a

dose-dependent manner (Figure 2.1). Similar effect was also seen with iron-loaded microglia (Figure 2.2). This study also found that E_2 exerts its anti-inflammatory effect on LPS-induced NO production in microglia in a dose-dependent manner (Figure 2.3). Similar results an obtained with iron-loaded, LPS-activated microglia (Figure 2.4).

3. Estrogen treatment affected gene expression of inducible nitric oxide synthase and pro-inflammatory cytokine in iron-loaded activated microglia differently.

Previous results suggest that cellular iron loading could influence inflammatory effector function of LPS-activated microglia. Therefore, these experiments were designed to further characterize the effect of iron loading on RNA expression of inducible nitric oxide synthase (iNOS) and two major pro-inflammatory cytokines, tumor necrosis factor TNF -α and interleukin IL-1β, in activated microglia. To begin the experiments, HAPI cells were exposed to LPS (1μg/ml) in the presence or absence of iron (FAC; 50 μg/ml) for 18h. By means of RT-PCR, to assess the expression of iNOS, TNF-α, and IL-1β in iron-loaded microglia and compared with that of non-iron loaded cells. As shown in Figure 3 (A-C; lane 1, 2), iron-loaded cells expressed iNOS, TNF-α, and IL-1β RNA at a similar level compared to that of non-iron loaded cells. LPS induced the expression of the iNOS, TNF-α, and IL-1β RNA in microglia (A-C; lane 3). Iron loading in LPS-activated cells was not affect the expression of the iNOS RNA, but it decreased and slightly increased the expression of the TNF-α, and IL-1β RNA, respectively, when compared with that of LPS-activated cells (A-C; lane 3, 4).

To examine anti-inflammatory effect of 17-β estradiol (E_2) on the expression of iNOS, TNF-α, and IL-1β RNA in non-activated and LPS-activated microglia in the absence or presence of iron. As shown in Figure 3 (A, B; lane 5-8), treatment with E_2 results in a decrease in the expression of the iNOS and TNF-α RNA in non-activated, as well as in LPS-activated microglia in a dose-dependent manner.

Similar results are obtained, when iron-loaded LPS-activated microglia are exposed to E_2 (Figure 3A, B; lane 9, 10). However, the higher dose of E_2 (10nM and 1nM) increased the expression of the IL-1β RNA in both non-activated and activated cells (Figure3C; lane5-8). Treatment with E_2 had no effect on the expression of the IL-1β RNA in iron-loaded activated microglia (Figure 3C; lane 9, 10).

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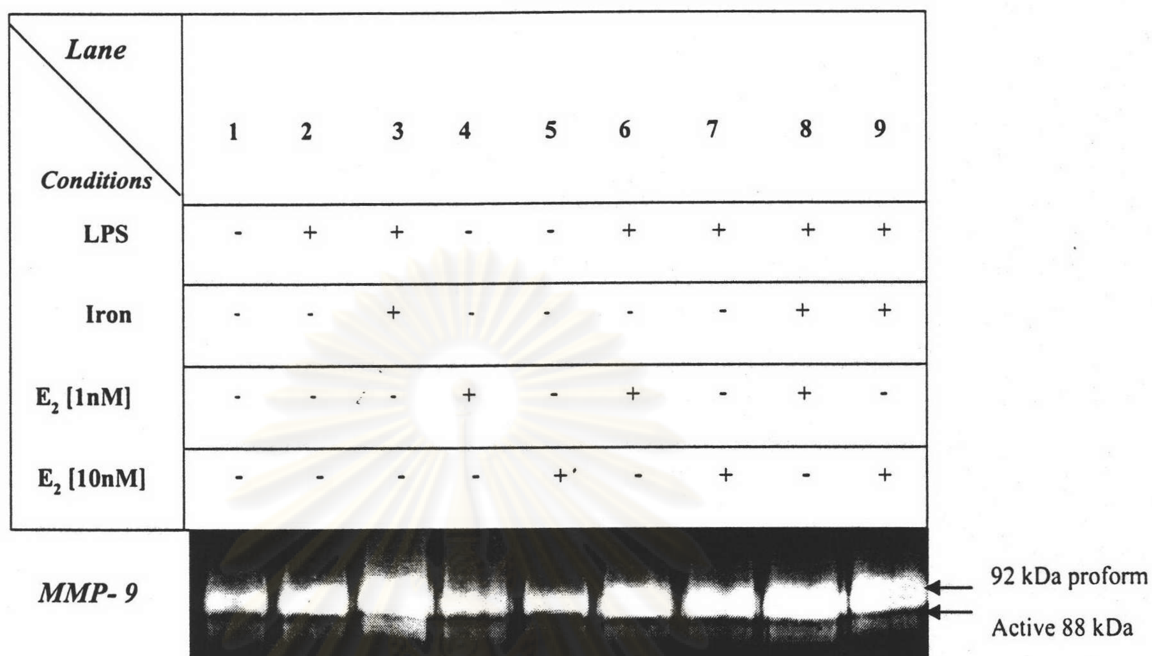

