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Induction of CMV-1 promoter by 4-hydroxy-2-nonenal in human embryonic kidney cells*

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Oxidative stress, i.e., excessive production of oxygen free radicals and reactive oxygen species, leads to lipid peroxidation and to formation of reactive aldehydes which act as second messengers of free radicals. It has previously been shown that oxidative stress may be involved in the transcriptional regulation of cytomegalovirus (CMV) immediate early promoter, involved in viral reactivation from latency. In the current study we used a plasmid containing the yellow fluorescent protein (YFP) gene under the control of CMV-1 promoter to monitor the influence of hydrogen peroxide and reactive aldehydes, 4-hydroxy-2-nonenal (HNE) and acrolein, on CMV-1 promoter activation in human embryonic kidney cells (HEK293). While acrolein was ineffective, hydrogen peroxide slightly (50 %) stimulated the CMV promoter. In contrast, HNE had a strong, up to 3-fold, enhancing effect on the CMV-1 promoter within four as well as after 24 h of treatment. The most effective was the treatment with 24 μM HNE. This effect of HNE suggests that stressful conditions associated with lipid peroxidation could lead to CMV activation.

Key words: 4-hydroxy-2-nonenal, oxidative stress, cytomegalovirus

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INTRODUCTION

Oxidative stress has been associated with the pathogenesis of many diseases. Oxidative stress is a balance shift of oxido-reductive reactions towards oxidation, resulting in excessive production of reactive oxygen species (ROS). Although most of the ROS are radical species like hydroxyl and superoxide radical, there are also non-radical species like hydrogen peroxide. ROS are normally formed in small quantities during metabolic processes, however, overproduction of ROS is cytotoxic and damages macromolecules (DNA, proteins, sugars and lipids) (Esterbauer *et al.*, 1991; Bartosz, 2009). Polyunsaturated fatty acids in membranes as well as storage lipids are subject to ROS-induced peroxidation resulting in the destruction of biomembranes. Final products of lipid peroxidation are reactive aldehydes like 4-hydroxy-2-nonenal (HNE), malondialdehyde, acrolein and other α,β-unsaturated aldehydes (Esterbauer *et al.*, 1991; Uchida, 2003). The lipid-derived aldehydes are more stable than ROS and therefore can diffuse to targets remote from the initial oxidative injury site. Among these aldehydes, HNE, also denoted as a "second messenger of free radicals", is of particular biochemical and biomedical relevance (Esterbauer *et al.*, 1991; Zollner *et al.*, 1991; Zarkovic *et al.*, 1999). Since HNE is formed during oxidative stress, it is not surprising that many deleterious effects have been attributed to it. These include a depletion of glutathione (Hartley *et al.*, 1995) and inhibition of DNA and protein synthesis (Esterbauer *et al.*, 1991). Furthermore, HNE has been shown to play a role in modulation of cell growth (Hartley *et al.*, 1995; Zarkovic *et al.*, 1993; 1994; 1995), differentiation (Cheng *et al.*, 1999; Rinaldi *et al.*, 2000; Borovic-Sunjic *et al.*, 2005), apoptosis (Cheng *et al.*, 1999; Uchida *et al.*, 1999; Sovic *et al.*, 2001; Borovic-Sunjic *et al.*, 2005), and cell signaling (Kreuzer *et al.*, 1998; Cheng *et al.*, 1999; Awasthi *et al.*, 2004). HNE is also very effective in binding to DNA or proteins leading to adduct formation, eliciting mutagenic or carcinogenic effects (Esterbauer *et al.*, 1991; Sovic *et al.*, 2001; Zarkovic, 2003).

Cytomegalovirus (CMV) is a widespread pathogen which latently infects the majority of adults and is responsible for generally asymptomatic and persistent infections in healthy people (Mocarski, 1996). However, primary infection or reactivation in immunosuppressed patients (e.g., after solid organ or bone marrow transplantation) or immunologically immature individuals may cause a severe disease and is associated with serious morbidity or mortality (Numazaki *et al.*, 1995). The mechanism by which CMV reactivates from latency in immunocompromised individuals is not well understood. Scholz and coworkers have reported that oxidative stress may contribute to the regulation of CMV replication, virus shedding, and the activation of endothelial cells by proinflammatory cytokines (Scholz *et al.*, 1996). The replication cycle of a human CMV is characterized by the expression of immediate early (IE), early (E), and late (L) gene regions. Since IE gene expression had previously been described to be of crucial relevance to CMV pathogenesis (Scholz *et al.*, 2001), the aim of this study was to investigate the impact of HNE on CMV-1 promoter induction. CMV-1 originates from the IE gene of human CMV (CMV-IE) and is one of the most commonly

