



Effect of the Lipid Peroxidation Product 4-Hydroxynonenal on Neuroinflammation in Microglial Cells: Protective Role of Quercetin and Monochloropivaloylquercetin

Mikroglial Hücrelerde Lipid Peroksidasyon Ürünü 4-Hidroksinonenalin Nöroinflamasyon Üzerine Etkisi: Kersetin ve Monokloropivaloilkersetinin Koruyucu Rolü

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ABSTRACT

Objectives: The lipid peroxidation-derived aldehyde 4-hydroxynonenal (HNE) has been implicated in a number of oxidative stress-induced inflammatory pathologies such as neurodegenerative diseases and aging. In this regard, we investigated the effects of HNE on neuroinflammatory responses by measuring cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) induction with cytokine production. In addition, we measured nuclear factor erythroid 2-related factor 2 (Nrf-2)/Kelch-like ECH-associated protein 1 (Keap1) signaling proteins, and antioxidant enzymes heme oxygenase-1 (HO-1) and nicotinamide adenine dinucleotide phosphate dehydrogenase, quinone 1 (NQO1), and compared the results with quercetin and monochloropivaloylquercetin (MPQ) pretreated microglial cells.

Materials and Methods: Cytotoxicity was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and production of cytokines was determined by cytokine array. Furthermore, intracellular Nrf2/Keap1 signaling proteins, HO-1, NQO1, and COX-2 expression were analyzed by western blot in 2.5 µM HNE treated BV-2 cells.

Results: Inducible nitric oxide synthase (iNOS) and COX-2 mRNA levels were measured with reverse transcription-quantitative polymerase chain reaction. HNE induced both COX-2 mRNA and protein levels, iNOS mRNA expression, and cytokine production. In addition, HNE markedly increased Keap1 levels and decreased cytoplasmic Nrf-2 expression with antioxidant enzyme HO-1 levels. Quercetin and monochloropivaloylquercetin treatment alleviated neuroinflammatory responses in microglial cells, by decreasing COX-2 mRNA expression. Monochloropivaloylquercetin decreased cytoplasmic Keap1 levels and increased nuclear translocation of Nrf-2 resulted in induction of HO-1 and NQO1 expression.

Conclusion: These results suggest that HNE could be a link between oxidative stress and inflammation in BV-2 microglia cells. In particular, monochloropivaloylquercetin alleviated inflammation, probably by decreasing the expression of proinflammatory genes and strengthening the antioxidant defense system.

Key words: 4-Hydroxynonenal, microglia, quercetin, inflammation, cyclooxygenase-2, hemeoxygenase-1

ÖZ

Amaç: Lipit peroksidasyonu ürünü aldehit, 4-hidroksinonenal (HNE), nörodejeneratif hastalıklar ve yaşlanma gibi oksidatif strese bağlı inflamatuvar patolojilerle ilişkilendirilmiştir. Bu bağlamda, HNE'nin nöroinflamatuvar cevap üzerine etkisini siklooksijenaz-2 (COX-2), indüklenebilir nitrik oksit sentaz (iNOS) ifadenmeleri ve sitokin üretimi üzerinden incelenmiştir. Bunlara ek olarak, nükleer faktör eritroid 2-ilişkili faktör 2 (Nrf2)/Kelch-benzeri ECH-ilişkili protein 1 (Keap1) sinyal proteinleri, antioksidan enzimler hemoksijenaz-1 (HO-1) ve nikotinamid adenin dinükleotit fosfat dehidrojenaz, kinon 1 (NQO1) ifadenme düzeyleri ölçüldü ve sonuçlar kersetin ve monokloropivaloilkersetin uygulanan hücrelerle karşılaştırılmıştır.

Gereç ve Yöntemler: Sitotoksikite MTT (3-(4,5-dimetiltiyazol-2-yl)-2,5-difeniltetrazolyum bromit) indirgenme yöntemi ile ölçüldü ve sitokin üretimi ise sitokin array ile belirlendi. Bununla birlikte, 2,5 µM HNE uygulanmış BV-2 hücrelerinde hücre içi Nrf2/Keap1 sinyal proteinleri, HO-1, NQO1

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ve COX-2 ifadeleri Western blot ile analiz edildi. iNOS ve COX-2 mRNA düzeyleri ters transkripsiyon-kantitatif polimeraz zincir reaksiyonu ile ölçülmüştür.

