

Introduction

Lipid peroxidation causes a generation in 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 reactions¹. The formation of these adducts (mostly irreversible) can lead to multiple deleterious events such as: inhibition of DNA, RNA, and protein synthesis; disruption of protein and cell membrane functions². Oxidative stress and associated membrane lipid peroxidation are involved in the pathogenesis of ageing³ and neurodegenerative diseases⁴, which include Alzheimer disease (AD) and others⁵. Previous studies supported the important role of HNE in development of AD^{6,7}. Thus, significant increase of free HNE in cerebrospinal fluid⁸, amygdala, hippocampus and parahippocampal gyrus⁹ were detected in brain of AD patients compared to control subjects. Central nervous system (CNS) is sensitive to oxidative stress because of the high levels of polyunsaturated lipids in neuronal cell membranes and poor antioxidant defense¹⁰. 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 the unique resident immune cells of the CNS acting as primary mediators of inflammation¹¹. Under physiological conditions, microglia display as small cell bodies (surveillance mode) and consist low levels of reactive free radicals. When hazardous signals excited microglial cells, cells pass to pro-inflammatory phase. The characteristics of inflammatory phase (M1 stage) are induction of stress-sensitive genes in NFκB signalling 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-10¹². 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 neuroinflammation¹³.

Nrf2 (NF-E2-related factor-2) transcription factor regulates oxidative stress response and also represses inflammation. Nrf2-deficiency causes an exacerbation of

inflammation and inducer of Nrf2 such as dimethyl fumarate has been approved for the treatment of multiple sclerosis ¹⁴ in part based on its anti-inflammatory function. In physiological condition, Nrf2 is sequestered by Keap1 (kelch like ECH associated protein 1). Keap1 is a key regulator of the Nrf2 signalling pathway and serves as a molecular switch to turn on and off the Nrf2/Keap1-ARE pathway. When oxidative modification of one of the Keap1 occurs, Nrf2 escapes from this proteolytic pathway, then translocates to the nucleus and binds to antioxidant response element (ARE). Nrf2 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 up-regulated after exposure to inflammatory stimuli, but the use of COX-2 inhibitors is a successful approach to dispose 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 properties¹⁸, and are able to regulate the immune responses¹⁹. Quercetin is the most common flavonoid in nature and fruits and vegetables, berries and onions, are the primary sources of naturally-occurring dietary quercetin derivatives. Quercetin bioavailability, which is generally poor and characterized by high intersubject variability²⁰. To find new analogs 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. (SR) according to Veverka et al. ²¹ Its antioxidant potential and enzyme inhibitory activity (aldose reductase, glucozidase and sarcoplasmic/endoplasmic reticulum calcium ATPases 1) were shown by Zizkova et al. ²² In addition to these, chloropivaloylquercetin has anti-inflammatory effect on lipopolysaccharide induced neuroinflammation by downregulating NFkB 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 cell) 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). Cells were cultured in either 75 cm² flasks and 6-well dishes containing Dulbecco's modified Eagle medium (DMEM) medium supplemented with 10% FBS, 2mM glutamine, 100 U/ml penicillin, 10 µg/ml streptomycin and grown in a 5% CO₂ atmosphere at 37°C.

Determination of cytotoxicity (MTT assay)

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 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).

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

Cells pretreated with N-acetyl-L-cysteine (NAC) (Sigma-Aldrich, St. Louis, MO), Quercetin (Sigma-Aldrich, St. Louis, MO) and monochloropivaloylquercetin (indicated concentrations for 3h) and treated with HNE (2,5 µmol/L, 6h). After treatment procedure cells were washed in ice cold PBS three times and lysed in RIPA 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 for prepare fractions according to the manufacturers instructions. Cell lysates were normalized for protein content using BCA reagent (Pierce, Rockford, IL, USA). Equal amounts (30µg) of protein were loaded onto 10 % PAGE gels and separated by standard SDS-PAGE procedure. Proteins were transferred to an PVDF membrane (Bio-Rad, Hercules, CA, USA) and blocked with 5 % non-fat dry milk in TBST. To detect protein expression, the blots were probed with the specific antibodies against HO-1, Keap-1, Nrf-2 (Bioss,

Woburn, MA, USA), COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA), NQO1 (Abcam, Cambridge, MA) 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 for an internal control. The immunoreactive proteins on the membrane were detected by chemiluminescence using the SuperSignal® West Pico (Pierce, Rockford, IL) on X-ray film.

