Cilostazol Attenuates 4-hydroxynonenal-enhanced CD36 Expression on Murine Macrophages via Inhibition of NADPH Oxidase-derived Reactive Oxygen Species Production

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Although anti-atherogenic effects of cilostazol have been suggested, its effects on the expression of SR in macrophages are unclear. This study investigated the role of cilostazol on CD36 expression of murine macrophages enhanced by HNE, a byproduct of lipid peroxidation. The stimulation of macrophages with HNE led to an increased expression of CD36, which was significantly attenuated by NAC, an antioxidant. Moreover, the increased production of ROS by HNE was completely abolished by NADPH oxidase inhibitors, DPI and apocynin, as well as by the 5-LO inhibitor, MK886, but not by inhibitors for other oxidases. This suggested that NADPH-oxidase and 5-LO were major sources of ROS induced by HNE. In addition, HNE-enhanced expression of CD36 was reduced by these inhibitors, which indicated a role for NADPH oxidase and 5-LO on CD36 expression. In our present study, cilostazol was a significant inhibitor of ROS production, as well as CD36 expression induced by HNE. An increase in NADPH oxidase activity by HNE was significantly attenuated by cilostazol, however cilostazol had no effect on HNE-enhanced 5-LO activity. Together, these results suggest that cilostazol attenuates HNE-enhanced CD36 expression on murine macrophages thorough inhibition of NADPH oxidase-derived ROS generation.

Key Words: Cilostazol, HNE, CD36, NADPH oxidase

INTRODUCTION

Oxidative stress is generally regarded as a key factor in atherogenesis, in that it is closely associated with inflammation and the formation of bioactive lipids (Berliner et al., 1995; Stocker and Keaney Jr, 2004; Leonarduzzi et al., 2005). Among the reactive aldehydes, 4-hydroxynonenal (HNE), a major end-product of lipid peroxidation (Esterbauer et al., 1991), is believed to promote the primary pathogenic effects of oxidative stress (Uchida, 2003; Lee et al., 2006; Sampey et al., 2007). In our previous study, elevated levels of HNE have been detected in atherosclerotic lesions (Yun et al., 2008), supporting an etiological role for HNE in the development of atherosclerosis.

One of the earliest events in atherogenesis is the accumulation of oxidized low density lipoprotein (oxLDL) in the intima layer of blood vessels and the subsequent uptake of modified lipoprotein by macrophages, leading to foam cell formation (Ross, 1999; Moore and Freeman, 2006). In macrophages, oxLDL is ingested by scavenger receptors (SR) including class A SR (SR-A) and class B SR (CD36), which are two of the most important SR for foam cell formation (Greaves and Gordon, 2005). Among these SR, the macrophage CD36 receptor was shown to be involved in the formation of foam cells and is highly expressed in atherosclerotic lesions (Nakata et al., 1999). Research studies indicate that the expression of CD36 is stimulated by phorbol esters, macrophage colony stimulating factor (M-CSF), and possibly ox-LDL (Yesner et al., 1996; Han et al., 1997; Yoshida et al., 1998). Moreover, our previous study demonstrated that HNE directly enhanced the expression of CD36 on murine macrophages via p38 MAPK-mediated activation of 5-lipoxygenase (5-LO), which leading to the increased uptake of oxLDL towards foam cell formation (Yun et al., 2008; Yun et al., 2009).

Kimura et al. (1985) reported that cilostazol [6-[4-(1-cyclohexyl-1H-tetrazol-5-yl) butoxy]-3,4-dihydro-2(1H)-quinolinone] increased intracellular cAMP by blocking its hydrolysis by type III phosphodiesterase (PDE3) (Kimura et al., 1985). This resulted in reduced platelet aggregation and in peripheral vasodilation. In our previous study, cilostazol inhibited NAD(P)H oxidase-dependent superoxide formation and the release of cytokines concomitant with the suppression of atheromeric plaque formation (Shin et al., 2004). In addition, cilostazol reduced atherosclerosis by inhibiting the formation of superoxide and TNF-α, and thereby reducing NF-κB activation/transcription of VCAM-1/MCP-1 ex-
pression, and monocyte recruitment of LDL receptor-null mice fed high cholesterol (Lee et al., 2005). Recently, Okatsu et al. (2009) reported that cilostazol inhibited the uptake of modified LDL and foam cell formation in mouse peritoneal macrophages. However, although anti-atherogenic effects of cilostazol have been suggested in the previous studies, the molecular mechanisms involved in macrophage foam cell formation have not been clarified.