Bulgular: HNE mikroglial hücrelerde COX-2 mRNA ve protein düzeylerini, iNOS mRNA düzeylerini ve bunlarla birlikte sitokin üretimini artırdı. Buna ek olarak, HNE uygulaması Keap1 düzeyinde belirgin bir artışla birlikte, sitoplazmik Nrf2 ifadeleri ve antioksidan enzim HO-1 düzeylerini azaltmıştır. Kersetin ve monokloropivaloilkersetin ile tedavi COX-2 mRNA ifadenme düzeylerini azaltarak nöroinflamasyonu hafifletmiştir. Monokloropivaloilkersetin sitoplazmik Keap1 seviyelerini azaltırken Nrf2'nin nükleer translokasyonunu artırmıştır, bu durum HO-1 ve NQO1 ifadenmelerini uyarımı ile sonuçlanmıştır.

Sonuç: Bu sonuçlar BV-2 mikroglial hücrelerde HNE'nin oksitativ stres ve inflamasyon arasında bağlayıcı olabileceğini göstermektedir. Özellikle monokloropivaloilkersetin, proinflamatuvar genlerin ifadenme düzeylerini azaltıp, antioksidan savunma sistemini güçlendirerek inflamasyonu hafifletmiştir.

Anahtar kelimeler: 4-Hidroksinonenal, mikroglia, kersetin, inflamasyon, siklooksijenaz-2, hemoksijenaz-1

INTRODUCTION

Lipid peroxidation causes generation of highly reactive aldehydes such as acrolein, malondialdehyde, and hydroxynonenal (HNE). This lipid by-product is capable of modifying nucleophilic side chains on amino acid residues (Cys, His, Arg, Lys) primarily through 1,4-Michael-type conjugate reactions.¹ The formation of these adducts (mostly irreversible) can lead to multiple deleterious events such as inhibition of DNA, RNA, and protein synthesis; and disruption of protein and cell membrane functions.² Oxidative stress and associated membrane lipid peroxidation are involved in the pathogenesis of ageing³ and neurodegenerative diseases,⁴ which include Alzheimer disease (AD) and others.⁵ Previous studies reported an important role of HNE in the development of AD.^{6,7} Thus, significant increases in free HNE in the cerebrospinal fluid,⁸ amygdala, hippocampus, and parahippocampal gyrus⁹ were detected in the brains of AD patients compared to control subjects. The central nervous system (CNS) is sensitive to oxidative stress because of the high levels of polyunsaturated lipids in neuronal cell membranes and poor antioxidant defense.¹⁰ HNE-mediated oxidative stress may indirectly contribute to brain damage by activating a number of cellular pathways, resulting in the expression of stress-sensitive genes and proteins to cause oxidative damage. Moreover, oxidative stress also may activate glia cell-mediated inflammation, which also causes secondary neuronal damage. Microglia are unique resident immune cells of the CNS acting as primary mediators of inflammation.¹¹ Under physiological conditions, microglia have small cell bodies (surveillance mode) and have low levels of reactive free radicals. When hazardous signals excite microglial cells, cells pass to the pro-inflammatory phase. The characteristics of the inflammatory phase (M1 stage) are induction of stress-sensitive genes in NFκB signaling and proinflammatory cytokines (IL-6, TNF-α, and IF-γ). The pro-inflammatory polarization of microglia is often followed by a long-lasting repair stage (M2 stage) The M2 program is activated by anti-inflammatory cytokines such as IL-4, IL-13, and IL-10.¹² Finally, activated glial cells are thus histopathological hallmarks of neurodegenerative diseases. When activated by proinflammatory stimuli, microglia release substantial levels of cytokines, chemokines, and other neurotoxins and mounting evidence suggests this contributes to neuronal damage during neuroinflammation.¹³

Nuclear factor erythroid 2-related factor 2 Nrf-2 (NF-E2-related factor-2) transcription factor regulates oxidative

stress response and represses inflammation. Nrf-2 deficiency causes an exacerbation of inflammation and an inducer of Nrf-2 such as dimethyl fumarate has been approved for the treatment of multiple sclerosis¹⁴ in part based on its anti-inflammatory function. In physiological condition, Nrf-2 is sequestered by Keap1 (kelch-like ECH-associated protein 1). Keap1 is a key regulator of the Nrf-2 signaling pathway and serves as a molecular switch to turn on and off the Nrf-2/Keap1-ARE pathway. When oxidative modification of one of the Keap1 occurs, Nrf-2 escapes from this proteolytic pathway, then translocates to the nucleus, and binds to the antioxidant response element (ARE). Nrf-2-mediated ARE activation leads to the expression of cytoprotective enzymes, such as NAD(P)H: quinone oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO-1).^{15,16} Cyclooxygenase-2 (COX-2) is mainly expressed in activated macrophages and other inflammatory cells and is upregulated after exposure to inflammatory stimuli, but the use of COX-2 inhibitors is a successful approach to counter the damaging effects of inflammation.¹⁷

