HNE-induced 5-LO expression is regulated by NF-κB/ERK and Sp1/p38 MAPK pathways via EGF receptor in murine macrophages

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Aims

5-Lipoxygenase (5-LO) has been suggested to be a modulator of atherosclerotic plaque instability and co-exists with 4-hydroxynonenal (HNE) in macrophages in atherosclerotic lesions. To determine the potential role for HNE in 5-LO expression, the molecular mechanisms of 5-LO expression were evaluated in HNE-stimulated macrophages.

Methods and results

A genomic sequence of the promoter 2.0 kb upstream of the transcription initiation site was amplified, and a series of sequentially deleted fragments were then fused to a luciferase reporter gene. The promoter region 213 bp upstream of the transcription start site was responsible for the HNE-enhanced transcriptional activity of 5-LO. Site-directed mutagenesis of this region showed that the transcription factors, including stimulating protein 1 (Sp1) and nuclear factor-κB (NF-κB), were associated with up-regulation of HNE-induced 5-LO transcription. Moreover, the role of Sp1 and NF-κB in HNE-induced 5-LO expression was confirmed by siRNA knockdown of Sp1 and NF-κB. The HNE-enhanced Sp1 and NF-κB activities were attenuated by SB203580, a p38 mitogen-activated protein kinase (MAPK) inhibitor, and PD98059, an extracellular signal-regulated kinase (ERK) inhibitor, respectively. In addition, the HNE-enhanced phosphorylation of p38 MAPK and ERK was inhibited by AG1478, an epidermal growth factor receptor (EGFR) antagonist, but not by AG1295, a platelet-derived growth factor receptor (PDGFR) antagonist.

Conclusion

5-LO expression by HNE was regulated at the transcriptional level by the EGFR-mediated activation of Sp1/p38 MAPK and NF-κB/ERK pathways in macrophages, which may lead to the development of therapeutic interventions for regulating 5-LO expression in atherosclerosis.

Keywords

HNE • 5-LO • Macrophages • Atherosclerosis

1. Introduction

Aldehydes generated endogenously during lipid peroxidation are involved in the pathogenesis of a large number of inflammatory and degenerative processes, including atherosclerosis. Among the reactive aldehydes, 4-hydroxynonenal (HNE) is one of the most abundant aldehydes formed during the oxidation of polyunsaturated fatty acids and is considered to be a sensitive marker of lipid peroxidation and oxidative stress. In our previous studies, an elevated level of HNE was detected in atherosclerotic lesions. Moreover, HNE directly increased the expression of CD36 scavenger receptor on murine macrophages, supporting an aetiopathological role for HNE in the development of atherosclerosis.

Several lines of evidence have implicated 5-lipoxygenase (5-LO) in the development and progression of atherosclerosis. An increased level of leukotrienes was detectable in atherosclerotic lesions, and implicated in the progression of atherosclerosis. Genetic studies also have identified variants of the 5-LO gene promoter and the 5-LO activating protein gene as risk factors in human atherosclerosis. Recently, several studies have indicated a link between 5-LO and atherosclerotic plaque instability.

The cellular activity of 5-LO is regulated in a complex manner that involves different signalling pathways, including p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK). Recent studies have reported that a possible strategy might involve the regulation of receptor tyrosine kinases, such as...
epidermal growth factors (EGF) and platelet-derived growth factors (PDGF), which subsequently activate members of the MAPK family. Reportedly, EGF receptor (EGFR) is commonly altered in atherosclerotic lesions leading to activation of excessive downstream signalling, the result of which is believed to contribute to the aggressive phenotype observed in atherosclerotic plaques.

Although EGFR and 5-LO pathways might have common roles in atherosclerosis, their relationship in the progression of atherosclerosis is still unknown.

In previous studies, an elevated level of 5-LO was detected and co-localized with HNE in macrophages accumulated in atherosclerotic lesions. Moreover, it has been demonstrated that HNE increases 5-LO activity in macrophages. Thus, it is probable that HNE might be responsible for the increased 5-LO production in atherosclerotic lesions. To identify the essential regulatory elements in the promoter that controls gene transcription, we cloned the 5-LO promoter regions from mouse genomic DNA sequence. In this study, the potential role for HNE on 5-LO expression in murine macrophages was investigated. In addition, we determined the transcription factors involved in the HNE-induced increase in 5-LO promoter activity and further investigated the signal pathways by which HNE activates transcription factors responsible for 5-LO expression.