Given that HNE is a sensitive marker for oxidative stress and increased CD36 expression on macrophages, it is likely hypothesized that cilostazol may regulate CD36 expression induced by HNE. In order to verify the role of cilostazol on CD36 expression, we determined 1) the stimulatory effects of HNE on CD36 expression of the murine macrophage, 2) the underlying mechanism(s) involved in HNE-dependent regulation of CD36 expression, and 3) the inhibitory effect of cilostazol on the mechanism regulating CD36 expression.

METHODS

Chemicals and antibodies

HNE was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). 2,7’-Dichlorodihydrofluorescein diacetate (DCFH-DA), lucigenin, N-acetylcysteine (NAC), NADPH, diphenylamine iodonium (DPI), rotenone, stigmatellin, allopurinol, indomethacin, nordihydroguaiaretic acid (NDGA), and arachidonic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Apocynin was supplied by Calbiochem (La Jolla, CA, USA). MK886 and baicalein were obtained from Biomol (Plymouth Meeting, PA, USA).

Cell culture and isolation of mouse peritoneal macrophages

J774A.1 macrophages (a murine macrophage cell line; ATCC, Rockville, MD, USA) were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and antibiotic-antimycotic at 37°C in 5% CO2. After reaching confluence, the cells were detached from the surface of T75 culture flasks by gentle scraping. The detached cells were then washed and resuspended in complete medium. Cells between passages 2 and 5 were used for experiments.

Immunoblot analysis

To determine the expression of CD36 at the protein level, 30 μg of protein extracted from macrophages were separated using 10% SDS-PAGE, then transferred electro-photographically to a nitrocellulose membrane (Hybond; Amersham Biosciences, Piscataway, NJ, USA). The membranes were incubated with anti-CD36 antibody (Santa Cruz Biotechnology; Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated IgG antibody (Santa Cruz Biotechnology) was used as the secondary antibody. The immunoblot was visualized using SuperSignal West Dura extended duration substrate kit (Pierce Chemical; Rockford, IL USA). As an internal control, this membrane was reblotted with anti-β-actin antibody (MP Biomedicals; Aurora, Ohio, USA). The blots were scanned using ScanJet 4C (Hewlett-Packard; Palo Alto, CA, USA) and analyzed using UN-SCAN-IT GEL™ software (version 5.1, Silk Scientific Inc; Orem, Utah, USA).

Analysis of mRNA expression

The oligonucleotide primers used to perform RT-PCR were 5’-TCG-GAA-CTG-TTG-GCT-CAT-TG-3’ and 5’-CTC-GCG-GTT-CCT-GAG-TTA-TAT-TT-3’ for CD36, and 5’-TTG-CCA-TTT-GCA-GTG-AAG-TGG-3’ and 5’-TTG-TCA-TGG-ATG-ACC-TTG-GCC-AAG-G-3’ for GAPDH. After macrophages were stimulated with HNE, total RNA from the cells was analyzed by RT-PCR using a Qiagen OneStep RT-PCR kit (Qiagen; Hilden, Germany).

Quantitating ROS generation

Changes in intracellular reactive oxygen species (ROS) levels were evaluated by measuring the oxidative conversion of DCFH-DA to fluorescent DCF as described previously (Amer et al., 2003). Cells grown in 12-well plates were loaded with 10 μM DCFH-DA for 45 min at 37°C, then incubated with HNE under the indicated conditions. The cells were washed with PBS and harvested by gentle scraping. DCF fluorescence of 10,000 cells was detected using FACScaliber flow cytometer (Becton Dickinson; San Jose, CA, USA) and analyzed using the CellQuest Software (version 3.3, Becton Dickinson).