Determination of COX-2 and iNOS mRNA levels with Quantitative Real-Time PCR

Total RNA isolation from cells was performed via phenol-guanidine thiocyanate extraction using RNeasy lysis reagent (Qiagen, Crawley, UK), according to the manufacturer's instructions. Total RNA (1 µg) was reverse-transcribed to cDNA using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) in a 20 µL reaction mixture. Real-time PCR was carried out using a Light Cycler Nano System (Roche Diagnostics GmbH, Mannheim, Germany). To quantify cDNA, qPCR was performed using FastStart Essential DNA probe master mix (Roche Diagnostics GmbH, Mannheim, Germany). The reaction mixture (15 µL) was prepared in LightCycler 8-tube strips (Roche Diagnostics GmbH, Mannheim, Germany) and included 10 µL of 2× Master Reaction Mix (Roche Diagnostics GmbH, Mannheim, Germany), 4 µL PCR grade water, 1 µL 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 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 (1×10^6) were treated with HNE (2,5 µmol/L, 6 h) and the released cytokines were determined semiquantitatively by mouse cytokine antibody array (Abcam, Cambridge, MA) according to the manufacturer's protocol. The density of the cytokine spots was analyzed by using Image J densitometric analyze programme. We used intensity of positive control spots for normalization of array results.

Statistical analyses

Possible associations between groups were analyzed with SigmaPlot 12. statistical software using t test. P values <0.05 were considered as statistically significant. Fold

increase or decrease of mRNA levels were also calculated by REST (relative expression software tool) software 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 incubation, HNE significantly reduced viability of cultured microglial cells at the conc.>2,5 $\mu\text{mol/L}$. Whereas 2,5 $\mu\text{mol/L}$ or lower concentrations of HNE decreased cultured cell viability, but the differences did not reach any statistical significance. Non-toxic 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 were 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 G-CSF, GM-CSF, 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, sTNFR1 were slightly increase in HNE treated cells, compared to those of untreated group. IL-12p70, MCP-5 and thrombopoietin expression patterns did not change in HNE treated cells, When compared with those of untreated group.

Changes on 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, 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 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 effect iNOS mRNA expression in HNE treated BV-2 cells, monochloropivaloylquercetin pretreatment significantly decreased COX-2 mRNA expression (Figure 4).

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

It has been 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 were 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 in cytoplasmic expression of Keap1 and decreased in cytoplasmic expression of Nrf-2. In addition, HNE treatment caused a slight alteration in HO-1 and NQO1 expression but the difference did not reach any statistical significance (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 antioxidant proteins; HO-1 and NQO1 expression, Quercetin pretreatment induced NQO1 expression with both molecules enforced 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. 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 augments cytokine production. Quercetin and monochloropivaloylquercetin exerted a significant antioxidant effect by enforcing 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 anti-neuroinflammatory agent by decreasing COX-2 expression against HNE-induced inflammatory process in BV-2 cells.