2. Methods

2.1 Chemicals and antibodies

HNE was obtained from Cayman Chemical Inc. (Ann Arbor, MI, USA). pGL3 basic vector, pRL CMV vector, and dual luciferase reporter assay kit were purchased from Promega (Madison, WI, USA). DNeasy Tissue Kit and QIAprep Spin Kit were supplied by Qiagen (GmbH, Germany). Various signal pathway inhibitors and phorbol 12-myristate 13-acetate (PMA) were acquired from Calbiochem (La Jolla, CA, USA) and Sigma (St Louis, MO, USA). 5-LO, stimulating protein 1 (Sp1), and nuclear factor-κB (NF-κB) (p50) antibodies were purchased from Santa Cruz Biotechnology Inc. (Beverly, MA, USA). MAPK and phosphospecific antibody against MAPK were obtained from Cell Signaling Technology (Beverly, MA, USA). ImProm-II Reverse Transcription Systems were supplied by Promega (Madison, WI, USA).

2.2 Cell culture

The murine macrophage cell line, J774A.1 (ATCC TIB-67, Rockville, MD, USA), was cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS), antibiotic–antimycotic, and L-glutamine (Invitrogen, NY, USA) at 37°C in a humidified atmosphere containing 5% CO2/95% air. After reaching confluence, the cells were detached from the T75 culture flasks by gentle scraping.

Mouse peritoneal macrophages (MPM) were harvested from C57BL/6J mice (C57BL/6J; Jackson Laboratories) at 4 days after intraperitoneal injection of 3% thioglycollate. The cells were washed, and resuspended in RPMI 1640 supplemented with 10% FBS and antibiotic–antimycotic. MPM were confirmed by CD11b staining and morphology.

The human monocytic cell line, THP-1, was obtained from ATCC, and maintained in RPMI 1640 medium supplemented with 10% FBS and 2 mmol/L L-glutamine. THP-1 cells were differentiated using 100 nM PMA for 3 days.

2.3 RT–PCR and real-time PCR

The mRNA expression of Sp1 and NF-κB was quantified by RT–PCR analysis, using GAPDH mRNA as an internal standard. The total RNA was extracted from the cells and reverse transcribed into cDNA using ImProm-II Reverse Transcription Systems. The cDNA was then used as a template for real-time PCR analysis using specific primers for 5-LO and GAPDH. The expression levels of 5-LO were normalized to the expression levels of GAPDH using the ∆∆CT method.

Figure 1 Effect of HNE on 5-LO expression in murine macrophages. (A) J774A.1 cells were stimulated with the indicated concentrations of HNE for 4 h. Total RNA isolated from the cells was analysed for 5-LO mRNA expression by real-time PCR. Each bar represents the mean ± SEM from five independent experiments. *P < 0.05, **P < 0.01 vs. value at concentration 0. J774A.1 cells and MPM were stimulated with 10 μM HNE for the indicated time (0–24 h). 5-LO mRNA (B) and protein (C) expression were analysed by real-time PCR and immunoblotting, respectively. Each bar represents mean ± SEM from four to six independent experiments. *P < 0.05, **P < 0.01 vs. value at time 0.
isolated and 1 µg of the total RNA was reverse transcribed into cDNA using an ImProm-II reverse transcription system (Promega, WI, USA), which was then amplified by PCR using the specific primers for Sp1 (forward, 5′-TTA CAC GTT CGG ACG AGC TTC AGA-3′; reverse, 5′-TAA TGG ACT GCA GCT CTG TCA CCT-3′), and NF-κB (forward, 5′-ATT GCT GTG CCT ACC CGA AAC TCA-3′; reverse, 5′-ATG GAG GAG AAG TCT TCA TCT CCG-3′). Equal amounts of RT–PCR products were separated on a 2% agarose gel and stained with ethidium bromide.