Flavonoids comprise a large group of compounds occurring widely throughout the plant kingdom and exert several biological activities, which are mainly related to their antioxidant properties,¹⁸ and they are able to regulate immune responses.¹⁹ Quercetin is the most common flavonoid in nature and fruits and vegetables, especially berries and onions, are the primary sources of naturally occurring dietary quercetin derivatives. Quercetin bioavailability is generally poor and characterized by high intersubject variability.²⁰ To find new analogues with increased bioavailability and enhanced pharmacological activity, researchers have attempted the chemical modification of quercetin.

Monochloropivaloylquercetin was prepared by Bel/Novamann International s.r.o. according to Veverka et al.²¹ Its antioxidant potential and enzyme inhibitory activity (aldose reductase, glycosidase, and sarcoplasmic/endoplasmic reticulum calcium ATPases 1) were shown by Žižková et al.²² In addition, chloropivaloylquercetin has an anti-inflammatory effect on lipopolysaccharide induced neuroinflammation by downregulating NFκB activity.²³

In the present study, we aimed to investigate the influence of HNE-mediated oxidative stress on the induction of inflammatory response in BV-2 (mouse microglia cells) and present some emerging therapeutic options for antioxidant/anti-inflammatory therapy, together with the therapeutic potential of quercetin and monochloropivaloylquercetin.

MATERIALS AND METHODS

Cell culture

BV-2 mouse microglial cells were kindly donated by Lucia Rackova PhD (Slovak Academy of Sciences, Slovakia). The cells were cultured in either 75 cm² flasks or 6-well dishes containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, and 10 µg/mL streptomycin and grown in a 5% CO₂ atmosphere at 37°C.

Determination of cytotoxicity (MTT assay)

The cytotoxic effect of HNE was evaluated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) reduction assay. BV-2 cells were treated with 0-50 µM of HNE without phenol red 1% FBS supplemented DMEM for 6 h and then rinsed three times with ice-cold phosphate-buffered saline (PBS). MTT was added to the final concentration of 0.5 mg/mL. After 4 h of MTT incubation, solubilization buffer (10% sodium dodecyl sulfate in 0.01 mol/L HCl) was added and the colored formazan crystals were gently resuspended. The absorbance at 570 nm was recorded with a microplate reader (Bio-Tek ELX800, BioTek Instruments Inc., Winooski, VT, USA).

Western blot analysis of antioxidant proteins (Nrf-2/Keap-1, HO-1, and NQO1)

Cells were pretreated with N-acetyl-L-cysteine (Sigma-Aldrich, St. Louis, MO, USA), quercetin (Sigma-Aldrich), and monochloropivaloylquercetin (indicated concentrations for 3 h) and treated with HNE (2.5 µmol/L, 6 h). After the treatment procedure the cells were washed in ice cold PBS three times and lysed in radio immunoprecipitation assay buffer supplemented with 2 mM Na₃VO₄ and protease inhibitor cocktail (Complete Mini™, Roche, Mannheim, Germany) at 4°C. The lysate was clarified by centrifugation at 10,000 rpm for 10 min at 4°C to remove insoluble components. We used Ne-PER (Pierce, Rockford, IL, USA) nuclear and cytoplasmic extraction reagents to prepare fractions according to the manufacturer's instructions. Cell lysates were normalized for protein content using bicinchoninic acid assay reagent (Pierce). Equal amounts (30 µg) of protein were loaded onto 10% PAGE gels and separated by standard SDS-PAGE procedure. Proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA) and blocked with 5% nonfat dry milk in TBST. To detect protein expression, the blots were probed with the specific antibodies against HO-1, Keap1, Nrf-2 (Bioss, Woburn, MA, USA), COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and NQO1 (Abcam, Cambridge, MA, USA) followed by the secondary antibodies coupled to horseradish peroxidase. The detection of GAPDH (Cell Signaling Technology Inc., Beverly, MA, USA) with a specific antibody was used as an internal control. The immunoreactive proteins on the membrane were detected by chemiluminescence using the SuperSignal® West Pico (Pierce) on X-ray film.

