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Original Article

Maprotiline inhibits LPS-induced expression of adhesion molecules (ICAM-1 and VCAM-1) in human endothelial cells

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Abstract

Regardless of the known anti-inflammatory potential of heterocyclic antidepressants, the mechanisms concerning their modulating effects are not completely known. In our earlier work, maprotiline, a heterocyclic antidepressants, considerably inhibited infiltration of polymorphonuclear cell leucocytes into the inflamed paw. To understand the mechanism involved, we evaluated the effect of vascular cell adhesion molecule (VCAM-1), intracellular adhesion molecule (ICAM-1) expression in stimulated endothelial cells. Endothelial cells were stimulated with lipopolysaccharide (LPS) in the presence and absence of maprotiline (10^{-8} to 10^{-6} M) and ICAM-1 and VCAM-1 expression were measured using real-time quantitative reverse transcription polymerase chain reaction. Maprotiline significantly decreased the LPS-induced expression of VCAM-1 at all applied concentrations. The expression of ICAM-1 decreased in the presence of maprotiline at 10^{-6} M concentration ($P<0.05$). Since maprotiline inhibits the expression of adhesion molecules, ICAM-1 and VCAM-1 in LPS-stimulated human endothelial cells , it can be a possible way through which maprotiline exerts its anti-inflammatory properties.

Keywords: Maprotiline; ICAM-1; VCAM-1; Endothelial cells

INTRODUCTION

Antidepressant drugs are widely used for the treatment of a range of medical disorders, such as depression, anxiety, panic and psychiatric disorders. The main classes of antidepressants include: tricyclic antidepressants, tetracyclic antidepressants, selective serotonin reuptake inhibitors (SSRIs) and serotonin/noradrenergic reuptake inhibitors (SNRIs) and monoamine oxidase inhibitors (1). They are different regarding their pharmacological effects. Besides their action as an inhibitors of serotonin and norepinephrine reuptake, interactions with receptors, like H_1 histamine receptors, muscarinic receptors, and adrenergic receptors, are variable (2).

Several studies have shown that analgesic activity of antidepressant is independent of their effect on mentality. Further, it has been stated that antidepressants exert anti-inflammatory effect both *in vitro* and *in vivo* conditions (3–6).

Many *in vitro* studies based on the anti-inflammatory effect of antidepressants have been performed on peripheral system (lymphocytes and monocytes) and brain (glia cells). Concordance studies have demonstrated the immune modulator effect of antidepressants in lymphocytes and monocytes. Furthermore, they showed that antidepressants inhibit the release of pro-inflammatory cytokines (7). Mechanism of their inhibitory effect on inflammation is not clear yet.

Maprotiline is a tetracyclic antidepressant and used for the treatment of mental depression like other antidepressants (8,9). It has less interference with autonomic system in comparison with tricyclic antidepressants (10,11). Maprotiline exhibits strong effects as a SNRI with only weak actions on the reuptake of serotonin and dopamine (12).

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Few studies till now have shown the anti-inflammatory effect of maprotiline. Even, there is no study *in vitro* to describe its effect on endothelial cells and factors involved in the inflammatory process. So in this study, we have made an attempt to understand the molecular mechanisms of action of maprotiline in inflammation using an *in vitro* model system of endothelial cells induced by LPS.

Endothelial cells play important roles in host responses to LPS during sepsis and inflammation (13). Leukocyte-endothelial interactions play a central role in inflammation. Adhesion is mediated by molecules belonging to different families such as selectins and integrins. Upregulation of integrin in neutrophils causes immobilization and attachment of neutrophils to the surface of the vascular endothelium. Majority of these cell surface integrins bind to cell adhesion molecules (CAMs) on endothelial cell surfaces like intracellular cell-adhesion molecules (ICAMs) and vascular cell-adhesion molecules (VCAMs). Resting endothelial cells have basal expression levels of ICAMs and VCAMs. Upon exposure to cytokines like tumor necrosis factor alpha (TNF-α), LPS, the level of ICAMs and VCAMs expression and cell surface distribution is enhanced which helps in attachment and recruitment of more circulating neutrophils and monocytes.

LPS is an inflammatory mediator who raises vascular permeability, expresses the CAMs and leads to edema formation in many inflammatory models (14). The inflammatory responses evoke the transcriptional activation of several pro-inflammatory genes, which leads to the release of pro-inflammatory cytokines, chemokines, and cell adhesion molecules (15,16).