Real-time RT–PCR for 5-LO mRNA expression was performed by ABI 7900 HT (Applied Biosystems, Foster City, CA, USA). Each reaction mixture consisted of SYBR Green Master Mix. The following oligonucleotide primers were used; forward, 5′-ATG TTG GCA TCT AGG TGC AGT GTG-3′ and reverse, 5′-ATC ATG GCT TCC TTC ACT GGC TTC-3′ for detecting 5-LO mRNA. The quantitative data were analysed using the sequence detection system software (SDS version 2.0, Applied Biosystems).

2.4 Western blot analysis
The cell lysates extracted from macrophages were separated on 8% sodium dodecyl sulphate (SDS)–polyacrylamide gels, and transferred electrophoretically onto a nitrocellulose membrane. The membranes were blocked with 5% skim milk in Tris-buffered saline with tween, followed by incubation with anti-5-LO (1:1000), Sp1 (1:1000), NF-κB (1:1000), and phosphor-MAPK (1:1000) in a blocking buffer. After the blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody, the chemiluminescence intensity was measured by LAS-3000 system (Fuji Photo Film, Japan).

2.5 Preparation of promoter constructs
Mouse genomic DNA was isolated from murine macrophages using a DNaseasy Tissue Kit. The 2 kb 5′-flanking promoter region from the genomic DNA was amplified by PCR using the upstream primer, 5′-ATT ACG ACT GCA GCT CTG TCA CCT-3′, and the downstream primer, 5′-ATT ACG GTG CCT ACC CGA AAC TCA-3′; reverse, 5′-ATG GAG GAG AAG TCT TCA TCT CCG-3′; underlined are SacI and BglII restriction enzyme sites. Both primers were designed based on the sequence retrieved from GenBank Accession Nos NM 009662 and AF 393814. The amplified 2034 bp fragment was cloned into luciferase vector pGL3 basic (Promega, WI, USA). The plasmids carrying different sizes of the 5′-flanking promoter region were created by PCR cloning using the specific forward primers (2112; 5′-AGA AGA GAG CTC ACT TAG ACT GTG GCA-3′, 2614; 5′-AGT CCG CTC TCA GCT AGC ATG CCA TTA-3′, 2409; 5′-ACC GTG GAG GTA GGA GCG GGC TTC-3′, 2213; 5′-TGA GGG ATA AAA GCT AGC TAG AGA TGG G-3′) containing the SacI and Nhel sites, and the downstream primer used for PCR of the 2034 bp fragment. The 5-LO promoter sequence was analysed for any transcription factor binding sites within the 5′-flanking promoter region using the sequence motif search program of GenomeNet (http://motif.genome.jp).

Figure 2 Cloning and characterization of 5-LO promoter region involved in HNE-induced 5-LO transcription. (A) J774A.1 cells were cotransfected transiently with various promoter constructs and an empty luciferase vector pRL CMV for 24 h, and then stimulated with 10 µM HNE for 4 h. The relative luciferase activity was represented as mean ± SEM from five independent experiments. *P < 0.05, **P < 0.01 vs. value in corresponding control. (B) Nucleotide sequence of the promoter region of the 5-LO gene. The 213 bp sequence of the 5′-flanking region of 5-LO is shown. The transcription start site is indicated by +1. The arrow indicates the translation start site. The underlined sequences are the possible transcription factor binding sites, as predicted by GenomeNet.
2.6 Transient transfection and luciferase assay

The plasmid DNA was prepared using a QIAprep Spin Kit. The cells were transfected with the luciferase reporter plasmids using Lipofectamine 2000 reagent (Invitrogen, CA, USA) according to the manufacturer’s protocol. The cell lysates were prepared using a passive lysis buffer from a Promega assay system (Promega) and used to measure the luciferase activity according to the manufacturer’s instructions for the dual luciferase Promega assay system (Promega) and used to measure the luciferase activity in J774A.1 cells transfected with the empty luciferase vector pRL CMV and promoter constructs, and then assayed for their relative luciferase activity. Data were represented as mean ± SEM from five independent experiments. *p < 0.05, **p < 0.01 vs. corresponding value in Wt5-LO.