Determination of COX-2 and inducible nitric oxide synthase mRNA levels with quantitative real-time polymerase chain reaction

Total RNA isolation from cells was performed via phenol-guanidine thiocyanate extraction using RNAzol isolation

reagent (Sigma-Aldrich), according to the manufacturer's instructions. Total RNA (1 µg) was reverse-transcribed to cDNA using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche) in a 20 µL reaction mixture. Real-time polymerase chain reaction (PCR) was carried out using a LightCycler Nano System (Roche). To quantify cDNA, qPCR was performed using FastStart Essential DNA probe master mix (Roche). The reaction mixture (15 µL) was prepared in LightCycler 8-tube strips (Roche) and included 10 µL of 2' Master Reaction Mix (Roche), 4 µL of PCR grade water, 1 µL of catalogue assay kit (kits consist mix of primers and probes for determination of iNOS, COX-2, β-actin), and 5 µL of cDNA. Real-time PCR was performed according to the following conditions: activation of Taq DNA polymerase and DNA denaturation at 95°C for 10 min, followed by 45 amplification cycles for 10 s at 95°C and for 30 s at 60°C. For each sample the level of target gene transcripts was normalized to β-actin.

Cytokine profiling of BV-2 cells by cytokine array

BV-2 cells (1×10⁶) were treated with HNE (2.5 µmol/L, 6 h) and the released cytokines were determined semiquantitatively by mouse cytokine antibody array (Abcam) according to the manufacturer's protocol. The density of the cytokine spots was analyzed by using the ImageJ densitometric analysis program. We used the intensity of positive control spots for normalization of array results.

Statistical analysis

Possible associations between groups were analyzed with SigmaPlot 12 statistical software using the t test. P values <0.05 were considered statistically significant. Fold increases or decreases in mRNA levels were also calculated by relative expression software tool developed for group-wise comparison and statistical analysis of relative expression results.

RESULTS

HNE induces microglial cell death

Firstly, we investigated the cytotoxic activity of HNE in BV-2 cells. As shown in Figure 1, HNE dose-dependently triggered cell death. After 6 h of incubation, HNE significantly reduced

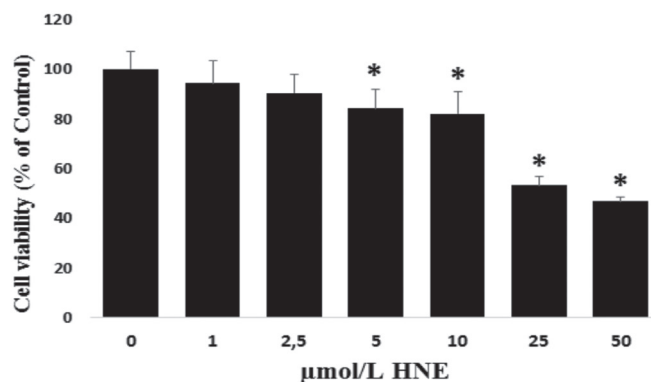


Figure 1. Effect of HNE on cell viability assessed by MTT reduction assay in BV-2 cell line

n=4, *p<0.05 vs. control (0)

HNE: 4-hydroxynonenal

the viability of cultured microglial cells at the conc. >2.5 μmol/L, whereas 2.5 μmol/L or lower concentrations of HNE decreased cultured cell viability, but the differences were not statistically significant. The nontoxic highest concentration of HNE was used for induction of neuroinflammation. Quercetin and monochloropivaloylquercetin were toxic over 10 μM (data not shown). Thus, for all the further studies these nontoxic doses of quercetin and monochloropivaloylquercetin were used, ranging from 2.5 to 5.0 μM.

Modulation of the cytokine secretion in BV-2 cells in response to HNE

We then examined the inflammatory effect of HNE on cytokine production in BV-2 cells. A mouse cytokine antibody array was applied to broadly observe the effects of HNE on cytokine secretion. After the cells were incubated with HNE for 6 h, the cytokine expression pattern in the treated cells was differentially compared to that in the control cells (Figure 2). According to the array results, under basal conditions (in control cells), IL-5, IL-17, SCF, and VEGF protein expressions were at undetectable levels. After HNE treatment, cytokine protein expressions of granulocyte colony stimulating factor, granulocyte/macrophage colony stimulating factor, IL-2, IL-3, IL-4, IL-5, IL-9, IL-10, IL-13, IL-17, IF-γ, SCF, p60/p70, VEGF, and TNF-α all were increased. Expressions of IL-6, RANTES, MCP-1, and sTNHR1 were slightly increased in HNE-treated cells, compared to those

of the untreated group. IL-12p70, MCP-5, and thrombopoietin expression patterns did not change in HNE-treated cells, when compared with those of the untreated group.