Figure1. Treatment with iron increased gelatinase activity of MMP-9 in activated microglia, whereas estrogen treatment decreases gelatinase activity. To analyze the amount of MMP-9 secreted by HAPI cells, total protein from each serum free conditioned medium (120 μg for MMP-9) at 24 h were separated on 10% polyacrylamide gel containing 1 mg/ml of gelatin substrate. The experiments were repeated three times.

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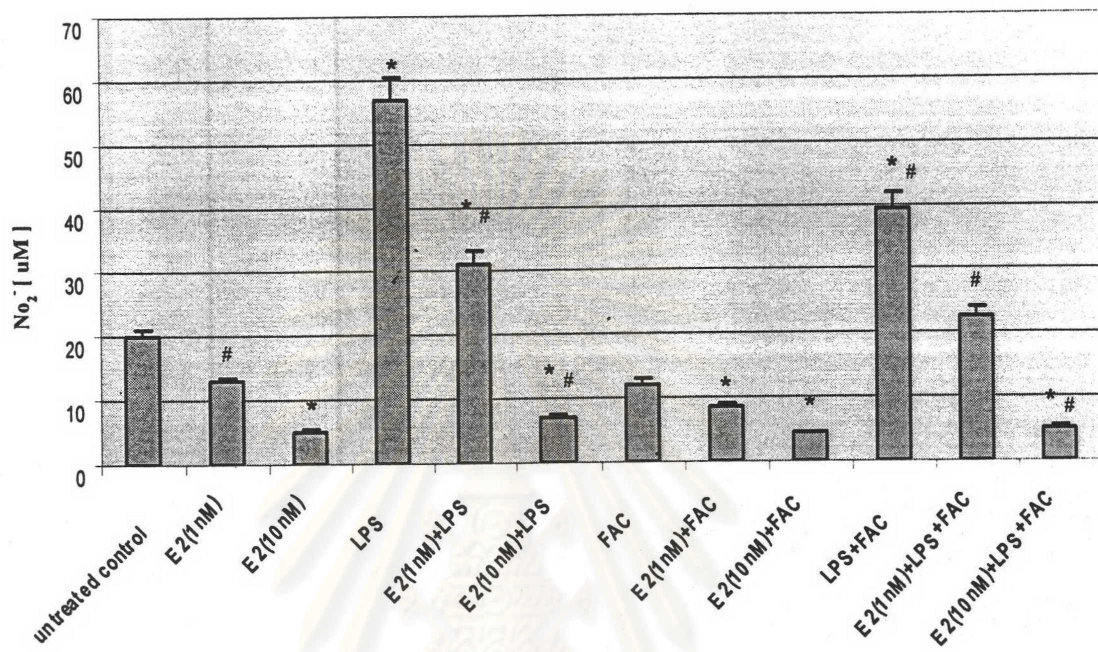

Figure2. **No_2^- levels were attenuated by Estrogen (E_2) and iron (FAC) in microglial cells culture.** Microglia were assayed in absence or presence of 1 μg/ml LPS, 1nM or 10nM 17β-estradiol (E2) and50ug/ml iron (FAC) by adding alone or together .Value present the mean ±SEM of three separate experiments.**p<0.05*, as compared with the control; # *p<0.05*, as compared with the LPS treated values, were calculated by ANOVA test, followed by LSD analysis.