HNE is believed to contribute to the dysfunction and death of the neurons in the pathogenesis of neurodegenerative diseases. On the other hand, oxidative stress also activates mechanisms that result in a glia cell-mediated inflammation that also causes secondary neuronal damage. Several studies have shown that when the oxidative stress induced by several agents brain cells like microglia and astrocytes release diverse inflammatory mediators^{24,25}. Moreover, oxidative stress indicators (ROS, reactive lipid peroxidation products) act as critical signaling molecule to trigger inflammatory responses in central nervous system through the activation of the 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 0,07-2,8 μM , while in diseased states and near to the core of lipid peroxidation sites, its concentration can be greatly increased (even more than 100 μM)³⁰. Treatment with low (1 and 10 μM) concentrations of HNE caused a significant induction in 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 period of treatment. Previous studies reported that HNE showed toxic effects in neuron cultures therefore, our data are in agreement with previous reports³³⁻³⁵. The goal of the present study was to identify whether HNE is a possible intracellular mediator between oxidative stress and inflammation in microglia cells. Therefore, 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, similarly, release of proinflammatory cytokines were significantly raised in response to HNE-mediated oxidative stress. Previous studies have been reported that, 4-HNE is an inducer of COX-2 expression in several types of cells including 3T3-L1 adipocytes³⁶, epithelial RL34 cells³⁷ and macrophages³⁸. Our data extend these results to BV-2 microglia cells. Given that lipid peroxidation reactions are 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 being generated. Other than hydroxynonenal, advanced lipid peroxidation end products (ALEs) induced proinflammatory response by activating proinflammatory gene expression in monocytes⁴⁰. In addition, activation of receptor for advanced glycation end product

(RAGE) mediated pathway on mononuclear phagocytes 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 (PDGF), and also insulin-like growth factor-1 (IGF-1). Moreover, it was emphasized in these studies that the HNE as a possible link between oxidative stress and inflammation. Our results are also in agreement with those of Kauppinen *et al.* who reported that 24 hr HNE treatment induced inflammasome signalling by increased inflammasome component NLRP3 (NLR family, pyrin domain containing 3) mRNA levels in retinal pigment epithelial cells (ARPE-19) raising the IL-1 β and IL-18 release⁴¹. Additionally, the inflammatory role of HNE was reported by Chen *et al.* who reported 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 chondrocytes⁴². Another key evidence for the inflammatory effect of HNE exemplified such HNE may contribute in 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 signalling pathway in osteoblasts⁴³. 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 signalling 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 Keap-1. The dissociation of Nrf-2 from Keap-1 is crucial for its nuclear translocation, followed by binding to antioxidant response element (ARE)⁴⁴. The innate immune response involves generation of ROS, which can act as second messengers activating proinflammatory signaling pathways such as NF κ B⁴⁵. Accordingly, inhibition of NF κ B herewith activation of Nrf-2 signalling pathway may be a beneficial strategy for reduction of deleterious effects of inflammation and it is well-known that anti-inflammatory agents suppress NF- κ B signaling while activating Nrf-2/ARE pathway⁴⁶⁻⁴⁸. Activation of Nrf-2 mediated signalling pathway resulting in up-regulation of HO-1 expression. HO-1, which is known to play an anti-inflammatory role due to carbon monoxide production and NF κ B inhibition⁴⁹. In our study, pretreatment with quercetin and monochloropivaloylquercetin activated Nrf-2/Keap-1 signalling by decreasing

cytoplasmic expression and inducing nuclear translocation of Nrf-2, resulting in up-regulation 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 the quercetin and monochloropivaloylquercetin. In this regard, there remains the possibility that induction of Nrf-2 target genes contribute both quercetin and monochloropivaloylquercetin's efficacy to inhibit COX-2 expression. Ramyaa et al. have already reported that, modulatory effect of quercetin on oxidative stress by upregulating Nrf-2 expression and downregulating NFκB and COX-2 expression⁵⁰. Anti-inflammatory properties of quercetin accompanied by an increase in HO-1 protein levels associated with elevated nuclear translocation of Nrf-2 was previously exhibited by Boesch-Saadatmandi et al in murine RAW 264.7 macrophages⁵¹. On the other hand, quercetin acts as an anti-inflammatory agent in lipopolysaccharide-induced acute lung injury by significantly reduced COX-2, iNOS expression, and NF-κB p65 phosphorylation⁵². In other study, intratracheal administration of quercetin affected protective agent by modulate HO-1 activity against lipopolysaccharide-induced acute lung injury⁵³. Quercetin was previously shown to down-regulate proinflammatory responses, involving iNOS expression and NO generation in BV-2 cells through inducing Nrf-2 mediated HO-1 expression⁵⁴. In contrary, quercetin pretreatment did not effect iNOS mRNA expression in our study it may be associated the dose of quercetin or incubation time.