Polymorphonuclear cell (PMN) adhesion to endothelial cells is the main event in acute inflammation and follows several steps (17). In previous report we showed that maprotiline considerably inhibited infiltration of PMN leucocytes into the inflamed paws (18) but the mechanism is unknown.

In this study we aimed to assess the effect of maprotiline on the expression of CAMs (ICAM-1 and VCAM-1) on the surface of endothelial cells stimulated by LPS.

MATERIALS AND METHODS

Chemicals

Human Umbilical Vein Endothelial Cell (HUVEC) was purchased from Pasteur Institute (Tehran, Iran). Dulbecco's minimal essential medium (DMEM), Roswell Park Memorial Institute medium (RPMI) 1640 phenol red free, Fetal Bovine Serum (FBS), Trypsin-EDTA and [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (MTT) were obtained from Gibco (Life Technology), LPS from *Escherichia coli* 055:B5 and Dimethyl Sulfoxide (DMSO) were obtained from Sigma-Aldrich. Maprotiline hydrochloride, was donated by Iran Daru Pharmaceutical Co. (Tehran, Iran) and was dissolved in phosphate buffer saline (PBS).

Endothelial cell culture

HUVECs were cultured in T-75 flasks in DMEM supplemented with 10% fetal calf serum and 1% antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. At 70–80% confluence, cells were washed with PBS solution pH 7.4, harvested with 0.025% trypsin–0.01% EDTA. For experiments, the cells were seeded in 6-wells and 96-wells plates and incubated for 24 h.

Different amounts of LPS were diluted in PBS and were added to the cells in 6-wells, to choose the suitable concentration of LPS for endothelial cells stimulation.

For the test, cells were treated with various concentrations of maprotiline from 10^{-6} M to 10^{-8} M. One hour later the proper concentration of LPS (1 µg/ml) for stimulation, was added. Cells were treated with LPS alone and control cells were also included. Control cells were incubated in DMEM alone (without LPS or component). Subsequently, cells were used for the assessment of cell viability by MTT assay and for the measurement of the mRNA levels of cell adhesion molecules (VCAM-1 and ICAM-1) by real-time quantitative reverse transcription polymerase chain reaction (real-time qRT-PCR) method.

Cell viability assay

To evaluate if the maprotiline and LPS at mentioned concentrations are toxic to

endothelial cells, we used MTT assay. The ability of the cells to convert MTT shows mitochondrial activity and cell viability (19). In this assay cells were plated in 96 wells plates at a concentration of 1×10^4 cells/ well. Cells were incubated with different concentrations of LPS and various amounts of maprotiline from 10^{-8} M to 10^{-6} M for 24 h at 37 °C. After incubation, the medium was removed and replaced with 100 µl RPMI 1640 phenol red free. Then 10 µl of (12 mM) MTT stoke was added to each well. The cells were incubated for 4 h at 37 °C. Finally the MTT crystals were dissolved by adding 50 µl of DMSO solution and the formation of formazan blue dye was measured using microplate reader (BioTek Instruments, Epoch, USA) at 570 nm.

Real-Time PCR

Real-time qRT-PCR was performed for the detection of the mRNA expressions of VCAM-1 and ICAM-1. Total RNA was isolated by Gene Jet RNA purification kit (Thermo Scientific, (EU) Lithuania) according to the manufacturer's instructions. The concentrations and quality of RNA preparations were determined by a spectrophotometer (BioTek Instruments, Epoch, USA) and gel electrophoresis. Standardized amounts of RNA were reverse-transcribed to cDNA using RevertAid first strand cDNA synthesis kit (Thermo Scientific, (EU) Lithuania) according to manufacturer's protocol.

The primers sequences for VCAM-1 and ICAM-1 and housekeeping gene 18srRNA were designed from the sequence list of GeneBank database (National Centre for biotechnology Information, NCBI) using Beacon designer 8 software and then blasted against GeneBank database sequences. The Primer sequences included for ICAM-1, forward: 5'-ACGGTGCTGGTGAGGAGAG-3' and reverse: 5'-TCGCTGGCAGGACAAAGGT-3'; for VCAM-1, forward: 5'-GCAAGTCTACATATCACCCAAG -3' and reverse: 5'- TCACAGAGCCACCTTCTT -3'.

Real-time PCR was performed using SYBRGreen (Thermo Scientific, (EU) Lithuania) detection in Corbett machine, Rotorgene 6000 (Australia). Master Mix in each reaction tube include, cDNA, H_2O, SYBR Green, forward and reverse primers of genes of interest.

