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HMG-CoA reductase inhibitors induce COX-2 gene expression in murine macrophages: role of MAPK cascades and promoter elements for CREB and C/EBPβ

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Abstract

Except functioning as lipid-lowering agents, HMG-CoA inhibitors, statins, are good tools to clarify the signaling role of small G proteins. In this study, we found in murine RAW264.7 macrophages, statins within 1-30 μM stimulated COX-2 gene transcription and PGE_2 formation, displaying potencies as lovastatin > fluvastatin > atorvastatin >> pravastatin. Transfection experiments with COX-2 promoter construct showed the necessity of c/EBPβ and CRE promoter sites, but not NF-κB promoter site. Effects of statins on the activation of COX-2 promoter, induction of COX-2 protein and PGE_2 production were all prevented by mevalonate and prenylated metabolites, FPP and GGPP. In consistent with the effect of statins, manumycin A, farnesyltransferase inhibitor, and geranylgeranyltransferase inhibitor increased PGE_2 production and COX-2 induction. Likewise, toxin B, an inhibitor of Rho family members, caused a prominent COX-2 induction. Results also indicated that tyrosine kinase, ERK, and p38 MAPK play essential roles in statin action. Taken together these results not only demonstrate a unique action of statins in the up-regulation of COX-2 expression in macrophages, but also suggest a negative role controlled by small G proteins in COX-2 gene regulation. Remove this

negative control by impairing G protein prenylation with statins leads to MAPKs activation and promotes COX-2 gene expression through the activation at CRE and c/EBPβ sites.

Key Words: Statins; Cyclooxygenase-2; MAPKs; G protein prenylation; Macrophages.

Introduction

Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are approved for treatment in hyperlipidemia. There is accumulating clinical and experimental evidence showing that statins have beneficial effects in thrombosis, stroke, inflammation, bone fractures, glucose tolerance, Th1-mediated autoimmune diseases, cardiac transplant rejection, and Alzheimer disease [1, 2]. Molecular mechanisms underlying pleiotropic properties of statins are independent of their classical actions on lipoproteins, but base on a concurrent reduction of mevalonate, farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP). The synthetic deficiency followed by depletion of cellular pools of GGPP and FPP causes profound effects on modulation of physiological functions of some small G proteins. In this aspect, Ras, Rho and Rac are the most well investigated targets. Translocation of these proteins from the cytoplasm to the plasma membrane, which is an essential step for inducing biological activity, is respectively dependent on farnesylation and geranylgeranylation [3-5]. For example, geranylgeranylation of Rho is involved in the up-regulation of ICAM-1 expression, cell proliferation, survival, migration, inhibition

of PPARα activation, and down-regulation of iNOS and eNOS [6-12]. The up-regulation of eNOS expression and NO production from vascular endothelial cells primarily account for the cardiovascular benefits of statins [1, 12].

Prostaglandins (PGs), produced by an inducible enzyme cyclooxygenase-2 (COX-2), have been implicated as key mediators of many inflammatory responses [13]. In the COX-2 gene, promoter elements for nuclear factor κB (NF-κB), CCAAT/enhancer-binding protein β (c/EBPβ) and a cAMP-responsive element (CRE) were found to be important in regulating transcription [14, 15]. Although there is transcriptional redundancy among the NF-κB, c/EBPβ, and CRE promoter sites in mediating COX-2 transcription in macrophages, maximal transcriptional activity requires cooperation among these elements [16]. Like other inflammatory mediators, positive regulation of COX-2 gene transcription by Rho proteins, including RhoA, Rac and/or Cdc42, has recently been identified in epithelial cells, renal mesangial cells, and fibroblasts [17-20]. However, results in human aortic smooth muscle cells using statins (mevastatin and lovastatin), selective inhibitors of geranylgeranyltransferase and modulators of Rho, demonstrated that statins can up-regulate COX-2 expression

through inhibition of Rho [21]. It thus appears existence of a cell-type difference in Rho-dependent modulation of COX-2 gene transcription.