Changes in proinflammatory gene expression in response to oxidative stress: effects of quercetin and monochloropivaloylquercetin pretreatment

After BV-2 cells were exposed to HNE for 0, 1, 3, 6, and 12 h, we performed mRNA and protein expression analysis of the COX-2 and mRNA expression of iNOS. Real-time PCR analysis confirmed that there was a time-dependent increase in the expressions of COX-2 and inducible nitric oxide synthase (iNOS) in the HNE-treated BV-2 cells compared with the control (Figure 3). In parallel to these results, HNE exposure increased COX-2 protein expression. Although pretreatment with quercetin and monochloropivaloylquercetin did not affect iNOS mRNA expression in HNE-treated BV-2 cells, monochloropivaloylquercetin pretreatment significantly decreased COX-2 mRNA expression (Figure 4).

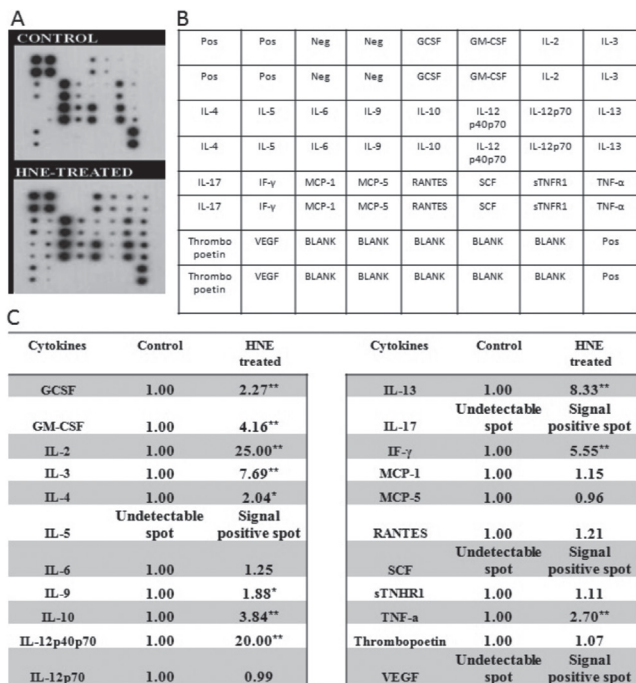


Figure 2. Effect of HNE treatment on cytokine production in BV-2 cells. (A) Image of cytokine array. (B) Gene map of cytokine array. All spots are in duplicate. (C) the quantification by image of cytokine secretion in nontreated control cells and HNE-treated BV-2 cells. Fold changes were given in the table after average spot signals of control cells were assumed as 1.00 *p<0.05, **p<0.01

Pos: Positive controls, Neg: Negative controls, HNE: 4-hydroxynonenal, GCSF: Granulocyte colony stimulating factor, GM-CSF: Granulocyte/macrophage colony stimulating factor

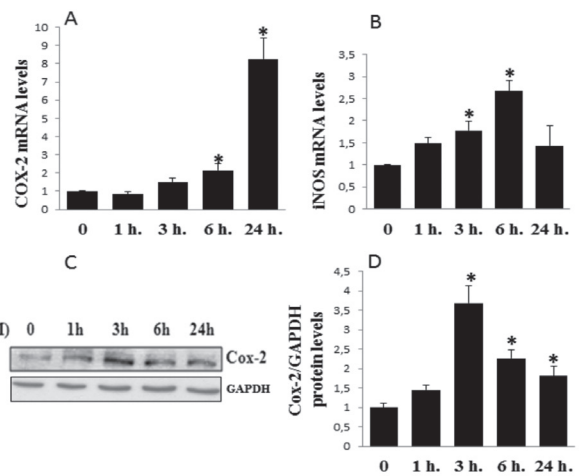


Figure 3. Time-dependent changes in COX-2 mRNA expression (A), iNOS mRNA expression (B), and COX-2 protein expression (C, D) in HNE-treated BV-2 cells

n=3, *p<0.05 vs. control (0)