The cycling conditions were as follows: initial denaturation at 95 °C for 3 min and amplification for 45 cycles (95 °C for 12 s for the denaturation, 60 °C for 45 s for the annealing and extension). The relative amount of gene expression, normalized to the internal control 18srRNA. Validation of the reference gene (18srRNA) and the amplification efficiencies of targets and reference were performed (20); the fold-change for each sample was analyzed using the $2^{-\Delta\Delta CT}$ method.

The $2^{-\Delta\Delta CT}$ values obtained from these analyses reflect the relative mRNA quantities for the specific gene in response to a precise treatment. A value higher than 1.0 indicates overexpression of the specific gene in stimulated endothelial cells in comparison with non-stimulated cells. Amplification of specific transcripts was further confirmed by obtaining melting curve profiles. All samples were run in duplicate.

Statistical analysis

The data are expressed as means ± S.E.M for gene expression and cell viability. Statistical analysis of data was performed using one-way ANOVA followed by the Tukey test. Differences were considered as significant for $P<0.05$. Statistical analysis was performed using the SPSS 19 software.

RESULTS

Cell viability

We aimed to determine the cytotoxic effect of maprotiline and LPS on HUVECs. Therefore, cells were treated with increasing concentrations of maprotiline (10^{-8} M to 10^{-6} M) and LPS (1 µg/ml and 10 µg/ml) for 24 h and cell viability was measured using MTT assay. We found that both 1 µg/ml and 10 µg/ml concentrations of LPS decreased the viability of HUVECs close to 20%; however, maprotiline with the concentrations of 10^{-8} M to 10^{-6} M did not change the cell viability in comparison with control cells, although maprotiline with indicated concentrations plus LPS (1 µg/ml) showed some moderate (up to 10%) cell death, and with LPS (10 µg/ml) showed a significant decrease in cell viability (Fig. 1). This result indicated that HUVECs are sensitive to LPS (10 µg/ml) besides maprotiline.

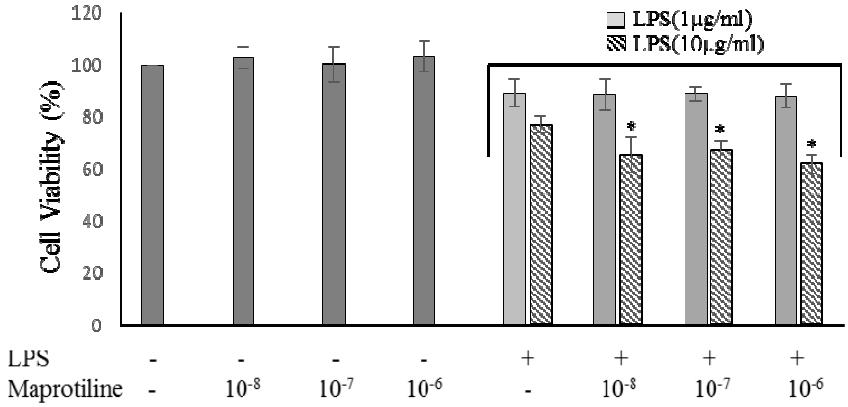

Fig. 1. Determination of cell viability. HUVECs were treated with increasing concentrations of maprotiline (10^{-8} M to 10^{-6} M) alone and besides LPS 1 and 10 µg/ml for 24 h. Mean ± SEM values are provided. **P*<0.05 compared with untreated cells.