To further address this controversial effects respect to of COX-2 regulation by small G proteins, we have performed experiments in statin-treated RAW264.7 macrophages. Using this cell type we have previously demonstrated the abilities of statins to block iNOS induction caused by endotoxin lipopolysaccharide (LPS) and interferon-γ (IFN-γ) [22]. This NO inhibitory action is mediated by the blockade of NF-κB and signal transducer and activator of transcription signaling. Surprisingly in this study we demonstrated an opposite effect of statins for COX-2 regulation. In the absence of stimulating factors, statins themselves are sufficient to induce COX-2 gene transcription and PGE_2 formation in RAW264.7 macrophages. This action is mediated by the MAPKs signaling cascades and activation of transcriptional factors acting on c/EBPβ and CRE sites of the COX-2 promoter.

Materials and Methods

Reagents

DMEM, fetal bovine serum, penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY). Polyclonal antibodies against COX-2, ERK, p38 MAPK and horseradish peroxidase-coupled anti-rabbit antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The ECL detection agents were purchased from Amersham Biosciences (Piscataway, NJ). Murine IFN-γ was purchased from R&D (Minneapolis, MN). Toxin B from *Clostridium difficile* was obtained from Calbiochem (La Jolla, CA). The Ras inhibitor manumycin A, lovastatin, phenol-extracted LPS (L8274) from *E. coli*, mevalonate, FPP, GGPP, farnesyltransferase inhibitor (FTI-277), geranylgeranyltransferase inhibitor (GGTI-298) and other chemicals were purchased from Sigma Aldrich (St. Louis, MO). Atorvastatin, fluvastatin and pravastatin were respectively provided by Pflizer Inc. (New York, USA), Novartis (Basel, Switzerland) and Sankyo Co., Ltd. (Tokyo, Japan). All materials for SDS-PAGE were obtained form Bio-Rad Laboratories (Hercules, CA).

Cell culture

Murine RAW264.7 macrophages obtained from American Type Culture Collection (Manassas, VA) were grown at 37°C in 5% CO_2 using DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin.

PGE_2 assay

RAW264.7 macrophages cultured in 24-well plate were stimulated with indicated agents and cultured for different periods. PGE_2 production was measured by commercial EIA kit (Cayman Chemical Company) according to manufacturer's instruction.

Immunoblotting analysis

After stimulation, cells were rinsed twice with ice-cold PBS, and 100 μl of cell lysis buffer (20 mM Tris-HCl, pH 7.5, 125 mM NaCl, 1% Triton X-100, 1 mM $MgCl_2$, 25 mM β-glycerophosphate, 50 mM NaF, 100 μM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin) was then added. Cell lysates with equal protein amount were subjected to SDS-PAGE and immunoblotted with the specific antibodies.

Reverse transcription-polymerase chain reaction (RT-PCR)

To amplify COX-2 mRNA, macrophages were homogenized with 1 ml of RNAzol B reagent (Gibco), total RNA was extracted by acid guanidinium

thiocyanate-phenol-chloroform extraction, and RT was performed using StrataScript RT-PCR Kit. The oligonucleotide primers used correspond to the mouse macrophages COX-2 (5'-CAG CAA ATC CTT GCT GTT CC-3' and 5'-TGG GCA AAG AAT GCA AAC ATC-3'), and mouse β-actin (5'-GAC TAC CTC ATG AAG ATC CT-3' and 5'-CCA CAT CTG CTG GAA GGT GG-3'). PCR was performed by an initial denature (1 min at 94°C), 35 cycles of amplification (94°C for 1 min, 58°C for 30 sec, and 72°C for 30 sec), and an extension (72°C for 7 min). PCR products were analyzed on 2% agarose gel. The mRNA of β-actin served as an internal control for sample loading and mRNA integrity.