COX-2: Cyclooxygenase-2, HNE: 4-hydroxynonenal, iNOS: Inducible nitric oxide synthase, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

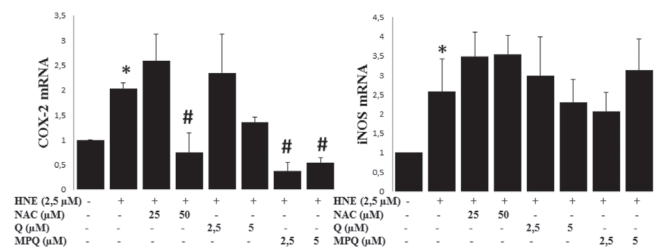


Figure 4. Effects of quercetin and monochloropivaloylquercetin on COX-2 mRNA expression and iNOS expression in HNE-treated BV-2 cells

n=3, *p<0.05 vs. control (0), #p<0.05 vs. HNE

COX-2: Cyclooxygenase-2, HNE: 4-hydroxynonenal, NAC: N-acetyl-L-cysteine, Q: Quercetin, MPQ: Monochloropivaloylquercetin, iNOS: Inducible nitric oxide synthase

Quercetin and monochloropivaloylquercetin induce HO-1 and NQO1 expression by inducing nuclear translocation of Nrf-2 in HNE-treated BV-2 cells

It is known that the redox-sensitive transcription factor Nrf-2 plays an important role in cellular defense against oxidative stress by inducing the expression of phase II genes (Figure 5). Western blot analysis was performed to determine the cytoplasmic expression and nuclear translocation of Nrf-2 in response to 2.5 $\mu\text{mol/L}$ HNE, quercetin, and monochloropivaloylquercetin. Although HNE did not cause any nuclear translocation of Nrf-2 at 6 h, it increased the cytoplasmic expression of Keap1 and decreased the cytoplasmic expression of Nrf-2. In addition, HNE treatment caused a slight alteration in HO-1 and NQO1 expression but the difference was not statistically significant (Figure 6). Pretreatment with quercetin and

monochloropivaloylquercetin markedly decreased cytoplasmic Keap1 levels with increased cytoplasmic Nrf-2 levels. At the same time, monochloropivaloylquercetin pretreatment induced expression of the antioxidant proteins HO-1 and NQO1, while quercetin pretreatment induced NQO1 expression with both molecules increased nuclear translocation of Nrf-2.

DISCUSSION

The aim of this study was firstly to test the effect of mild-type oxidative/electrophilic stress on neuro-inflammatory response in microglial cells induced by HNE, and secondly to investigate the protective ability of quercetin and monochloropivaloylquercetin. We found that HNE induced inflammatory response by increasing both COX-2 mRNA and protein expression as well as iNOS mRNA levels in a time-dependent manner and augmenting cytokine production. Quercetin and monochloropivaloylquercetin exerted a significant antioxidant effect by strengthening the antioxidant defense system via induced nuclear translocation of Nrf-2 and decreased Keap1 expression in addition to increased NQO1. Additionally, monochloropivaloylquercetin induced HO-1 expression and behaved as an anti-neuroinflammatory agent by decreasing COX-2 expression against HNE-induced inflammatory in BV-2 cells.

HNE is thought to contribute to the dysfunction and death of neurons in the pathogenesis of neurodegenerative diseases. Oxidative stress also activates mechanisms that result in glia cell-mediated inflammation, also causing secondary neuronal damage. Several studies have shown that when the oxidative stress is induced by several agents, brain cells like microglia and astrocytes release diverse inflammatory mediators.^{24,25} Moreover, oxidative stress indicators [reactive oxygen species (ROS), reactive lipid peroxidation products] act as critical signaling molecules to trigger inflammatory responses in the CNS through activation of redox sensitive transcription factors.^{26,27} The ability of HNE to exert a number of toxicological effects has been attributed to its electrophilic α , β -unsaturated carbonyl moiety that can react through 1, 2 and 1, 4 additions with nucleophiles such as cysteine, lysine, and histidine residues.^{28,29} The normal, physiological level of HNE in human tissues and plasma range from 0.07 to 2.8 μM , while in diseased states and near the core of lipid peroxidation sites, its concentration can be greatly increased (even above 100 μM).³⁰ Treatment with low (1 and 10 μM) concentrations of HNE caused significant induction of cell death.^{31,32} As expected, in our study, starting with the concentration of 5 $\mu\text{mol/L}$ HNE, a significant drop in BV-2 cell viability was assessed by MTT ($84.19 \pm 7.61\%$ of control) after 6 h of treatment. Previous studies reported that HNE showed toxic effects in neuron cultures and therefore our data are in agreement with those studies.³³⁻³⁵