Conclusion

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.

Conflict of interest

All authors declare no conflict of interest.

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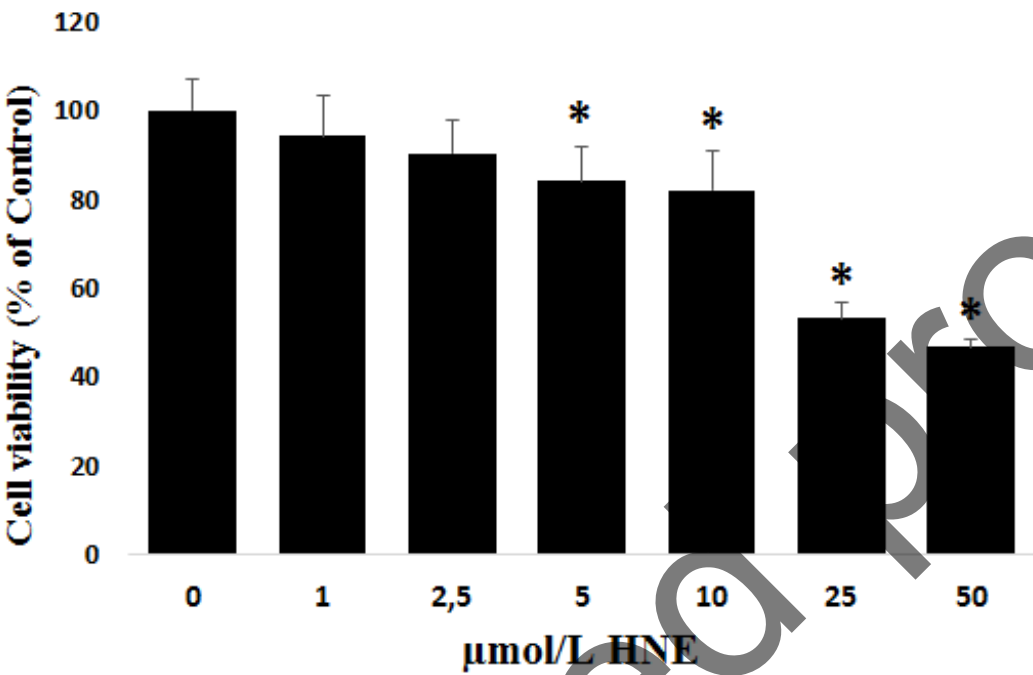
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Uncorrected proof

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).



Uncorrected Proof

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. Pos; positive controls. Neg; negative controls. **C:** the quantification by Image J of cytokines secretion in non-treated control cells and HNE-treated BV-2 cells. Fold changes were given in the table after average spot signals of control cell were assumed as 1.00 *p<0.05, **p<0.01