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Abbreviations: CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; HEK293, 293 human embryonic kidney cells; HNE, 4-hydroxy-2-nonenal; HNE-DMA, 4-hydroxy-2-nonenal-dimethylacetal; LD_{50}, lethal dose 50 %; RFU, relative fluorescence units; ROS, reactive oxygen species; TLR4, Toll-like receptor 4; YFP, yellow fluorescent protein.

used promoters. The CMV-IE promoter region contains a high density of transcription factor-binding sites. These sites include multiple 18-, 19- and 21-bp repeated motifs that bind the transcription factors NF-κB/rel, CREB/ATF and Sp1, respectively. In addition, it contains binding sites for AP1, retinoic acid, ETS and SRE. The promoter regulates downstream major IE genes which have a critical role in reactivation from latency and acute infection.

Since HNE is known to affect most of the respective signaling pathways and expression of several other genes relevant to the cellular stress response and growth control (Zarkovic, 2003; Leonarduzzi *et al.,* 2004), the aim of our study was to analyze if this particular aldehyde could also affect the CMV-1 promoter.

MATERIALS AND METHODS

Cell cultures and transfection. The 293 human embryonic kidney cells (HEK293), a kind gift from Dr. Andreja Ambriovic Ristov, were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, Gibco BRL/Life Technologies, New York, NY, USA) and 1% penicillin/streptomycin (Gibco) in a humidified atmosphere.

In the transfection experiment we used the pCDNA3 plasmid with a fusion gene encoding Toll-like receptor 4 (TLR4) with yellow fluorescent protein (YFP) that was cloned under the control of CMV-1 promoter (pCMV1-TLR4-YFP, a kind gift from Dr. Alberto Visintin). TLR4 is a transmembrane receptor which detects lipopolysaccharides derived from bacteria and activates innate immunity. Given that the main goal of this work was to elucidate the HNE influence on CMV-1 promoter induction, we used the fusion protein since HNE has no stimulating but inhibiting effect on the TLR4 activation (Kim *et al.*, 2009).

Transient transfection was performed using Lipofectamine™ reagent according to the manufacturer's procedures (Invitrogen). Cells were plated at 7×10^4 cells per well in 96-well microcytoplates and incubated for 24 h. Cells were washed once in PBS (phosphate-buffered saline) and transfected for 3 h in 70 μL of serum-free DMEM medium containing 0.5 μL of Lipofectamine and 0.075 μg of pCMV1-TLR4-YFP (Flo *et al.*, 2002). Afterwards the transfection medium was removed, replaced with DMEM containing 10% FCS and left over night. The plasmid used was isolated using QIA prep Spin Miniprep Kit (Qiagen). The next day the cells were treated with acrolein (Sigma, USA), HNE, hydrogen peroxide or left untreated as control at 37 °C in a humidified air atmosphere with 5% CO_2 for 4 h and 24 h.

HNE, acrolein and H_2O_2 treatment. Prior to treatment the cells were left in serum-free medium for 1 h. HNE, acrolein or H_2O_2 were freshly prepared before each experiment and used in several concentrations in DMEM medium (2% FCS). The concentrations used are expressed as equivalent per 2×10^4 cells.

HNE, in the form of 4-hydroxy-2-nonenal-dimethylacetal (HNE-DMA), was kindly provided by the Institute of Molecular Biology, Biochemistry and Microbiology (Graz, Austria). Prior to the experiment, it was activated with 1 mM HCl (Kemika, Croatia) for 2 h. The concentration of HNE was determined by spectrophotometry (Shimadzu UV-1601 spectrophotometer) (Zivkovic *et al.*, 2005).

Transfected HEK293 cells were treated with four different concentrations of HNE (8, 16, 24 and 32 μM), acrolein (8, 16, 32 and 48 μM) and H_2O_2 (10, 25, 50 and 100 μM) for 4 h and 24 h at 37 °C in a humidified air atmosphere with 5% CO_2. Transfected HEK293 cells treated with DMEM medium served as control.