The goal of the present study was to identify whether HNE is a possible intracellular mediator between oxidative stress and inflammation in microglia cells. Our results reveal that oxidative stress is an iNOS and COX-2 activator in BV-2 microglia cells. Indeed, COX-2 expression was significantly increased and similarly release of proinflammatory cytokines

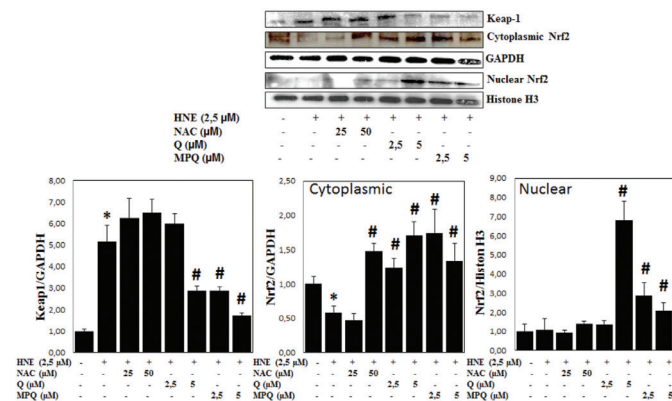


Figure 5. Effects of quercetin and monochloropivaloylquercetin on cytoplasmic Nrf-2/Keap1 protein expression and nuclear Nrf-2 translocation in HNE-treated BV-2 cells

n=3, *p<0.05 vs. control (0), #p<0.05 vs. HNE

HNE: 4-hydroxynonenal, NAC: N-acetyl-L-cysteine, Q: Quercetin, MPQ: Monochloropivaloylquercetin, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

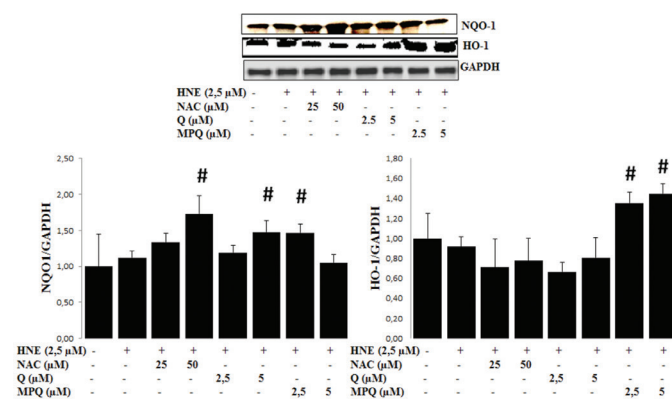


Figure 6. Effects of quercetin and monochloropivaloylquercetin on phase II protein NQO1 expression and antioxidant HO-1 protein expression in HNE-treated BV-2 cells

n=3, *p<0.05 vs. Control (0), #p<0.05 vs. HNE

COX-2: Cyclooxygenase-2, HNE: 4-hydroxynonenal, NAC: N-acetyl-L-cysteine, Q: Quercetin, MPQ: Monochloropivaloylquercetin, GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