Real-time RT-PCR with SYBR green detection

Real-time RT-PCR was performed using an ABI Prism 7700 (Applied Biosystems). Real-time RT-PCR fluorescence detection was performed in 96-well plates using platinum SYBR green qPCR superMix-UDG (Invitrogen). Each 25-μl PCR reaction contained cDNA, platinum SYBR green qPCR superMix-UDG, forward and reverse primers (COX-2: CTTTGCCCAGCACTTCA, CAGTCTAGAGTTTCACCGTAA; actin: CGGGGACCTGACTGACTACC, AGGAAGGCTGGAAGAGTGC), and the

Passive Reference dye (ROX) to normalize the SYBR green/double-stranded DNA complex signal during analysis to correct for well-to-well variations. Primer concentrations were optimized to yield the lowest concentration of primers that yielded the same C_t values as recommended by Invitrogen. A no RT control RNA sample was used with each real-time RT-PCR experiment containing β-actin primers to verify no genomic DNA contamination. Amplification parameters were UDG incubation, for one cycle at 50°C for 2 min to prevent amplification of carryover DNA; denaturation, UDG inactivation at 95°C for 2 min; amplification, 40 cycles of 95°C/15 s and 60°C/30 s. Amplification products using SYBR Green detection were routinely checked using dissociation curve software (Perkin-Elmer Life Sciences) and by gel electrophoresis on a 1% agarose gel then visualized under UV light following staining with 0.05% ethidium bromide to confirm the size of the DNA fragment and that only one product was formed. Samples were compared using the relative (comparative) C_t method to analyze results. The C_t value, which is inversely proportional to the initial template copy number, is the calculated cycle number where the fluorescence signal emitted is significantly above background levels. The fold induction by real-time RT-PCR was

measured in triplicate relative to time-matched vehicle-treated controls and calculated after adjusting for β- actin using $2^{-\Delta\Delta Ct}$, where ΔC_t = target gene C_t – β-actin C_t, and $\Delta\Delta C_t$ = ΔC_t treatment – ΔC_t control.

Transient transfection and luciferase assay

Murine COX-2 promoter with wild type construct (native -966/+23), deletion constructs (-350/+23, and -98/+23), and mutant constructs cloned into pGL3-basic vector (Promega) were kindly provided by Dr. Byron Wingerd (Michigan State University, East Lansing, MI). Wild-type CRE sequence ACGTCA was changed to AATTCA. Wild-type c/EBPβ site 2 sequences TTGCGCAAC was changed to CCGCTCAAC. Wild-type NF-κB sequence GGGGATTCCC was changed to GGGCCTTCCC. We also constructed a COX-2 promoter where both CRE and c/EBPβ sites were mutated. All DNAs were prepared using endotoxin-free plasmid preparation kits (Qiagen). Using electroporation (280 volts, 1070 μF, 0.4 msec time constant), RAW264.7 cells (2×10^7 cells/cuvette) were co-transfected with 0.25 μg of either an expression vector or empty plasmid, and 0.25 μg of β-galactosidase expression vector (pCR3lacZ; Pharmacia, Sweden). After electroporation, transfected cells were cultured

in 24-well plate at 2 x 10^6 cells/well and incubated in 10% FBS DMEM for 24 h. Subsequently, cells were treated with agents for 24 h and using Promega kit the luciferase activity was assayed by a microplate luminometer (Packard, Meriden, CT). Luciferase activity values were normalized to transfection efficiency monitored by β-galactosidase expression, and was presented as the percentage of luciferase activity measured without statin stimulation.

p38 MAPK kinase assay

After stimulation cells were washed twice with ice-cold PBS, lysed in 1 ml lysis buffer and centrifuged. The supernatant was collected, and anti-p38 MAPK antibody with protein A/G-agarose beads was added, and then stored at 4°C overnight. The immunoprecipitates were washed three times with lysis buffer and immune-complex kinase assays were performed on the antibody immunoprecipitates at 30°C for 30 min in 20 μl kinase reaction buffer (25 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 0.1 mM Na_3VO_4, 2 mM dithiothreitol, 100 mM ATP, 10 μCi [γ-^{32}P]ATP) containing 50 μg/ml myelin basic protein (MBP). The reaction was terminated with 5X Laemmli sample buffer and samples were separated on SDS-PAGE followed by autoradiography.