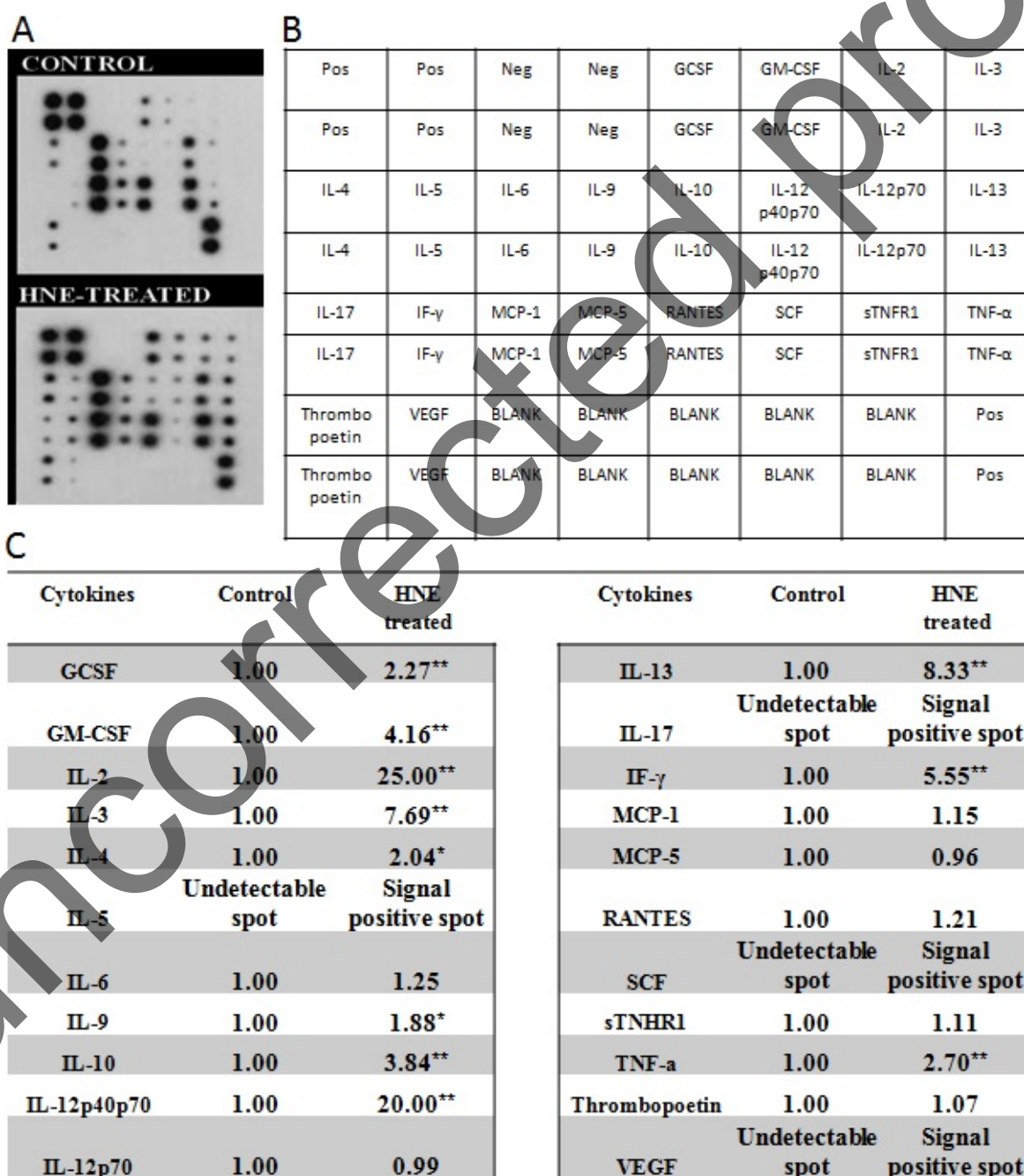


Figure 3. Time-dependent changes in COX-2 mRNA expression (A), iNOS mRNA expression (B) and COX-2 protein expression (C and D) in HNE treated BV-2 cells. n=3, *p<0.05 vs. Control (0)