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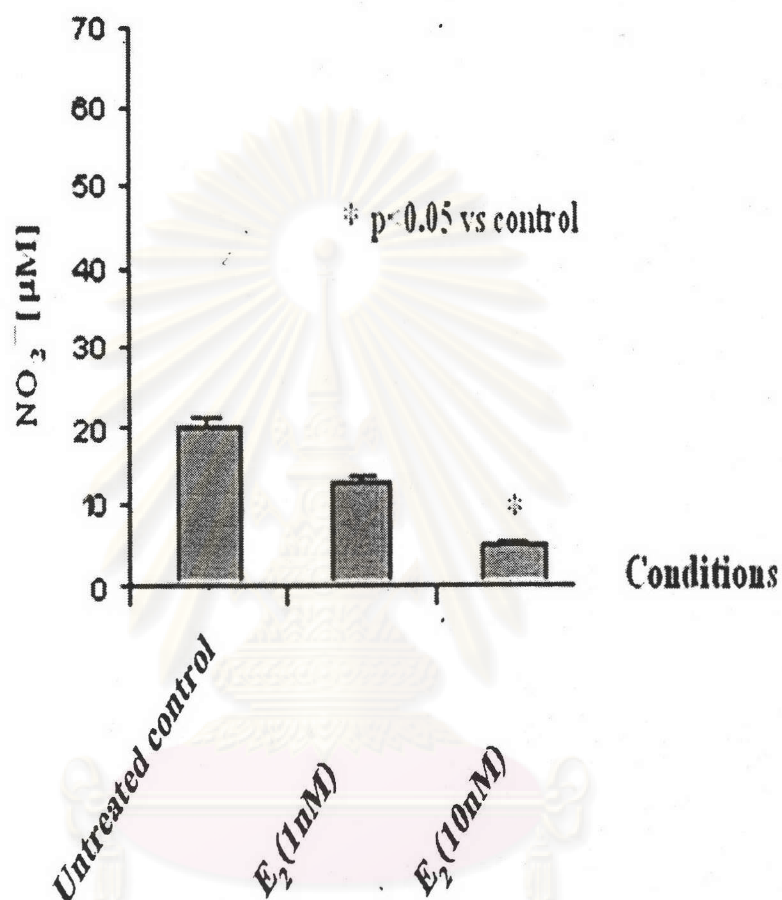

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Figure 2.1 NO_2^- productions in the absence or presence of 17 β –estradiol (E_2) from microglia cells. Values represent the mean ±SEM of three separate experiments.*$p<0.05$, as compared with the control, were calculated by ANOVA test, followed by LSD analysis.