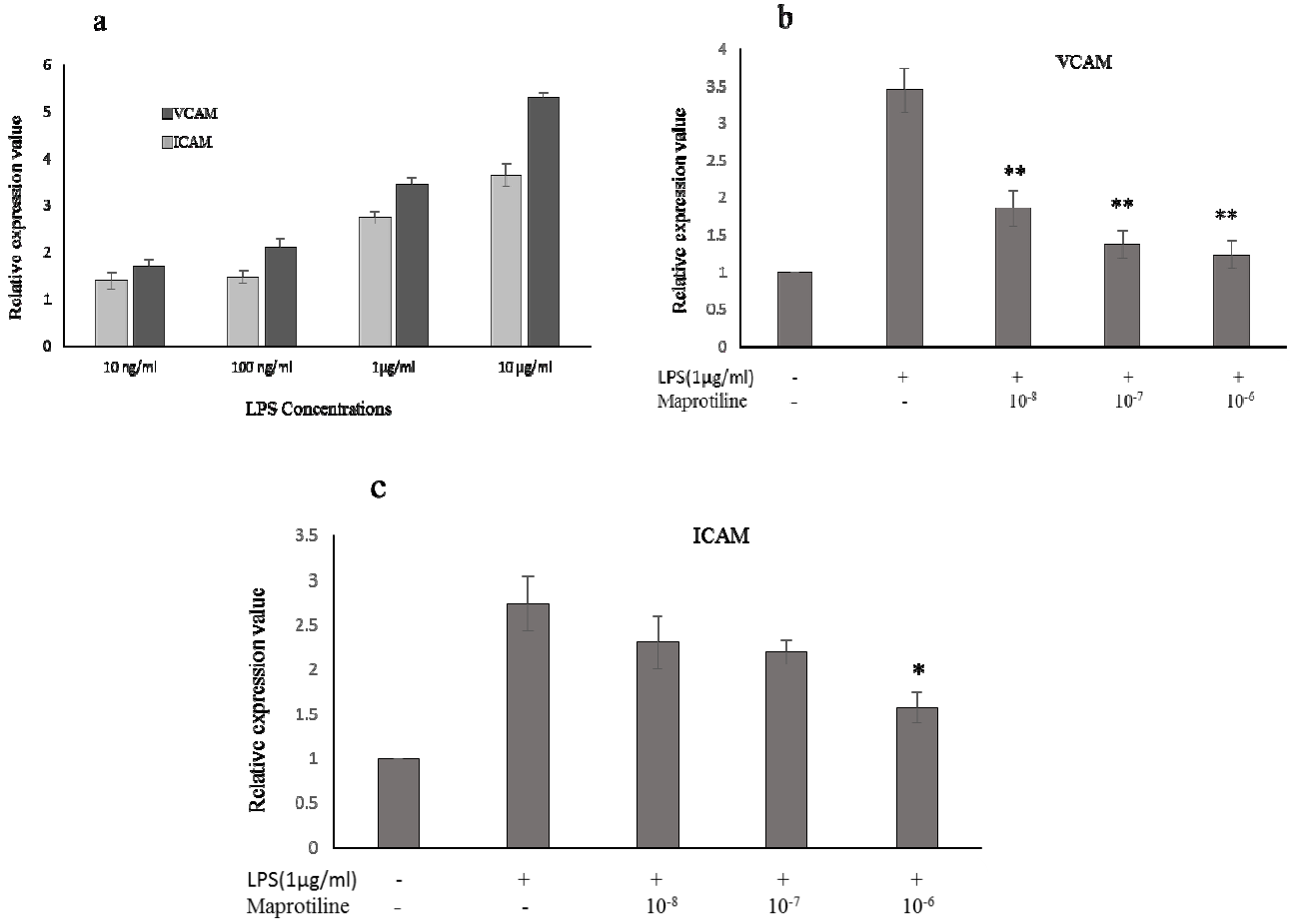

Fig. 2. Effect of maprotiline on LPS-induced endothelial cells expression of ICAM and VCAM. a; Endothelial cells were stimulated with increasing concentrations of LPS for 6 h and the amount of expression was measured by RT-PCR. b and c; Cells pretreated with the indicated amounts of maprotiline for 1 h, and then cells were activated with LPS (1 µg/ml). After 6 h, RNA was purified from cells and transcription of VCAM-1 (b) and ICAM-1 (c) was determined by quantitative RT-PCR. The mRNA expression data normalized to the 18srRNA signal. Fold changes relative to control are presented. Mean ± SEM values of experiments are shown. **P*<0.05, ***P*<0.01 compared with control group.

Inhibition of LPS induced mRNA expression of ICAM-1 and VCAM-1 by maprotiline

We examined the effect of maprotiline on the expression of these two related genes. First, we examined the effect of increasing concentrations of LPS on VCAM-1 and ICAM-1 genes expression, as it is shown in Fig. 2a, LPS enhanced the expression of ICAM-1 and VCAM-1 genes in a concentration dependent manner. LPS (1 μg/ml) increased significantly the basal expression of ICAM-1 and VCAM-1 by 2.7 and 3.4 folds ($P<0.05$), so we chose this concentration for the following tests. Maprotiline decreased the LPS induced endothelial expression of VCAM-1 at a concentration range from 10^{-8} to 10^{-6} M ($P<0.001$) (Fig. 2b). The expression of ICAM decreased significantly in the presence of maprotiline only at concentration 10^{-6} M ($P<0.05$) (Fig. 2c) in LPS induced endothelial cells.