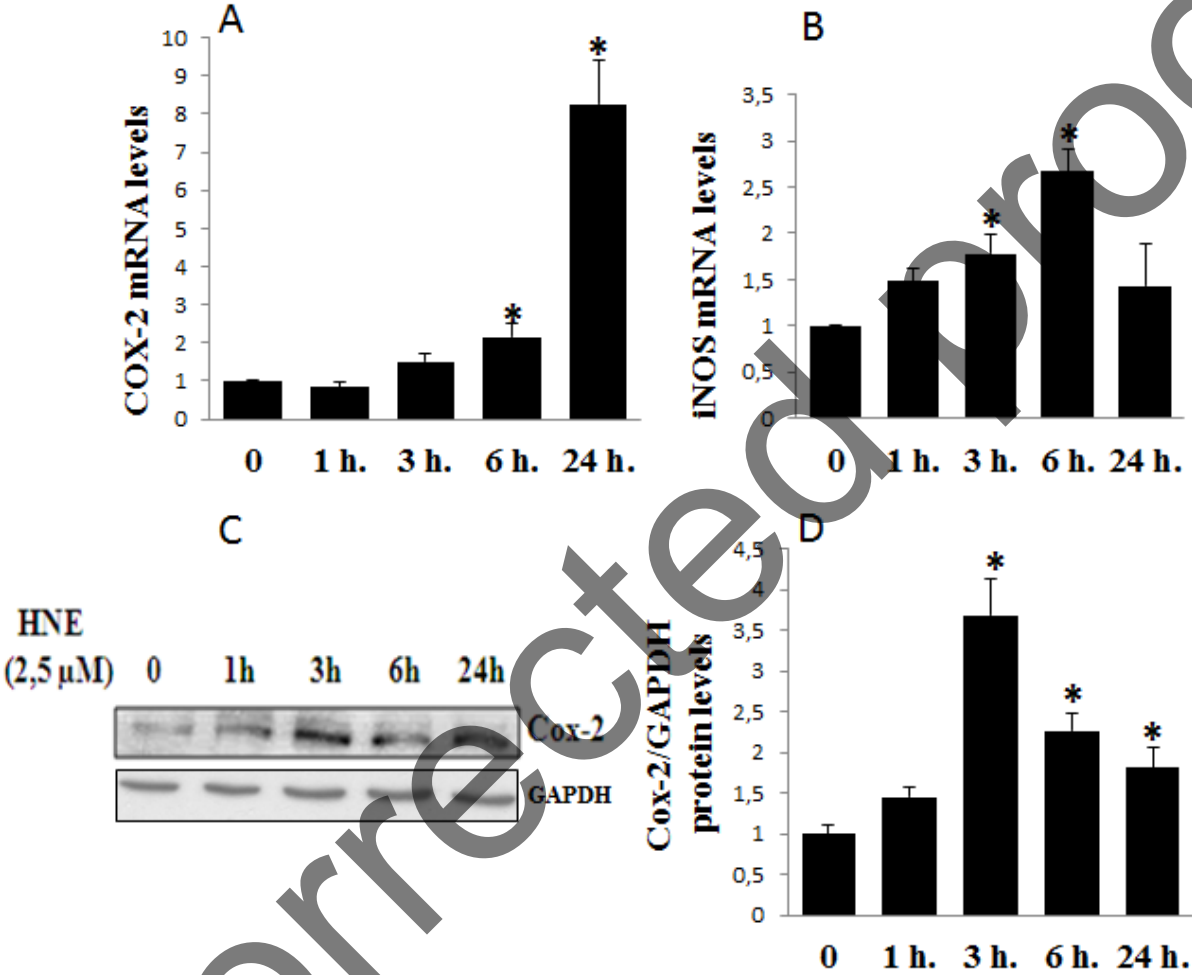
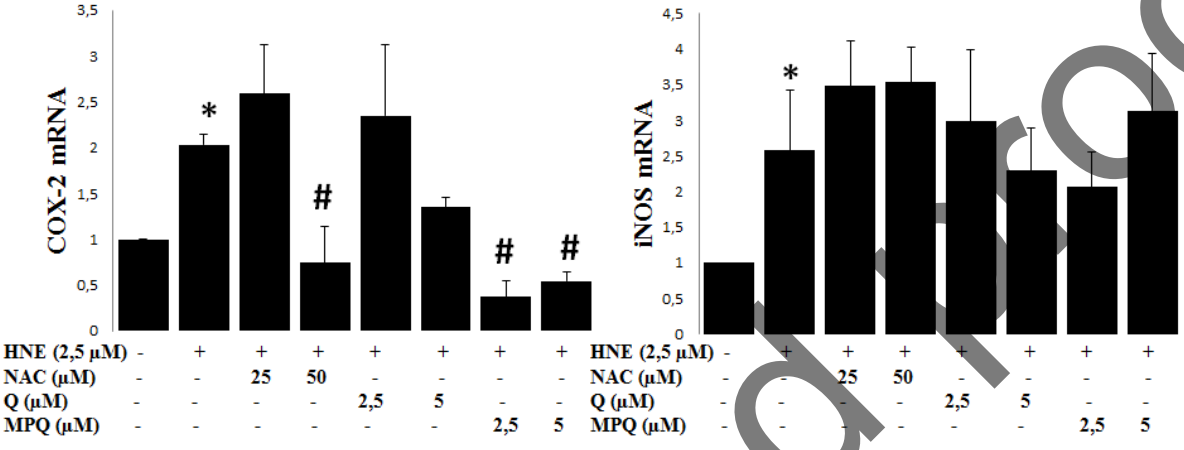