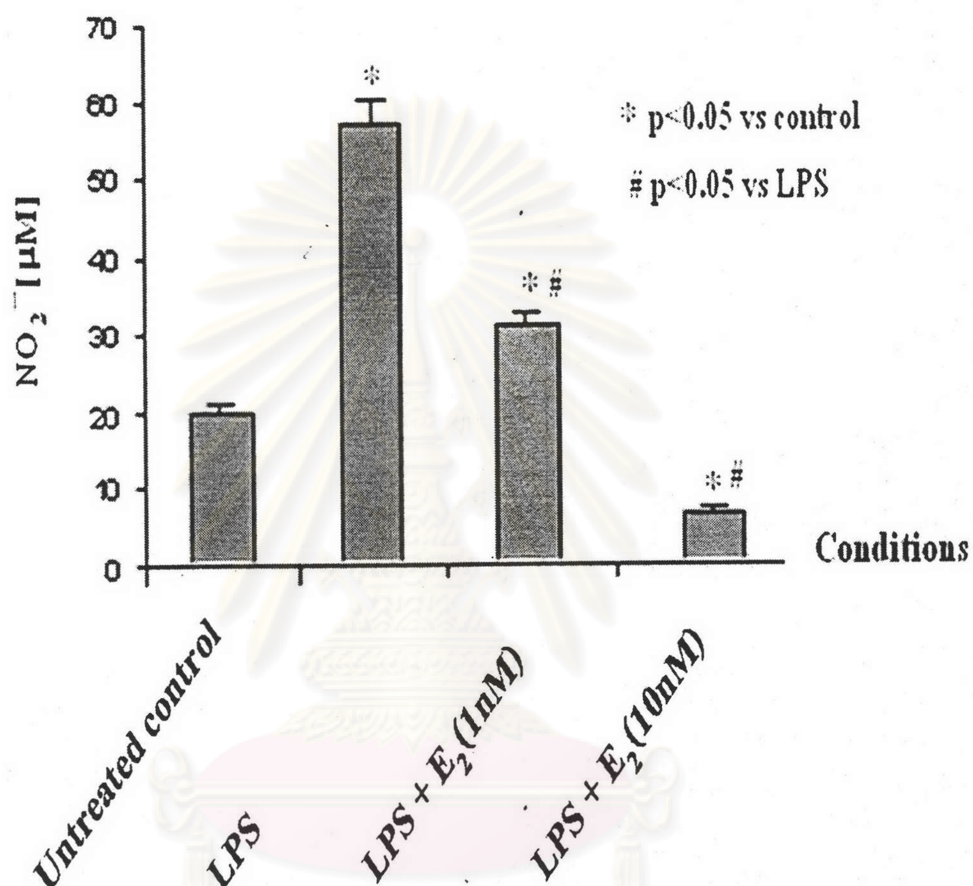

Figure 2.2 LPS induced NO_2^- productions were attenuated by 17 β –estradiol (E_2) in microgial cells. Values represent the mean ±SEM of three separate experiments.*$p<0.05$, as compared with the control; # $p<0.05$, as compared with the LPS treated values, were calculated by ANOVA test, followed by LSD analysis.

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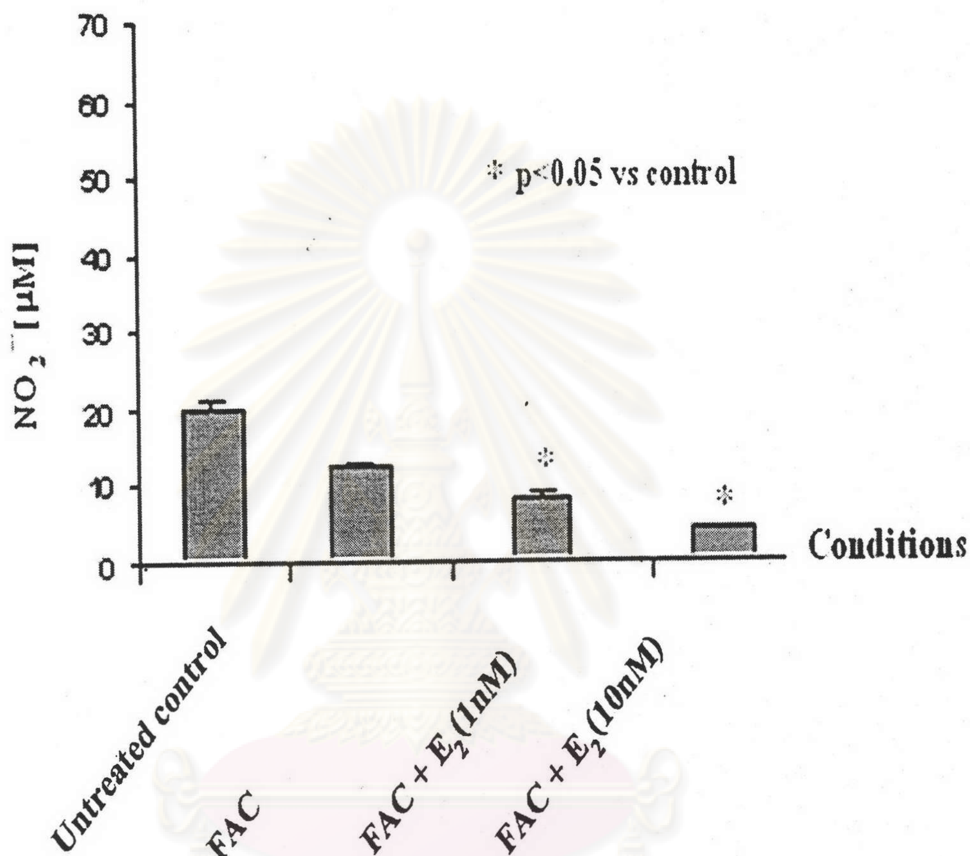

Figure 2.3 NO_2^- productions in the presence of iron (FAC) or by treating with17 β – estradiol (E_2) from microglia. Values represent the mean ±SEM of three separate experiments.*$p<0.05$, as compared with the control, were calculated by ANOVA test, followed by LSD analysis.

