CHAPTER III

MATERIALS AND METHODS

1. Cultures and treatments of microglial cells

To elucidate the anti-inflammatory effect of estrogen on iron-loaded activated microglia, HAPI cells, an immortalized rat microglial cell line possessing the characteristics of microglia/brain macrophages as previously described (Cheepsunthorn et al., 2001) were selected, as an *in vitro* model for these studies. HAPI cells were plated in 24-well plates at the density of $5x10^4$ cells/well and grown in 5% fetal bovine serum (FBS) supplemented Dulbecco's modified Eagle's medium (DMEM). Both DMEM and FBS were purchased from HyClone. When cells reached 80% confluence, the medium was removed and cells were then allowed to grow in serum-free medium for 24 h before the experiments. To begin the treatments, the medium in each well was completely removed, cells in each well were added with freshly prepared serum-free DMEM containing 50 μg/ml ferric ammonium citrate (FAC) (Sigma), or 1 μg/ml lipopolysaccharide (LPS) (Sigma), or 1 or 10 nM 17β-estradiol (Sigma), or LPS in the presence of FAC, or LPS in the presence 1 or 10 nM 17β-estradiol, or LPS in the presence of both FAC and 1 or 10 nM 17β-estradiol. Cells in serum-free DMEM served as the untreated control. Times for treatments were indicated in each experiment (see below).

2. RNA Isolation

After 18 h treatments, total mRNAs from each condition was isolated using Trizol reagent (Invitrogen). Briefly, the medium in each well was completely removed. The cells were washed once with phosphate-buffered saline (PBS), pH 7.4. Then, Trizol reagent (300 μl) was added into each well. The homogenates were placed on a low speed rotator for 5 min at room temperature. This is to allow the complete

dissociation of nucleoprotein complexes. Next, the homogenates were transferred into steriled 1.5 ml tubes. Then, chloroform (60 µl) was added into each tube followed by vortexing vigorously for 15 seconds. The mixture was allowed to stay at room temperature for 5 min before centrifugation at 12,000 g for 15 min at 4°c. RNAs in the colorless upper aqueous phase was carefully transferred to a fresh tube. Then, isopropanol (150 µl) was added into each tube. Each sample was stored at room temperature for 10 min and centrifuged at 12,000 g for 10 min at 4°c. RNAs will precipitate and form a white pellet at the bottom of the tube. The supernatant was carefully removed before adding iced-cold 75% ethanol (300 µl) into each tube to wash RNA pellet for 5 min. Subsequently, the pellet was centrifuged at 10,000 g at 4°c for 5 min. At the end of procedure, the RNA pellets were allowed to air dry briefly. It is important not to let the RNA pellet dry completely as it will greatly decrease its solubility. Dissolve the RNA pellets in RNase free water. The amount of RNA obtained by spectophotometry was calculated using the formular below.

RNA (µg) = Absorbance at 260 nm x 40 x dilution factor

The solution containing RNA sample can be stored at -80°c until use.

3. Reverse transcription (cDNA synthesis)

A volume of 2 µg total RNA from each sample was added into the reaction mixture containing 10x reaction buffer, 25mM $MgCl_2$, dNTPs, random primer, ribonuclease inhibitor, AMV reverse transcriptase and RNase free water. All reagents were purchased from Promega. Each sample was kept at room temperature for 10 min incubated at 42°c for 60 min followed by inactivation at 99°c for 5 min. Then, distilled water was added into each sample to a final volume of 100 µl. At this point, each sample can be kept at -20°c until use. See appendix A for preparing the reaction mix.

4. Polymerase Chain Reaction (PCR)

The PCR reaction was performed in a total volume of 25 µl. A 5 µl of cDNA (as described above) was use as templates for subsequent PCR reaction in the Perkin Elmer Thermocycle. PCR reaction was composed of distilled water, PCR buffer, dNTP mixed, Taq DNA polymerase (Promega) and primer pairs (see Table 1). To ensure there was no genomic DNA contamination in the RNA samples, the PCR reaction was performed without reverse transcription. As a control reaction, the RNase free water was replaced the RNA samples in the PCR protocol. Subsequently, amplified PCR products were visualized by gel electrophoresis. Briefly, a 10 µl of each PCR product was mixed with 2 µl of loading buffer, vortexed, spined, and then loaded on to 1.5 % agarose gel containing ethidium bromide. The gel was run at 95 volts until front dye reaches the end of gel. Estimate size of PCR products was compared to the DNA marker (100 bp ladder, Fermentas). No PCR products were observed in any control reactions (data not shown). See appendix A for preparing the reaction mix.