Quantitating NADPH oxidase activity

NADPH-oxidase activity was measured using a lucigenin-enhanced chemiluminescence assay (Manea et al., 2007). Cells were washed briefly with PBS, and then harvested. After low spin centrifugation, the pellet was lysed in phosphate buffer (20 mM monobasic potassium phosphate [pH 7.0], 1 mM EGTA, 10 μM apotinin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, and 0.5 mM phenyl-methylsulfonyl fluoride [PMSF]). The cellular lysates were centrifuged for 10 min at 13,000 rpm and the supernatant was used for the assay. The total protein concentration was determined using a bicinchoninic acid protein assay kit (Sigma Chemical Co.). The reaction mixture comprised a Krebs/HEPES buffer, pH 7.0, lucigenin (5 μM) as the electron acceptor, and NADPH (100 μM) as the substrate. The reaction was initiated by the addition of 25 μg protein, and photon emission was measured every second for 10 min time period using a microtiterplate luminometer (Microlumat LB96P; EG and G Berthold, Germany). The activity was expressed as relative light units (RLU) per second per milligram of total protein (RLU/sec/mg protein).

Quantitation of LTB4 production

Leukotriene B4 (LTB4) production was measured in cell-free supernatants using a commercially available LTB4 assay kit (R&D Systems; Minneapolis, MN, USA). Briefly, after macrophages were stimulated with HNE (10 μM) in the presence of exogenous arachidonic acid (40 μM), the conditioned media was harvested and then concentrated using Vivaspin 2 (Sartorius Biolab; Goettingen, Germany). The amount of LTB4 in the concentrated media was measured using ELISA (Bio-Tek Instrument Inc; Winooski, VT, USA) following the manufacturer’s instructions.

Statistical analysis

The results were expressed as means±SE. Statistical sig-
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RESULTS

HNE enhanced CD36 expression in J774A.1 macrophages

SR family proteins play an important role in cellular lipid accumulation and in macrophage foam cell formation. Therefore, we first investigated whether HNE could modulate the expression of CD36, a major SR, in J774A.1 macrophages. The effect of HNE on CD36 mRNA expression was examined by semi-quantitative RT-PCR analysis. As shown in Fig. 1A, HNE (10 μM) significantly increased CD36 mRNA in a time-dependent manner for up to 8 hrs. Moreover, immunoblot analysis showed that CD36 protein levels were enhanced significantly in murine macrophages upon exposure to HNE for the indicated time (Fig. 1B).

Role of ROS on HNE-induced CD36 expression

To determine the involvement of ROS in HNE-induced expression of CD36, we pretreated macrophages with NAC, an antioxidant, followed by stimulation with HNE (10 μM) for the indicated time. As shown in Fig. 2, the increase in HNE-induced CD36 expression at the mRNA and protein levels was attenuated significantly by treatment with NAC, indicating participation of ROS in HNE-enhanced CD36 expression.

HNE enhanced ROS production in J774A.1 macrophages

To examine the level of intracellular ROS by treatment...
with HNE in J774A.1 macrophages, cells were labeled with DCFH-DA, and then incubated with HNE for 45 min. This was followed by flow cytometric analysis. As shown in Fig. 3A, after stimulation with increasing concentrations of HNE ranging from 0.1 to 30 μM, macrophages showed a concentration-dependent increase in the level of intracellular ROS. To further elucidate enzymatic sources of HNE-induced ROS generation, macrophages were stimulated with 10 μM HNE for 45 min in the presence of various inhibitors for ROS-generating enzymes. As shown in Fig. 3B, HNE-induced ROS generation was almost completely inhibited by DPI and apocynin, specific inhibitors for NADPH-oxidase, as well as NDGA, a LO inhibitor, but not by inhibitors of other enzyme source including xanthine oxidase and mitochondrial oxidases (Fig. 3B). Furthermore, since NDGA was not selective to individual LO because it inhibited most of eicosanoids including 5-LO, 12-LO, and cyclooxygenase (COX), we examined the individual role of eicosanoid pathways in HNE-induced ROS production. As shown in Fig. 3C, HNE-induced ROS generation was sig-

**Fig. 3. Effects of inhibitors of NADPH oxidase or lipoxygenase on HNE-induced ROS production in J774A.1 macrophages. (A) Cells were treated with different concentrations of HNE for 45 min, and then ROS generation was measured by flow cytometry (FACS) using DCF fluorescence. Fluorescence intensity was analyzed by CellQuest Software. Data was presented as mean±SE from 5 independent experiments. **p<0.01 vs. concentration 0. (B) Cells were incubated with 10 μM HNE for 45 min after pre-treatment with various inhibitors for ROS including N-acetylcysteine (Nac, 5 mM), diphenylamine iodonium (DPI, 10 μM), apocynin (Apo, 300 μM), nordihydroguaiaretic acid (NDGA, 10 μM), allopurinol (Allo, 100 μM), or rotenone (Ro, 1 μM) and then stimulated with 10 μM HNE for 45 min. ROS generation was analyzed by FACS. Data was presented as mean±SE from 5 independent experiments. **p<0.01 vs. control. (C) Cells were pre-treated with eicosanoid inhibitors including MK886 (10 μM), baicalin (10 μM), or indomethacin (Indo, 100 μM) for 30 min, and then stimulated with 10 μM HNE for 45 min. ROS generation was analyzed by FACS. Data was presented as mean±SE from 5 independent experiments. **p<0.01 vs. veh (vehicle).**