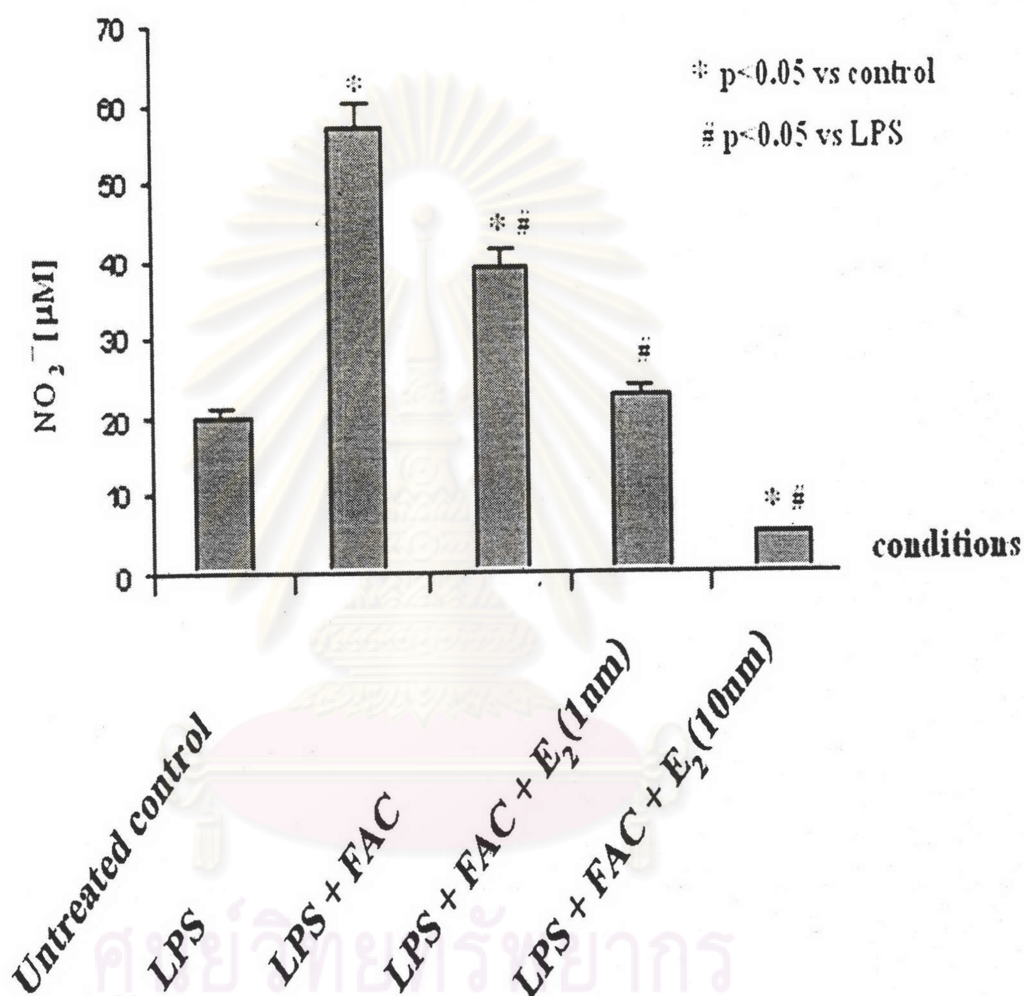

Figure 2.4 LPS induced NO_2^- productions were attenuated by iron (FAC) and 17β –estradiol (E_2) in microglial cells. Values represent the mean ±SEM of three separate experiments.*$p<0.05$, as compared with the control; # $p<0.05$, as compared with the LPS treated values, were calculated by ANOVA test, followed by LSD analysis.