EMSA was performed using nuclear extracts from J774A.1 cells stimulated with HNE (10 μM) for the indicated time. Each blot is representative of five independent experiments.

2.7 Site-directed mutagenesis

Mutagenesis of Sp1 and NF-κB in the 5-LO promoter region of the pGL3-5LO plasmid was generated using a Quick change site-directed mutagenesis system (Stratagene, CA, USA). The primer pairs for Sp1 and NF-κB were as follows: Sp1: wild type, 5′-CAGGGGCGAGCC-3′; mutant, 5′-CAGTGTATAGCC-3′. NF-κB: wild type, 5′-TGAGGACTGGCA-3′; mutant, 5′-TATGCTGATGA-3′. PCR was performed using two anti-parallel primers with the required nucleotide substitutions and pGL3-5LO plasmid as a template, and the PCR product was then treated with DpnI endonuclease. The sequences of the mutation constructs were confirmed by bidirectional DNA sequencing.

2.8 Electrophoretic mobility shift assay (EMSA)

DNA-protein binding assays were carried out by “The LightShift™ Chemiluminescent EMSA kit” (Pierce) that uses a non-isotopic method. Biotin end-labelled DNA duplex oligonucleotides encompassing the Sp1 and NF-κB binding sites in 5-LO promoter region were synthesized.
the two groups or by ANOVA with a Bonferroni correction for comparisons of multiple groups. A P-value < 0.05 was considered significant.

3. Results

3.1 HNE increases 5-LO expression in murine macrophages

Stimulation of J774A.1 cells with various concentrations of HNE caused an increase in 5-LO mRNA expression in a concentration-dependent manner (Figure 1A). In the time-course studies, the levels of 5-LO mRNA (Figure 1B) and protein expression (Figure 1C) in J774A.1 cells and MPM were increased slightly after 1 h of HNE stimulation, and further increased in a time-dependent manner. The maximum expression of 5-LO mRNA and protein occurred at 4 and 8 h after HNE treatment, respectively.

3.2 Cloning and characterization of the 5-LO promoter involved in HNE-induced 5-LO transcription

As shown in Figure 2A, the luciferase reporter activity of p5-LO-2034 after HNE stimulation was 2.4(±0.4) times higher than that in control. To locate the approximate regions responsible for HNE-induced 5-LO transcription within 2034 nt 5-LO promoter, five constructs with different promoter sizes were prepared and transfected transiently into murine macrophages, and then luciferase activity was measured. The promoters containing the progressive 5′ deletions from nt −2034 to nt −213 remained strongly inducible in response to HNE. The luciferase reporter activity of HNE-stimulated p5-LO-213 was 3.8(±0.6) times higher than the corresponding control, and approximately two to three times higher than that in the promoter constructs carrying more than 409 nt of the 5-LO promoter. This suggests that the region between nt −213 and nt +1 is responsible for HNE-enhanced 5-LO promoter activity in murine macrophages. Sequence analysis within the region between nt −213 and nt +1 demonstrated the presence of consensus elements for transcription factors including Sp1 and NF-κB (Figure 2B).

3.3 Involvement of Sp1 and NF-κB in HNE-induced 5-LO transcription

To validate the effect of Sp1 and NF-κB on 5-LO transcription, the predicted transcription factor binding sites for Sp1 and NF-κB were mutagenized. The mutation of two consensus sites for Sp1 and
NF-κB decreased HNE-enhanced 5-LO promoter activity by 30–60%. Double mutations of Sp1 and NF-κB binding sites led to an almost 90% reduction in reporter activity (Figure 3A), highlighting the importance of these cis-acting elements and their involvement in 5-LO promoter activation by HNE in murine macrophages.

To directly assess whether HNE increases binding of Sp1 and NF-κB to the corresponding binding sites within the −213 and +1 region of 5-LO promoter, EMSA analyses were carried out. Stimulation of cells with HNE increased the formation of complex between protein and DNA probe (Figure 3B), providing evidence that Sp1 and NF-κB binding to specific sites in 5-LO promoter was increased by HNE. Moreover, the increased 5-LO protein expression by HNE was markedly attenuated in cells transfected with siRNA for Sp1 and NF-κB (Figure 4A and B), demonstrating a role for Sp1 and NF-κB in HNE-induced 5-LO expression.