DISCUSSION

In the present study, we determined the effect of maprotiline on the expression of ICAM-1 and VCAM-1 in endothelial cells. For this purpose, we stimulated HUVECs with LPS. To find the optimal concentration for HUVECs stimulation, increasing concentrations of LPS were used and the expressions of ICAM-1 and VCAM-1 were assessed in comparison with non-stimulated cells. In agreement with previous studies, the expression of these genes was depended on LPS concentrations and increased at higher concentrations (21). Because LPS at a concentration of 10 μg/ml significantly reduced the HUVECs viability, we chose the 1 μg/ml concentration for expression test.

One of the primary events in the inflammatory process is an attraction of monocytes toward the endothelial cells (22) that follows multistep process in which leukocytes interact with the endothelium. This process includes in order, capture on, rolling along and adhesion to the micro vascular endothelium. Leukocytes attachment and their migration into the intimal spaces, mediated by adhesive molecules (VCAM and ICAM) are expressed by activated endothelial cells (23,24).

Stating that maprotiline inhibited the expression of ICAM-1 and VCAM-1 which mediated leukocyte- endothelial strong adhesion and subsequently migration across endothelial cells, in this study we demonstrated that maprotiline has an anti-inflammatory effect by inhibiting the expression of adhesion molecules and subsequent adhesion of leukocyte to the endothelial cells.

Our previous investigations confirmed this study and presented that administration of maprotiline as an antidepressant, decreased paw edema four hour after subplantar injection of carrageenan (25). This drug considerably inhibited the infiltration of PMN leukocytes into the carrageenan-treated paws, according to pathological examination and the activity of myeloperoxidase in the inflamed paw tissues. Also, maprotiline inhibited the release of IL-1β and TNF-α (18,26).

Gurgel and colleagues performed a similar study. They investigated the effect of three antidepressant amitriptyline, clomipramine, and maprotiline on neutrophil migration and mast cell degranulation. They suggested an important anti-inflammatory role of heterocyclic antidepressants, which is dependent on the modulation of neutrophil migration and mast cell stabilization (27).

The only study that investigated the effect of antidepressant drugs on adhesion molecules as an inflammatory agent has been achieved by Lekakis and coworkers (28). In accordance with our results they showed that SSRIs (fluvoxamine, fluoxetine and citalopram) decreased the TNF-α-induced endothelial expression of VCAM-1 and ICAM-1.

In another study amitriptyline was tested for the treatment of chronic renal disease using the mouse model of unilateral ureteral obstruction. It was shown in this study that amirtiptiline reduced the ICAM expression in progressive tubulointerstitial injury (29).

The mechanisms governing this anti-inflammatory action of maprotiline on endothelial cells are not clear. Based on inhibitory effect of maprotiline on release of IL-1β and TNF-α and expression of ICAM-1 and VCAM-1, it seems this drug has some effect on NF-κB pathway. NF-κB is an important transcription factor that is activated

by some signals in cells and has a crucial role in the expression of pro-inflammatory cytokines and proteins like TNF-α, IL-1β and adhesion molecules (CAMs) (30,31). LPS causes the nuclear translocation of p65 subunit of NF-κB thorough IκB degradation. Hwang and colleagues showed some antidepressants like clomipramine and imipramine inhibited IκB degradation, nuclear translocation of the p65 subunit of NF-κB, and phosphorylation of p38 mitogen-activated protein kinase in the lipopolysaccharide-stimulated microglia cells (32).

In the present study, maprotiline probably interferes with NF-κB pathway to inhibit ICAM-1 and VCAM-1 expression, and it is proposed for the next step to evaluate the effect of maprotiline on IκB expression and the degradation.

In our opinion, this is the first study that examines the effect of antidepressant maprotiline *in vitro* on endothelial cells, one of the main cells involved in inflammatory process, and on adhesion molecules expression.

Furthermore, we assessed the viability of HUVECs in the presence of maprotiline. The result showed that maprotiline with examined concentration did not change the cell viability. Previous studies on Burkitt lymphoma and prostate cancer cells demonstrated that maprotiline modulates the cell proliferation and reduces cell viability (33,34), in the current study perhaps because of low concentration of maprotiline, it could not reduce the viability of HUVECs.

Some mechanistic aspects concerning the anti-inflammatory activity of antidepressants at the molecular level have yet to be elucidated. One of this aspect include how leukocyte trafficking is affected both in acute and in chronic inflammation.

CONCLUSION

In conclusion, since maprotiline inhibits the expression of adhesion molecules, ICAM-1 and VCAM-1 in human endothelial cells stimulated with an inflammatory mediator and because these two inflammatory endothelial markers have an effect on leukocyte migration and inflammation process, this can be one of the ways through which maprotiline exerts its anti-inflammatory properties.

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