Statistical evaluation

Values were expressed as the mean ± S.E.M. of at least three experiments, which was performed in duplicate. Analysis of variance (ANOVA) was used to assess the statistical significance of the differences, and a “p” value of less than 0.05 is considered statistically significant.

Results

Statins transcriptionally increase COX-2 gene expression and PGE_2 formation in RAW264.7 macrophages

Exposure of RAW264.7 macrophages to LPS (1 μg/ml) and IFN-γ (10 ng/ml) for 24 h led to a significant increase in PGE_2 production compared with basal conditions. PGE_2 level was increased from 1145±84 pg/ml (n=11) in untreated cells to 6346±521 pg/ml (n=11) and 3048±287 pg/ml (n=6) of cells incubated with LPS and IFN-γ, respectively (Fig. 1A). Simultaneous addition of lovastatin (10 μM) together with LPS or IFN-γ led to a higher increase of PGE_2 production than LPS or IFN-γ treatment alone. We found that this PGE_2 increase was resulting from stimulating effect of lovastatin, which by itself increased PGE_2 release to 325±50% of control at 10 μM (n=4). In agreement with the extent of PGE_2 increase, COX-2 protein was markedly induced in lovastatin-stimulated cells as compared to cells without stimulation. The induced COX-2 level of 10 μM lovastatin was 64±8 % of that of LPS (1 μg/ml), and 121±15 % of that of IFN-γ. Moreover, COX-2 level induced by LPS and IFN-γ was accordingly

enhanced by lovastatin (Fig. 1B). Under these conditions, no change of COX-1 expression was observed (data not shown). To clear the possible effect on COX-2 directly, we measured COX-2 enzyme activity *in vitro*. COX-2 activity assay with kit purchased from Cayman (Cat. No 760151) did not reveal direct effect of lovastatin (30 μM) on COX-2 enzyme activity (data not shown).

Since lovastatin was able to stimulate COX-2 protein expression and PGE_2 production, we extended this action to other statins for understanding the drug specificity. As shown in Fig. 2A, four statins were compared and showed different potencies. In terms of PGE_2 production, lovastatin was the most potent statin tested, which induced a concentration-dependent stimulation at a concentration ranging from 1 to 30 μM. Examining the stimulatory efficacies achieved at 30 μM (shown in parentheses), the potency among these statins was in the order of lovastatin (360%) > fluvastatin (250%) > atorvastatin (250%) >> pravastatin. At 30 μM, pravastatin did not stimulate PGE_2 production. In agreement with the extents for PGE_2 production, COX-2 protein was accordingly induced by statins except pravastatin (Fig. 2B). Compared to the response of LPS, lovastatin (30 μM) and fluvastatin (30 μM) displayed 74±11 %

and 51±7 % efficacy, respectively (Fig. 2C). The time-dependent effect of lovastatin on COX-2 expression was shown (Fig. 2D). Consistent to our previous results [22], no significant cell toxicity was observed following treatment of each statin at these concentrations for 24 h (data not shown).