**Fig. 4. Effect of inhibitors for NADPH oxidase or 5-lipoxygenase on HNE-induced CD36 expression in J774A.1 macrophages.** Macrophages were treated with diphenylamine iodonium (DPI, 10 μM), apocynin (Apo, 300 μM), or MK886 (10 μM) for 30 min prior to HNE application. Cells were stimulated with 10 μM HNE for the indicated time. The levels of CD36 mRNA and protein were determined by RT-PCR (A) and Western blot analysis (B), respectively. Each fig is the representative of 5 independent experiments. Data was presented as mean±SE from 5 independent experiments. **p<0.01 vs. control; #p<0.05; ##p<0.01 vs. veh (vehicle).**
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Effects of cilostazol on the expression of CD36 in J774A.1 macrophages. Effects of cilostazol on the expression of CD36 mRNA (A) and protein (B) were analyzed using RT-PCR and Western blot, respectively. Each fig is the representative of 4 independent experiments. Data was presented as mean±SE from 4 independent experiments. **p < 0.01 vs. control; ##p < 0.01 vs. HNE alone.

Role of NADPH oxidase and 5-LO on HNE-induced CD36 expression

Next, we investigated whether enzymes involved in HNE-induced ROS generation play a role in regulation of HNE-enhanced CD36 expression. Similar to the effects of HNE-induced ROS generation, inhibitors for NADPH oxidase and 5-LO attenuated the increase in HNE-induced CD36 expression (Fig. 4). These results suggested that both NADPH oxidase and 5-LO were essential for HNE-induced expression of CD36 in murine macrophages.

Cilostazol inhibited NADPH oxidase activity, not 5-LO activity

Then, we evaluated the effect of cilostazol on the activities of NADPH-oxidase and 5-LO enhanced by HNE. After J774A.1 macrophages were exposed to HNE (10 μM) for 45 min, NADPH oxidase activity was measured by lucigenin-enhanced chemiluminescence assay. Our data showed that the activity of NADPH-oxidase was increased significantly in response to HNE, which was concentration dependently suppressed by cilostazol (Fig. 7A). Furthermore, to determine the activity of 5-LO, we measured a 5-LO metabolite, LTB4, as a marker for 5-LO activity. As shown in Fig. 7B, the incubation of macrophages with HNE (10 μM) for 30 min in presence of 40 μM arachidonic acid as exogenous substrate (to circumvent the need for phosphatase activity) significantly elevated 5-LO activity. However, cil-
ostazol had no effect on HNE-induced 5-LO activation. These results suggested that the inhibitory effect of cilostazol on HNE-mediated expression of CD36 was mediated by inhibition of NADPH-oxidase, but not by 5-LO.

**DISCUSSION**

The present study demonstrated that HNE-enhanced ROS produces a subsequent increase in CD36 expression in murine macrophages. This response was blunted by NAC, an antioxidant, as well as by inhibitors of NADPH-oxidase and 5-LO. Linked to these results, cilostazol significantly inhibited ROS production, as well as CD36 expression in murine macrophages stimulated with HNE. An increase in NADPH-oxidase activity by HNE was attenuated significantly by cilostazol, however, cilostazol had no effect on HNE-enhanced 5-LO activity. Thus, it is suggested that cilostazol attenuates HNE-enhanced CD36 expression in murine macrophages through inhibition of NADPH-oxidase-derived ROS generation.