Figure 4. Effects of quercetin and monochloropivaloylquercetin on COX-2 mRNA expression and iNOS expression in HNE-treated BV-2 cells. NAC; N-acetyly-L-cysteine, Q; quercetin, MPQ; monochloropivaloylquercetin. n=3, *p<0.05 vs Control (0), #p<0.05 vs. HNE.



Uncorrected proof

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. NAC; N-acetyly-L-cysteine, Q; quercetin, MPQ; monochloropivaloylquercetin. n=3, *p<0.05 vs Control (0), #p<0.05 vs HNE.

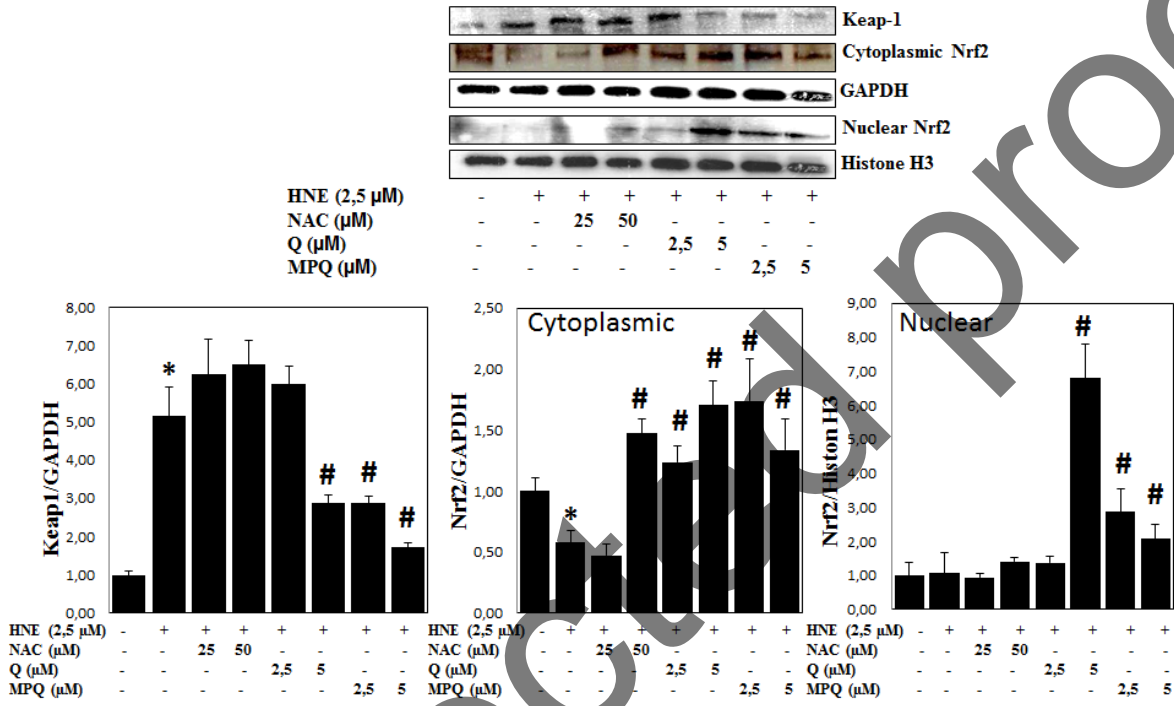


Figure 6. Effects of quercetin and monochloropivaloylquercetin on phase II protein NQO1 expression and antioxidant HO-1 protein expression in HNE treated BV-2 cells. NAC; N-acetyly-L-cysteine, Q; quercetin, MPQ; monochloropivaloylquercetin. n=3, *p<0.05 vs Control (0), #p<0.05 vs HNE.