Table 1 Specific primer for TNF-α, IL-1β, iNOS and GAPDH

Gene	Primer sequences (5′ ⟶ 3′)	bp.	PCR Protocols
TNF-α	ATC CGA GAT GTG GAA CTG GCA CCG GAC TCC GTG ATG TCT AAG T	659	1
IL-1β	GCT ATG GCA ACT GTC CCT GAA C TGA GTG ACA CTG CCT TCC TGA A	200	2
iNOS	ATC CCG AAA CGC TAC ACT TCC GGC GAA GAACAA TCC ACA ACT C	311	3
GAPDH	AAG CTC ACT GGC ATG GCC TTC C TTG GAG GCC ATG TAG GCC ATG AG	300	4

Protocol 1 Pre denature at 94°c for 4 min followed by 35 cycles of 94°c for 1 min, 60°c for 1 min, 72°c for 2 min with final extension at 72°c for 4 min

Protocol 2 Pre denature at 94°c for 4 min followed by 32 cycles of 94°c for 1 min, 60°c for 1 min, 72°c for 2 min with final extension at 72°c for 4 min

Protocol 3 Pre denature at 94°c for 4 min followed by 35 cycles of 94°c for 1 min, 58°c for 1 min, 72°c for 2 min with final extension at 72°c for 4 min

Protocol 4 Pre denature at 94°c for 4 min followed by 35 cycles of 94°c for 1 min, 61°c for 1 min, 72°c for 2 min with final extension at 72°c for 4 min

5. Zymography

The presence of MMP-9 in the serum-free supernatant collected from cultures of microglia under tested conditions described above was measured by zymography according to the protocol previously described (Chauvet et al., 2001). The serum free medium from each condition was collected at 24 h and centrifuged at 1,500 g for 15 min to remove cellular debris. The protein content in the supernatant was concentrated approximately 25 folds by centrifugation at 12,000 g in centriprep-10 concentrators. Total Protein concentration in the concentrated media was determined using the Bio-Rad protein determination assay. Samples (120 μg/lane for MMP-9) were mixed with 2x non-reducing sample buffer and incubated for 10 min at room temperature. Protein samples were separated on 10% SDS- polyacrylamide gel electrophoresis containing 1 mg/ml gelatin (Sigma). After eletrophoresis, gels were washed in renaturing buffer for 45 min to remove SDS and restore enzymatic activity. The gels were then incubated overnight at 37°c in developing buffer The gelatinolytic activity, after staining with Coomessie blue, appeared as a clear band against a dark blue background of gelatin gel. The relative molecular weights of the clear bands were

determined using prestained protein markers (Pierce). See appendix B for buffer preparations.

6. Quantitative Analysis of Nitric Oxide

HAPI cells were plated in 24-well plates at the density of $5x10^4$ cells/well and grown in 5% fetal bovine serum (FBS) supplemented Dulbecco's modified Eagle's medium (DMEM). Both DMEM and FBS were purchased from HyClone. When cells reached 80% confluence, the medium was removed and cells were then allowed to grow in serum-free medium for 24 h before the experiments. To begin the treatments, the medium in each well was completely removed, cells in each well were added with freshly prepared serum-free DMEM containing 50 μg/ml ferric ammonium citrate (FAC) (Sigma), or 1 μg/ml lipopolysaccharide (LPS) (Sigma), or 1 or 10 nM 17β-estradiol (Sigma) , or LPS in the presence of FAC, or LPS in the presence 1 or 10 nM 17β-estradiol, or LPS in the presence of both FAC and 1 or 10 nM 17β-estradiol. Cells in serum-free DMEM served as the untreated control 24 h for treatments were indicated in this experiment.

NO_2^- production in the cell supernatants was determined by measuring a stable end-product nitrite according to the protocol previously described (Cheepsunthorn et al., 2001). Each supernatant sample was assayed using Griess reagent (Molecular Probes). Optical density was read at 550 nm using microplate reader. The concentration of NO was determined by a standard curve generated from known concentration of nitrites (0-150 μM). These experiments were performed in triplicate.

7. Statistical analysis

All data were presented as means and SD or standard error of means (mean ± SEM). One way analysis of variance (ANOVA) followed by LSD test was used to compare the significance between treatment. The p-value of less than 0.05 was set for the significant difference.