An increasing number of studies have suggested the potential pathophysiological role of HNE, a major electrophilic end-product of lipid peroxidation in atherosclerotic lesion formation (Leonarduzzi et al., 2005). In support of these hypotheses, an immunohistochemical analysis of atherosclerotic lesions demonstrated that intense HNE immunoreactivity in atherosclerotic lesions was associated with cells, primarily macrophages (Kumagai et al., 2004; Yun et al., 2008). In our previous study, consistent with the report in which HNE enhanced CD36 expression in murine macrophages (Ishii et al., 2004), in addition, HNE caused an increased expression of CD36 in murine macrophages with an increased foam cell formation (Yun et al., 2008). This was mediated via p38-MAPK pathway activated by 5-LO metabolites in macrophages (Yun et al., 2009). Although HNE was suggested as an inducer of macrophage foam cell formation, however, the precise mechanism involving the expression of scavenger receptors on macrophages remains unclear.

Consistent with other reports showing that lipid peroxidation products, including HNE and other reactive aldehydes, stimulated ROS formation in various types of cells (Knobel et al., 2006; Lee et al., 2006; Raza and John, 2006; Forman et al., 2008), a significant increase in ROS generation by HNE was observed in J774A.1 macrophages.

Although ROS has been reported to be generated by a variety of enzymatic sources (Thannickal et al., 2000), our present study demonstrates that ROS production in HNE-stimulated macrophages was attenuated by inhibition of either NADPH-oxidase or 5-LO, but not by inhibitors of other pro-oxidant enzymes, including xanthine oxidase and mitochondrial oxidases. Thus, it was suggested that HNE-induced ROS generation in macrophages occurred exclusively through activation of both NADPH oxidase and 5-LO. Similar to the effects of HNE-induced ROS generation, inhibitors for NADPH oxidase and 5-lipoxygenase attenuated the increase in HNE-induced CD36 expression. These results suggest that both NADPH oxidase and 5-LO were essential for HNE-induced expression of CD36 in murine macrophages. Considering the facts that both these enzyme inhibitors had similar magnitudes of inhibition during ROS generation and CD36 expression, it was also suggested that there was an interaction between these 2 enzymes in ROS generation in macrophages.

Arachidonic acid is converted to LT by 5-LO, and the major products formed in the neutrophils are 5-HETE, LTA4 (the precursor of LTB4) and cysteinyl LT (CysLT), such as LTC4, LTD4 and LTE4 (Shimizu et al., 1984; Samuelsson et al., 1987; Funk, 2001; Peters-Golden and Brock, 2003). In contrast to prostaglandin F2a (PGF2a), a COX metabolite, which stimulates NADPH-oxidase through transcriptional upregulation of NOX-1, a subunit of NADPH oxidase (Katsuyama, 2007; Cevik, 2008), LTB4 activated NADPH oxidase through phosphorylation and translocation of p47phox to the membrane, a process that was dependent on PKC activity (Woo et al., 2002; Serezani et al., 2005). These observations suggest both a short-term and a delayed activation of the NADPH-oxidase system by eicosanoids. The short-term activation may involve membrane translocation of cytosolic subunits by 5-LO-derived LTB4, formation, while the delayed long-term activation may involve a transcriptional up-regulation of the NADPH oxidase sub-units by PGF2a. In this regard, our present study is of particular interest, as it shows the HNE-enhanced synthesis and release of LTB4 in murine macrophages. However, since indomethacin, an unspecific COX inhibitor, had no influence on ROS formation by HNE, it was suggested that PGF2a formation might not be involved in the early phase of NADPH-oxidase activation.

The previous report noted that cilostazol strongly inhibited the uptake of modified LDL by decreasing the pro-
tein expression of SR-A, but not CD36 (Okutsu et al., 2009). However, in the present study using J774A.1 macrophages stimulated with HNE, cilostazol significantly suppressed HNE-induced CD36 expression. Moreover, cilostazol attenuated HNE-induced activation of NADPH oxidase in murine macrophages, as well as an increased generation of ROS. This is supported by previous reports in which cilostazol reduced atherosclerosis by inhibition of NAD(P)H oxidase-dependent superoxide production, thereby reducing NF-κB activation/transcription of VCAM-1/MCP-1 expressions, and monocyte recruitments in LDL receptor-null mice fed a high cholesterol diet. (Shin et al., 2004; Lee et al., 2005).

In conclusion, the present study demonstrates that cilostazol suppresses HNE-stimulated CD36 expression in a significant manner, as well as ROS generation in murine macrophages through inhibition of NADPH-oxidase. Moreover, the identification of a role for cilostazol in the activation of NADPH-oxidase by HNE suggests that cilostazol has an anti-atherogenic action in which ROS might be responsible for vascular pathophysiology.

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REFERENCES

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