Since COX-2 is an inducible gene product, we determined whether the effect of statins results from the increased gene transcription. Using RT-PCR analysis, we found that COX-2 mRNA was time-dependently increased by 30 μM lovastatin and fluvastatin, but not by pravastatin (Fig. 2E). Moreover, real-time PCR analysis confirmed this action of lovastatin (Fig. 2F).

c/EBPβ and CRE promoter elements mediate statin-induced COX-2 transcription

Following identification of transcriptional regulation of COX-2 by statins, the relative contribution of the different promoter elements in mediating COX-2 transcription was elucidated. Previous study has identified the DNA binding sites for NF-κB (-402/-393), c/EBPβ (site 1, -93/-85 and site 2, -138/130) and CRE (-59/-52) in COX-2 promoter [14, 15]. Studies with LPS stimulation revealed the necessity of CRE

and c/EBPβ site 2, but not of distal NF-κB and c/EBPβ site 1 [15]. According to this finding, we performed transient transfection experiments using reporter constructs harboring deleted and mutated variants of the COX-2 promoter to characterize the promoter region(s) regulating lovastatin-mediated transcription. Transfection experiment with a –966/+23 COX-2 promoter-luciferase reporter construct showed that lovastatin was able to increase luciferase activity in a concentration-dependent manner (Fig. 3A). Deletion of the NF-κB site (-350/+23 reporter) did not affect lovastatin- and LPS-induced reporter activity. However, deletion of both c/EBPβ site 2 and NF-κB (-98/+23 reporter) diminished the effects of lovastatin and LPS by 39% and 69%, respectively (Fig. 3B). These results suggest that like the effect of LPS, both c/EBPβ site 2 and CRE site at murine COX-2 promoter are required for lovastatin-induced expression in macrophages. To further investigate the contribution of these transcription binding sites, we compared cells transfected with COX-2 reporter plasmids of wild-type or mutant form. The results indicated that mutation of the NF-κB site did not attenuate LPS- or lovastatin-induced activity. In contrast, mutation of the c/EBPβ site 2 and CRE site strongly repressed the responses of LPS and lovastatin. When both c/EBPβ and

CRE sites were mutated, a complete abolishment was seen (Fig. 3C).

COX-2 stimulation by statins is dependent on protein isoprenylation

To further identify the product of HMG-CoA reductase reaction necessary for the effect of statins, we incubated cells with mevalonate, FPP, or GGPP in the presence of statins. Mevalonate is a cholesterol precursor, and its metabolites FPP and GGPP are involved in farnesylation and geranylgeranylation of proteins, respectively. Figure 4A showed that mevalonate (100 μM) as well as GGPP (10 μM) almost completely reversed the effects of lovastatin and fluvastatin on PGE_2 production. FPP (10 μM), on the other hand, only induced a partial prevention by 35-50%. Meanwhile, mevalonate, FPP and GGPP alone did not modulate the basal and LPS-dependent PGE_2 production (Fig. 4A). In consistent with PGE_2 production, the induced protein level and reporter activity of COX-2 by lovastatin, but not by LPS, were concomitantly suppressed (Fig. 4B and 4C). These observations suggest that the effect of statins on COX-2 expression is ascribed to the reduction of protein isoprenylation, which negatively regulates COX-2 gene expression.

To further verify the involvement of reduced isoprenylation of signaling proteins in statin-induced COX-2 expression, we studied the effects of manumycin A, FTI and GGTI. Manumycin A is a potent and selective inhibitor of farnesyltransferase (FT, IC_{50}=5 μM) compared to geranylgeranyltransferase (GGT, IC_{50}=180 μM), and acts as an inhibitor of Ras function [23-25]. FTI and GGTI can respectively block FT and GGT to transfer farnesyl moiety and geranylgeranyl moiety to the carboxy terminus of Ras and Rho [26, 27]. Our results showed that after 24 incubation, GGTI (10 μM), FTI (10 μM) and manumycin A (10 or 30 μM) caused a weak but significant increase of PGE_2 production and COX-2 expression (Fig. 5A & B). Moreover, simultaneous presence of FTI and GGTI led to additive responses in PGE_2 production and COX-2 protein expression. Likewise direct inhibition of Rho family proteins with toxin B (400 pM) caused COX-2 induction, with level being comparable to lovastatin (Fig. 5B). Moreover when combination with LPS (1 μg/ml), manumycin A enhanced LPS-induced PGE_2 production and COX-2 expression in a concentration-dependent manner (Fig. 5C). Because of cytotoxicity occurrence at 100 μM manumycin A., we did not further determine higher concentrations.