Cell viability assay. Cell viability was determined after the treatment with HNE, acrolein or H_2O_2. The MTT-related colorimetric assay (EZ4U; Biomedica, Austria) was used to determine cell growth and viability, according to the manufacturer's instructions. The method was based on the fact that living cells are capable of reducing less colored tetrazolium salts into intensely colored formazan derivatives. This reduction process requires functional mitochondria, which are inactivated within a few minutes after cell death.

In order to determine LD_{50} (dose that kills 50% of cells), the cells were treated with HNE, acrolein or H_2O_2 for 4 h prior to MTT assay. The HNE, acrolein and H_2O_2 concentrations corresponding to the respective LD_{50} values were further used in experiments with YFP expression. HNE concentrations below LD_{50} were also further used in the induction experiment. Therefore, the viability of cells was also analysed after 24 h of treatment for the cultures treated with respective LD_{50} doses of HNE, acrolein or H_2O_2 and for cultures treated with HNE concentrations below LD_{50} to evaluate the effects of these treatments on the CMV-1 expression in the viable cells. Briefly, after the treatment the medium was removed and 200 μl of fresh Hanks' balanced salt solution (HBSS) and 20 μl of the tetrazolium agent were added to each well. After 2 h of incubation, plates were scanned in a microplate reader (Easy-Reader 400 FW, SLT Lab Instruments GmbH, Austria) at 450 nm.

Effect of HNE, acrolein and H_2O_2 on CMV-1 promoter activation. After four or 24 h of treatment the effect of LD_{50} of HNE, acrolein or H_2O_2 on CMV-1 promoter activity was checked. Fluorescence intensity was measured with a Cary Eclipse fluorescence spectrophotometer (Varian) with excitation at 514 nm and emission detection at 527 nm. Arbitrary relative fluorescence units (RFU) were based directly on fluorescence intensity.

The fluorescence intensity was also checked for treatments with HNE concentrations below LD_{50}.

Statistics. The results obtained are shown as the mean ± SE for quadruplicates of cultures. The statistical significance was assessed using the Student's *t*-test and Chi-square test. Differences with values of $P < 0.05$ were considered statistically significant.

RESULTS

HEK293 cells were transiently transfected with pCMV1-TLR4-YFP with transfection efficiency above 80%. Cell viability of transfected HEK293 cells was determined 4 h after HNE, acrolein or H_2O_2 treatment (Fig. 1). HNE and H_2O_2 decreased the cell viability in a concentration-dependent manner. In contrast, low concentrations of acrolein (8 and 16 μM) significantly increased the viability of transfected HEK293 cells ($P < 0.05$). However, after the treatment with higher acrolein concentrations the viability of cells was decreased ($P < 0.05$) when compared to control untreated cultures. The obtained results allowed determination of the LD_{50} concentrations for each treatment. Accordingly, the following LD_{50} concentrations were further used to treat

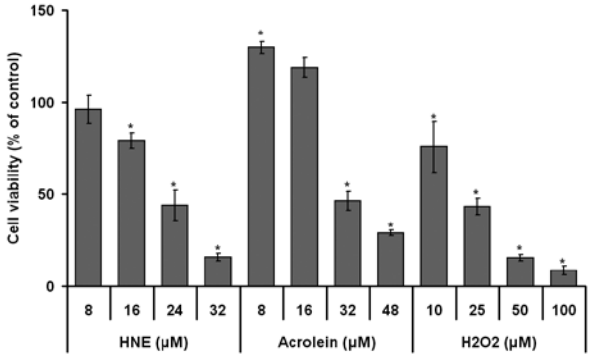

Figure 1. Cell viability of transiently transfected HEK293 cells after 4 h treatment with HNE, acrolein or H_2O_2.
Cell viability is presented as percentage of control untreated transfected HEK293 cells. Mean values (±SE) for triplicates are given: (*) significance $P<0.05$ in comparison to control untreated HEK293 transfected cells.