3.4 Involvement of MAPK pathways in HNE-induced 5-LO expression

To investigate whether MAPK pathways were involved in HNE-induced 5-LO expression, macrophages were pre-treated with MAPK inhibitors including PD98059 (an ERK inhibitor), SP00125 (a JNK inhibitor), and SB203580 (a p38 MAPK inhibitor), and then stimulated with HNE. As shown in Figure 5A and B, both HNE-enhanced 5-LO promoter activity in J774A.1 cells and MPM, and protein expression in J774A.1 cells and THP-1 cell-derived macrophages were significantly inhibited by PD98059 and SB203580, but not by SP00125, in a concentration-dependent manner. Moreover, the increased 5-LO protein expression by HNE was markedly attenuated in cells transfected with siRNA for ERK and p38 MAPK (Figure 5C). To investigate whether MAPK pathways were involved in the activation of Sp1 and NF-κB, macrophages were stimulated with HNE in the presence of various MAPK inhibitors, and then activities of Sp1 and NF-κB were determined. As shown in Figure 5D, the increased activities of Sp1 and NF-κB by HNE were attenuated by pretreatment with SB203580 and PD98059, respectively. This suggests that HNE-induced 5-LO expression is mediated by p38 MAPK and ERK, which are essential for activating Sp1 and NF-κB, respectively.

3.5 Involvement of EGF receptor in HNE-induced 5-LO expression

To determine whether HNE-mediated activation of ERK and p38 MAPK pathways was mediated through receptor tyrosine kinases including PDGF and EGF receptors, macrophages were stimulated with HNE in the presence of AG1295, a PDGF receptor antagonist and AG1478, an EGF receptor antagonist. As shown in Figure 6A and B, both HNE-enhanced 5-LO promoter activity in J774A.1 cells and protein expression in J774A.1 cells and THP-1 cell-derived macrophages were significantly attenuated by AG1478, not by...
AG1295. In addition, HNE-induced 5-LO protein expression was markedly inhibited by siRNA for EGF receptor (Figure 6C), demonstrating a role for EGF receptor in 5-LO expression induced by HNE. Moreover, the increased phosphorylation of ERK and p38 MAPK by HNE was also attenuated by pretreatment with AG1478 in J774A.1 cells as well as in MPM, suggesting a potential role for EGF receptor in the activation of ERK and p38 MAPK by HNE.

4. Discussion

The present study demonstrated that HNE enhanced the activity of Sp1 and NF-κB in murine macrophages, which increased the expression of 5-LO mRNA and protein. These increased Sp1 and NF-κB activities were attained by inhibiting p38 MAPK and ERK pathways, respectively. Furthermore, the HNE-enhanced phosphorylation of p38 MAPK and ERK was attenuated by an EGFR antagonist, but not by a PDGFR receptor (PDGFR) antagonist. These results support the hypothesis that HNE enhances 5-LO expression in macrophages through EGFR-mediated activation of p38 MAPK/Sp1 and ERK/NF-κB pathways.

An abundance of 5-LO in atherosclerotic lesions was demonstrated in macrophages and macrophage-derived foam cells, in which the HNE adducts were highly accumulated, suggesting a possible relationship between HNE and 5-LO expression. To investigate the potential role for HNE on 5-LO expression in macrophages, we determined the effect of HNE on 5-LO production. Our results clearly showed that HNE increased the levels of 5-LO mRNA and protein expression as well as the activity of 5-LO promoter. Based on our experimental results with other reports in which 5-LO expression might be regulated at the transcriptional and translational levels, it was suggested that 5-LO expression by HNE might be regulated at the transcriptional level. Therefore, an attempt was made to identify the transcription factors involved in 5-LO expression.