Signaling pathways of statin-induced PGE_2 formation

Numbers of pathways have been implicated in transmitting the extracellular signals to the nuclei for COX-2 gene expression. To investigate the signal transduction pathway(s) involved in regulating COX-2 expression in response to statin, we examined the effects of chemical inhibitors of signaling intermediates on PGE_2 levels. In addition, since previous reports indicated the participation of tyrosine phosphorylation in signaling pathway underlying simvastatin-induced cell death in L6 myoblasts [28, 29], we examined the effects of herbimycin A (a non-selective inhibitor of tyrosine kinases) and PP2 (a selective inhibitor of Src tyrosine kinases). The effects of these kinase inhibitors on LPS action were simultaneously determined for comparison. As shown in Fig. 6, we found that treatment of cells with the MEK inhibitor (PD98059), p38 inhibitor (SB203580), ~~JNK inhibitor (SP600125),~~ and tyrosine kinase inhibitors (herbimycin A and PP2) inhibited lovastatin-, fluvastatin-, and LPS-induced PGE_2 formation. Rho kinase inhibitor (Y27632) did not have any effect. In contrast to the inhibition on LPS response, PKC inhibitors (Ro318220 and GF109203X) and PKA

inhibitor (KT5720) failed to change the responses of lovastatin and fluvastatin. These results suggest the participation of ERK and p38 MAPK, but not PKC, PKA or Rho kinase, in COX-2 response of statins.

Next we used kinase assay as well as immunoblotting to verify the role of p38 MAPK and ERK. Fig. 7A showed the stimulating effects of lovastatin (30 μM) and LPS (1 μg/ml) on p38 MAPK activity. This effect of lovastatin can maintain for at least 3 h. Furthermore Fig. 7B showed lovastatin is able to induce ERK phosphorylation, while pravastatin cannot.

Discussion

COX-2 is a prostaglandin endoperoxide synthase that is dramatically induced by multiple extracellular stimuli and oncogene products. Given the pivotal role of inducible prostaglandin production in many normal and abnormal processes, particularly contributing to the development of inflammatory responses and cancer, the transcriptional regulation of inducible COX-2 expression is the subject of intense interest. Our results demonstrate that HMG-CoA reductase inhibitors induce the activation of COX-2 promoter and increase COX-2 gene expression in murine macrophages. These effects are the result of decreased isoprenylation of small GTPase proteins subsequent to the blockade of mevalonate pathway by statins. These findings raise a novel link between small G proteins and negative regulation on COX-2 gene transcription.

Protein isoprenylation by mevalonate-derived isoprenoids is important for the post-translational modification of protein functions. Several lines of evidence suggest that farnesylation of Ras, geranylgeranylation of Rho family proteins, and possibly isoprenylation of other small G proteins are essential for full activation of their

downstream effectors [5, 27, 30]. For example, following activation of these small G proteins by cytokines, COX-2 gene transcription was induced [18-20, 31-33], while inhibition of G proteins by statins prevented cytokines effect [34]. Not only in the down-regulation of COX-2 gene expression through uncoupling small G proteins, statins also attenuated stimuli-induced upregulation of inflammatory mediators with similar action mechanism. The targeted genes negatively modulated include TNF-α, IL-1β, MCP-1, IP-10, MMPs, ICAM, IL-6, IL-8, plasminogen activator inhibitor-1, and VEGF [34-39]. All these actions account for the anti-inflammation and therapeutic benefits of statins.