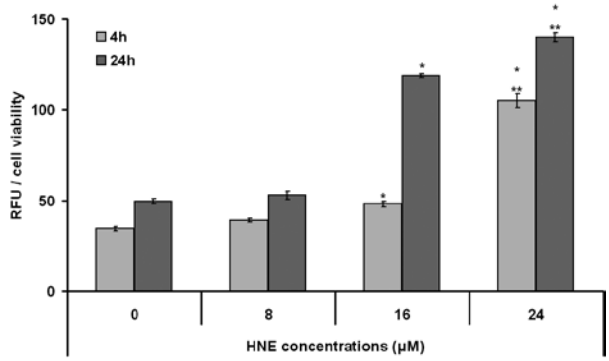

Figure 3. CMV-1 induction by various HNE after 4 h or 24 h.
Results are presented as RFU normalized to cell viability (RFU/cell viability). Mean values (±SE) for the triplicates are given: (*) significance $P<0.05$ in comparison to control untreated HEK293 transfected cells, (**) significance $P<0.05$ in comparison to results obtained for16 μM HNE.

the cells: HNE — 24 μM, acrolein — 32 μM and H_2O_2 — 25 μM.

The effects of LD_{50} concentrations of HNE, acrolein or H_2O_2 on the activation of CMV-1 promoter are shown in Fig. 2. The results are presented as RFU normalized to cell viability (RFU/cell viability). It can be seen that the LD_{50} concentration of HNE had the strongest effect ($P<0.05$) on CMV-1 promoter activation resulting in a three-fold increase in YFP expression after 4 h or 24 h of treatment. Hydrogen peroxide at LD_{50} increased slightly (50%) but also significantly ($P<0.05$) the YFP expression after 4 h and 24 h. However, treatment with acrolein LD_{50} did not significantly affect YFP expression.

The induction efficiencies obtained in the experiments using HNE concentrations below LD_{50} (8 and 16 μM HNE), in which the viability of the transfected cells was not strongly affected, are shown in Fig. 3. As it can be seen, treatment with 8 μM HNE had no influence ($P>0.05$) on CMV-1 promoter induction. However, treatment with 16 μM HNE slightly (12%) but significantly ($P<0.05$) induced the CMV-1 promoter after 4 h while after 24 h it had a very prominent effect ($P<0.05$) on CMV-1 promoter activation resulting in a two-fold increase of YFP expression.

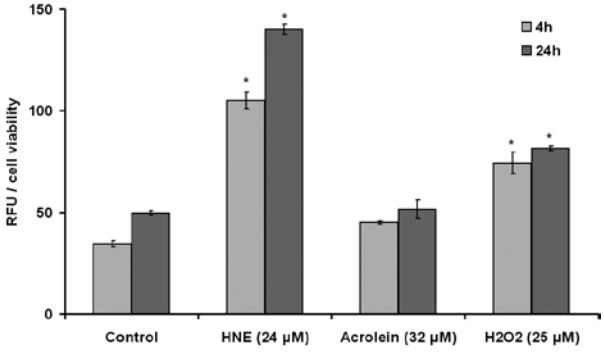

Figure 2. Effect of HNE, acrolein or H_2O_2 on CMV-1 promoter activation after 4 h or 24 h of treatment.
Results are presented as RFU normalized to cell viability (RFU/cell viability). Mean values (±SE) for triplicates are given: (*) significance $P<0.05$ in comparison to control untreated HEK293 transfected cells.

DISCUSSION

The induction of CMV promoter by H_2O_2 shown here is consistent with the previous results reported by Speir (2000). That study showed that H_2O_2 treatment can induce MIEP (major immediate early promoter) transcription in smooth muscle cells. This was abolished by the addition of antioxidants, supporting the hypothesis of ROS involvement as important signaling mechanism of CMV promotion.