To identify the essential regulatory element in the promoter that controls gene transcription, a 2.0 kb fragment of 5′ of the 5-LO open reading frame was cloned using PCR with the primer set. The promoter activity of p5LO-2034 after HNE stimulation was 2.4±0.4 times higher than that in control, and the promoters containing progressive 5′ deletions from nt –2034 to nt –213 remained highly inducible in response to HNE. Moreover, the promoter activity of HNE-stimulated p5LO-213 was ~3.8±0.6 times higher than those of the untreated controls. This suggests that the region between nt –213 and nt +1 contains enhanced transcription factors for HNE-induced 5-LO promoter activity in murine macrophages.

Using the sequence motif search of GenomeNet (http://motif.genome.jp), the putative transcription factor binding sites were identified in the region between –213 and +1 bp relative to the transcriptional initiation site in the 5-LO promoter, which is involved in signal-induced transcriptional activation. Sequence analysis demonstrated the presence of consensus binding sites for Sp1 and NF-κB. This result was confirmed by the observations from a site-directed mutagenesis study, in which mutations of the Sp1 and NF-κB binding site in the 5-LO promoter completely abolished the responsiveness of macrophages to HNE. In addition, the knockdown of Sp1 and NF-κB using siRNA greatly decreased the HNE-induced expression of the 5-LO protein. This shows that Sp1 and NF-κB is an essential transcription factor involved in HNE-induced 5-LO transcription. Interestingly, promoter constructs containing the region from nt –214 and nt –409 inhibited the promoter activity in response to HNE compared with nt –213. This suggests that the promoter region upstream of nt –213 might contain some inhibitory transcription factor(s). However, this will require more study.

The activation of 5-LO in intact cells involves phosphorylation events because phosphorylated 5-LO was isolated from the nucleus of ionophore-stimulated HL-60 cells. Among the different MAPks, the p38 MAPK-regulated MAPK-activated protein kinases (MKs) and ERK phosphorylate 5-LO in vitro at Ser271 and Ser663, respectively, and mediate the cellular activity of 5-LO. Our previous study also demonstrated that p38 MAPK and ERK pathways were critical steps in HNE-induced activation of 5-LO in VSMC. In line with these studies, the results in the present study clearly showed that HNE activated p38 MAPK and ERK in macrophages in a time-dependent manner. Moreover, HNE-enhanced promoter activity and protein expression of 5-LO in macrophages were markedly attenuated by SB203580 (a p38 MAPK inhibitor) and PD98059 (an ERK inhibitor). Furthermore, HNE-enhanced Sp1 and NF-κB transcriptional activity were also significantly inhibited by SB203580 and PD98059, respectively. These results implicated that HNE-induced 5-LO expression was mediated by p38 MAPK and ERK pathways, which were essential for activating Sp1 and NF-κB, respectively.

To determine the involvement of receptor tyrosine kinase pathways in HNE-induced p38 MAPK and ERK phosphorylation, we stimulated macrophages with HNE in the presence of pharmacological inhibitors for EGFR and PDGFR pathways. In contrast to previous reports, in which PDGFR acts as a sensor for HNE in VSMC, the present study demonstrated that HNE-induced phosphorylation of p38 MAPK and ERK was dependent on EGFR activation but not on the PDGFR pathway. These results were consistent with the previous reports in which EGFR-induced cyclooxygenase expression was mediated by the p38 MAPK/Sp1 pathway in human gliomas. Based on our results with other reports, it was suggested that EGFR was a major element for the intracellular signal transduction in 5-LO expression in HNE-treated macrophages via activation of p38 MAPK/Sp1 and ERK/NF-κB pathways.

In summary, 5-LO expression was significantly increased in HNE-stimulated macrophages, and was associated with increased 5-LO promoter activity. Sp1 and NF-κB were essential transcription factors involved in HNE-induced 5-LO transcription. In addition, the increased Sp1 and NF-κB activities by HNE were attenuated by inhibiting p38 MAPK and ERK pathways, respectively. Moreover, inhibition of the EGFR pathway attenuated p38 MAPK and ERK phosphorylation, as well as the expression of 5-LO evoked by HNE. Collectively, these results suggested that HNE-induced 5-LO transcription might be attributed to the EGFR-mediated activation of p38 MAPK/Sp1 and ERK/NF-κB pathways, which might provide novel options for therapeutic interventions aimed at regulating 5-LO transcription in atherosclerosis.

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