Based on above findings, it is predictable to observe an inhibition of COX-2 gene transcription by statins. Nevertheless, our present results oppose this prediction, but are consistent with previous study showing the abilities of lovastatin and mevastatin to up-regulate COX-2 in human aortic smooth muscle cells [21]. To our knowledge, current study performed in murine macrophages and that observed in human aortic smooth muscle cells are the only two reports demonstrating the stimulatory effect of statins on COX-2 induction. To explain these controversial data, we suggest the

complexity and integration of upstream signaling pathways involved for COX-2 gene regulation. The outcome of COX-2 expression might depend on microenvironmental factors, cell types and/or species. It is interesting to note that COX-2 induction is not the only proinflammatory effect of stains. Studies have shown the increased expression of iNOS, TNF-α and other cytokines production following statin stimulation [9-11, 40, 41].

Although upregulation of COX-2 expression by statins was reported in cell cultures, it seems not to appear in atherosclerotic condition. High expression of COX-2 in vascular smooth muscle cells has been implicated in cell proliferation and enhanced release of active matrix metalloproteinase, which contributes to cell hyperplasia and plaque instability associated with pathological condition detected in atherosclerosis [42]. Clinical and animal studies confirm the ability of statins to down-regulate increased COX-2 expression in atherosclerotic lesions, and ameliorate inflammatory responses [34, 43]. Supporting this notion, inhibition of COX-2 expression by atorvastatin was demonstrated in cytokine IL-1β/TNF-α stimulated rat vascular smooth muscle cells [34]. Collectively statins have inhibitory effects on COX-2 expression in inflammatory states,

where several potent cytokines, such as IL-1β and TNF-α, are released and play crucial roles for chronic inflammation progress.

Extending to understand whether this action of statins may be of potential clinical relevance, we tested human monocytes and vascular smooth muscle cells. We found, in contrast to the effect in murine macrophages, statins themselves do not change basal level of COX-2 in primary monocytes and THP-1 monocytic cells. Nevertheless, they induce COX-2 expression in rat and human vascular smooth muscle cells (data not shown). We thus conclude stimulation of COX-2 gene expression by statins is species and tissue dependent. It is interesting to note that the cell-specific action of statins also appears in their regulation of iNOS expression. In contrast to the inhibition of stimuli-induced iNOS expression in astrocytes, microglia, macrophages and endothelial cells, statins could up-regulate stimuli-induced iNOS induction in vascular smooth muscle cells, airway epithelial cells, fibroblasts, and cardiac myocytes [9-11, 22, 44-46]. All these controversial results in various cell types might attribute to their actions on multiple signaling targets (small G proteins), which play differential roles and timely cross-interaction in gene transcription [22, 47].

In this study we showed that statin-induced COX-2 expression requires the binding of transcription factors to the c/EBPβ element located at the positions -138/-130 and CRE element located at the positions -59/-52. Both promoter elements, as previously have been demonstrated obligatorily involved in determining COX-2 expression by LPS [15], were required for statin action. However, distal NF-κB site is not required for LPS- [15] or statin-dependent COX-2 induction. This NF-κB-independent action of statins is consistent with previous findings demonstrating their inhibitory effect on IKK/NF-κB activation [22, 37, 39, 48]. Regarding to the upstream signaling pathways induced by statins, we showed the necessity of ERK and p38 MAPK signaling pathways. Moreover, biochemical analyses consistent with pharmacological evidence further prove lovastatin on ERK and p38 MAPK activation. Although studies on different cell systems have shown that statins inhibit stimuli-induced ERK activity [49-51], our system displays distinct manner. A recent study in macrophages also demonstrated lovastatin can prolong LPS-induced ERK activation, and as a result increase TNF-α production [40]. Again it is suggested that cell types and stimulation conditions determine the outcome of signaling cascades, and

dual functionality of small G proteins might exist depending on the situation of cells. In addition, contribution of ERK and p38 MAPK activation in COX-2 mRNA transcription through CRE and c/EBPβ promoter elements has been evidenced [16, 52, 53]. For LPS action, our results are in agreement with previous findings indicating that ERK, p38 MAPK and PKC converge to mediate COX-2 gene expression via integration of transcriptional factors [15, 26, 52, 54, 55].