In the present paper we further show strong effects of the major bioactive marker of lipid peroxidation, the aldehyde HNE, on CMV promoter induction. This particular effect of HNE probably occurred due to the HNE-specific induction of transcription factors and was not observed for acrolein, which is another bioactive aldehyde produced by lipid peroxidation. It is well known that HNE can induce activation of several transcription factors including activator protein 1 (AP-1), a well known redox-sensitive transcription factor that regulates the activation of transcription of a variety of genes (Liu *et al.*, 2001). The activity of AP-1 is regulated by the phosphorylation of MAPKs that include JNK. It has also been reported that HNE may activate JNK by its direct binding (Parola *et al.*, 1998) or through the redox-sensitive MAPK kinase cascade (Uchida *et al.*, 1999). However, the influence of HNE on NF-κB, another redox-sensitive transcription factor, remains unclear. Some researchers have shown that HNE can inhibit NF-κB activation through inhibition of IκB phosphorylation by directly reacting with IκB kinase (IKK) (Camandola *et al.*, 1997; 2000; Page *et al.*, 1999; Vaillancourt *et al.*, 2007), while others have reported its stimulatory effect (Ruef *et al.*, 2001; Lee *et al.*, 2008). While HNE is of continuous interest among researchers studying cell signaling and epigenomic aspects of oxidative stress (Zarkovic N, 2003), a detailed mechanism of HNE action as a regulatory factor has yet to be elucidated.

We cannot infer yet which of the above-mentioned transcription factors is involved in this HNE-induced CMV promoter activation because this promoter has binding sites for AP-1 and NF-κB both. Therefore, in the continuation of this research we will mutate binding sites for these transcription factors in the CMV-IE promoter to distinguish which one is crucial for the observed enhancing effects of HNE.

It should also be mentioned that the transfection procedure is always a stress for the cell, hence it might also be associated with mild, non-toxic oxidative stress resulting in HNE production due to peroxidation of membrane lipids, which could consequently affect the CMV promoter. Since CMV promoter is commonly used in transfection studies, it seems likely that this bioactivity of HNE might also be relevant for other transfection studies. Such CMV stimulation by HNE could influence the transcription of other target genes, in particular if occurring in cells with weak antioxidant capacities or if combined with additional stressors. Yet, this assumption has to be further evaluated in more detail. In the case of our study, the main goal was to elucidate the influence of exogenous HNE on CMV-1 promoter induction in HEK293 cells so we may conclude with certainty that the effects of HNE treatments were specific to the CMV-1 promoter and were not influenced by endogenous HNE. We used the fusion protein of TLR4 because HNE does not stimulate its expression (Kim *et al.*, 2009). However, it is impossible to completely exclude the possible influence of HNE on specific activation of TLR4 since we did not use a vector encoding solely the YFP-reporter protein. When used at the low concentration of 8 μM, which is still above a physiological one for cultured cells even if mildly stressed by lipid peroxidation (Zarkovic, 2003; Borovic *et al.*, 2006), HNE was not effective. This indicates that only supraphysiological levels of HNE are able to induce CMV promoter. This also suggests that in case of latent CMV infection conditions that might activate the CMV, such as chemotherapy, radiotherapy, ischemia/reperfusion or inflammatory processes (Scholz *et al.*, 1996; Spier, 2000; Vereecque *et al.*, 2003), induce the CMV because of their pathophysiology involving oxidative stress and increased HNE production. This possibility has to be further studied, in particular since recent data also indicate that CMV infection itself provokes cellular oxidative stress inducing a vicious circle. Hence, possible antioxidant treatments were also proposed as adjuvant therapy for CMV infections (Cintal *et al.,* 2000; Dhaunsi *et al.*, 2003; Gnana-Prakasam *et al.*, 2003).

It could also be possible that HNE could have a dual, concentration-dependent effect on the CMV-IE promoter. Namely, the dependence of the CMV promoter activity on cAMP concentration was reported several years ago (Zhang *et al.*, 2003). Cyclic AMP is also a well known second messenger involved in cell signaling cascades, which activates various effector proteins. This suggestion is further supported by the fact that HNE has diverse effects depending on its concentration, also affecting adenylate cyclase if used at micromolar concentrations (Paradisi *et al.,* 1985). It has been shown that low levels of HNE promote proliferation (Ruef *et al.*, 1998; Cheng *et al.*, 1999) while higher concentrations induce differentiation and apoptosis (Kruman *et al.*, 1997; Cheng *et al.*, 1999; 2001; Dianzani *et al.*, 1999; Soh *et al.*, 2000). In the study presented here, we tested only the LD_{50} concentration and several lower concentrations, so further studies should be done before making final conclusions about this assumption.

In conclusion, our results suggest that transfection experiments should consider possible effects of transfection-induced stress and consequential membrane lipid peroxidation-generating HNE. Stimulation of the CMV promoter by HNE may be of practical importance also for better monitoring and treatment of CMV infections.

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