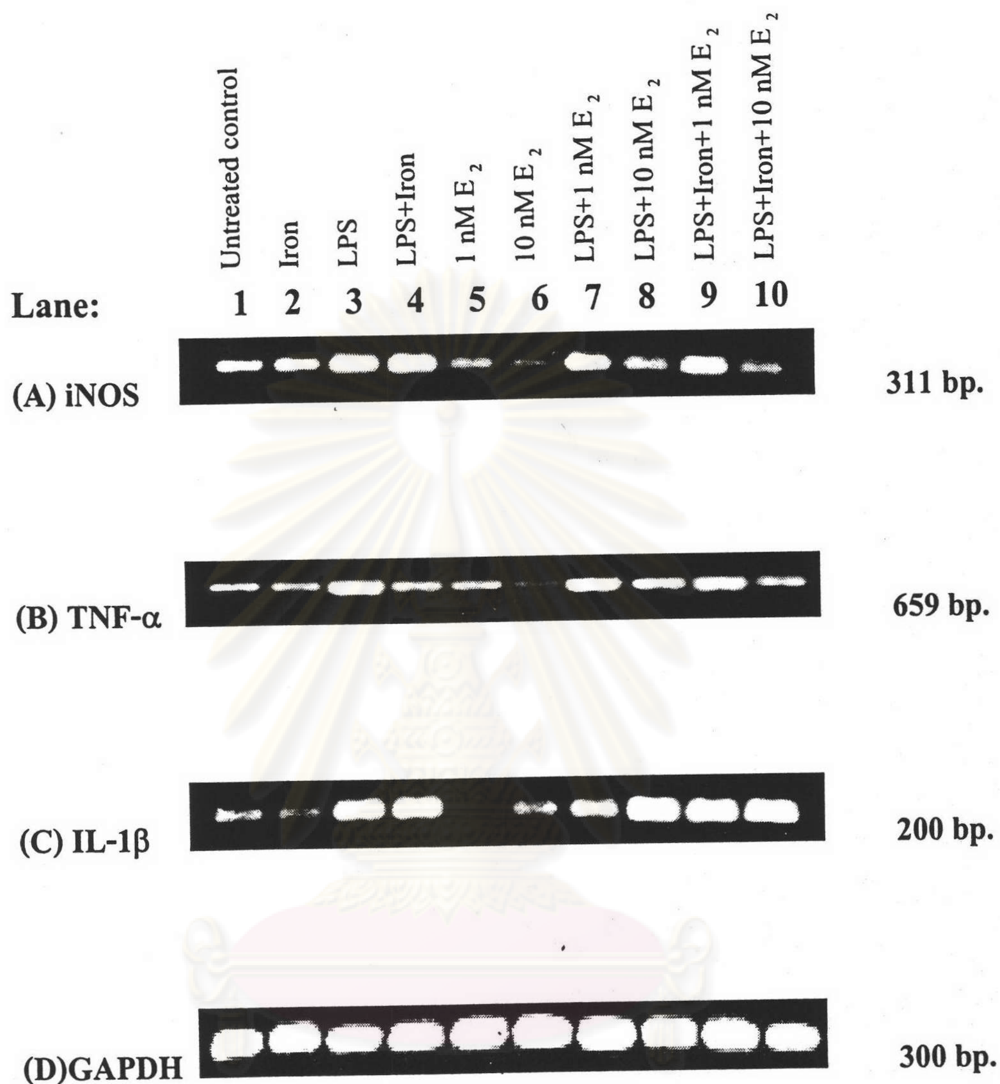

Figure3. Estrogen treatment affected gene expression of inducible nitric oxide synthase (iNOS) and pro-inflammatory cytokine TNF-α, and IL-1β in iron-loaded activated microglia. HAPI cells were exposed to LPS (1μg/ml) in the presence or absence of iron (FAC; 50 μg/ml) and 17-β estradiol (E_2; 1nM, 10nM) for 18h.The expression of GAPDH, a housekeeping gene, was served as an internal control.