was significantly raised in response to HNE-mediated oxidative stress. Previous studies have reported that 4-HNE is an inducer of COX-2 expression in several types of cells including 3T3-L1 adipocytes,³⁶ epithelial RL34 cells,³⁷ and macrophages.³⁸ Our data extend these results to BV-2 microglia cells. Given that lipid peroxidation reactions are an important source of advanced glycation end products (AGEs),³⁹ the production of lipid peroxidation-derived aldehyde fragments such as HNE may indicate a mechanism by which AGE epitopes are generated. Other than HNE, advanced lipid peroxidation end products induced proinflammatory response by activating proinflammatory gene expression in monocytes.⁴⁰ In addition, activation of the receptor for advanced glycation end product mediated pathway on mononuclear phagocytes has been reported to give rise to this phenotype of activated macrophages that is manifested by the induction of some proinflammatory cytokines (IL-1 β and TNF- α), platelet-derived growth factor, and insulin-like growth factor-1. Moreover, it was emphasized in these studies that HNE is a possible link between oxidative stress and inflammation. Our results are also in agreement with those published by Kauppinen et al.⁴¹ who reported that 24-h HNE treatment induced inflammasome signaling by increased inflammasome component NRLP3 (NLR family, pyrin domain containing 3) mRNA levels in retinal pigment epithelial cells (ARPE-19), raising the IL-1 β and IL-18 release. Additionally, the inflammatory role of HNE was reported by Chen et al.⁴², who stated that single treatment with 10 μ M HNE induced proinflammatory prostaglandin E₂ (PGE₂) release as well as COX-2 and microsomal PGE₂ synthase-1 (mPGES-1) expression in osteoarthritic chondrocytes. Another key piece of evidence for the inflammatory effect of HNE was that HNE may contribute to osteoarthritis development via its ability to alter cellular phenotype and metabolic activity of osteoblasts by modulating inflammatory processes. HNE induced PGE₂ release and COX-2 expression and COX-2 promoter activity with activated MAPK signaling pathway in osteoblasts.⁴³ Although quercetin and its derivatives have been shown to have anti-inflammatory and antioxidant effects in several *in vivo* and *in vitro* studies, the effect of a new quercetin derivative monochloropivaloylquercetin on the HNE-stimulated activation of inflammatory COX-2 expression and Nrf-2/HO-1 and NQO1 antioxidant signaling pathway, which may be involved in the neuroinflammation and immune response, has not been studied before. Nrf-2 is sequestered in the cytoplasm with its cytosolic repressor Keap1. The dissociation of Nrf-2 from Keap1 is crucial for its nuclear translocation, followed by binding to the ARE.⁴⁴ The innate immune response involves generation of ROS, which can act as second messengers activating proinflammatory signaling pathways such as NF κ B.⁴⁵ Accordingly, inhibition of NF κ B and herewith activation of the Nrf-2 signaling pathway may be a beneficial strategy for reduction of the deleterious effects of inflammation and it is well known that anti-inflammatory agents suppress NF κ B signaling while activating the Nrf-2/ARE pathway.⁴⁶⁻⁴⁸ Activation of the Nrf-2 mediated signaling pathway results in upregulation of HO-1 expression. HO-1 is known to play an anti-inflammatory role due to carbon monoxide

production and NF κ B inhibition.⁴⁹ In our study, pretreatment with quercetin and monochloropivaloylquercetin activated Nrf-2/Keap1 signaling by decreasing cytoplasmic expression and inducing nuclear translocation of Nrf-2, resulting in upregulation of downstream genes HO-1 and NQO1 in HNE-treated BV-2 cells. Additionally, COX-2 mRNA stimulation by 4-HNE was inhibited when BV-2 cells were preincubated with quercetin and monochloropivaloylquercetin. In this regard, there remains the possibility that induction of Nrf-2 target genes contributes to both quercetin and monochloropivaloylquercetin's efficacy to inhibit COX-2 expression. Ramyaa et al.⁵⁰ have already reported the modulatory effect of quercetin on oxidative stress by upregulating Nrf-2 expression and downregulating NF κ B and COX-2 expression. The anti-inflammatory properties of quercetin accompanied by an increase in HO-1 protein levels associated with elevated nuclear translocation of Nrf-2 were previously exhibited by Boesch-Saadatmandi et al.⁵¹ in murine RAW 264.7 macrophages. On the other hand, quercetin acts as an anti-inflammatory agent in lipopolysaccharide-induced acute lung injury by significantly reducing COX-2 and iNOS expression, and NF κ B p65 phosphorylation.⁵² In another study, intratracheal administration of quercetin affected the protective agent by modulating HO-1 activity against lipopolysaccharide-induced acute lung injury.⁵³ Quercetin was previously shown to downregulate proinflammatory responses, involving iNOS expression and NO generation in BV-2 cells through inducing Nrf-2 mediated HO-1 expression.⁵⁴ In contrast, quercetin pretreatment did not affect iNOS mRNA expression in our study; this may be associated with the dose of quercetin or incubation time.

CONCLUSIONS

Our data provide new evidence for the inflammatory role of HNE, an oxidative stress-related product, in the physiopathology of neuro-inflammation and we propose that the activation of the Nrf-2/HO-1 pathway by quercetin and monochloropivaloylquercetin may mediate its anti-inflammatory properties.

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