In contrast to the activation of ERK and p38 MAPK, we cannot observe JNK activation following lovastatin incubation up to 6 h (data not shown). Nevertheless LPS causes a transient and rapid JNK activation (data not shown), which as previous proved is required for LPS-induced COX-2 gene transcription [15]. Given that elevation of intracellular cAMP enhances COX-2 transcription through a PKA/CREB/CRE pathway [56-58], and there is existence of a positive autocrine PGE_2/PKA loop in regulating COX-2 promotion [59], attenuation of LPS-mediated PGE_2 synthesis by PKA inhibitor is expected as shown in this study. Moreover not only the Ser/Thr protein kinases, protein tyrosine kinases, including Src families, are necessary for mediating MAPKs pathway for the expression of COX-2 [60]. Here our data revealed the involvement of

tyrosine protein kinases and two types of MAPKs, but not PKA, in statin-induced up-regulation of COX-2.

Our results indicated the effect of statins is associated with inhibition of protein farnesylation and geranylgeranylation, because three small G proteins modifying agents (manumycin A, FTI, GGTI) were capable of increasing PGE_2 production, regardless the less efficacy. Another data to strength the action owing to G protein inhibition is the reversal effects by FPP and GGPP, and the mimic by manumycin A and toxin B. Manumycin A is a Ras inhibitor, while clostridial toxin B is a Rho-modifier, which can non-specifically inhibit Rho, Rac and Cdc42 by glucosylation [61, 62]. These results suggest one or more small GTPase proteins exert a negative effect on COX-2 gene expression in unstimulated murine macrophages by tonic inhibition of ERK and p38 MAPK activity. In this aspect, several possible candidates of G proteins have been proposed. A study found that decreased activity in the Rac1 and Cdc42 resulted in ERK activation in fibroblasts [63]. In a model of malignant transformation, RhoB has been shown to inhibit constitutive activation of ERK [64]. Another study showed inhibiting Rho kinase (activated by RhoA) increased ERK phosphorylation [65]. Thus the identity

of G proteins and signaling intermediates in murine macrophages responsible for the negative regulation on COX-2 gene are currently unclear and require future investigation.

The four statins we tested in this study are competitive inhibitors of HMG-CoA reductase. Although each statin functions by a similar mechanism of action but maintains unique binding affinities, pharmacokinetics and dosing levels [66-68]. The binding affinities (Ki values) in rat microsomal HMG-CoA reductase are 0.6 nM of lovastatin, 0.3 nM of fluvastatin, and 2.3 nM of pravastatin. The pharmacokinetics are disparate and largely dictated by their lipophilic nature, structure form (active acid form as fluvastatin, atorvastatin and pravastatin, or prodrug lactone form as lovastatin), hepatic metabolism, and half-life (2.8 h of lovastatin, 0.5-1.5 h of fluvastatin, 2-4 h of atorvastatin, and 0.9-1.6 h of pravastatin). In a number of cultured cells, all statins except pravastatin effectively inhibit cholesterol synthesis to a similar degree and their IC_{50} values are not of much difference. Our current result of pravastatin is in agreement with this notion. The lower effect of pravastatin in extrahepatic cells or tissues is ascribed to its hydrophilicity, which hinders its cellular permeation and actions. The

efficacy of pravastatin in inhibiting cholesterol synthesis in hepatocytes and in clinical therapy to a degree comparable to other statins, however, suggests that specific uptake occurs in hepatocytes. This suggestion was also evidenced by presence of a carrier that facilitates the entry of statins into hepatocyes. The therapeutic dosage for hypocholesterolemia is quite similar for these statins (20-80 mg/day for lovastatin and fluvastatin, 10-40 mg/day for pravastatin and 10-80 mg/day for atorvastatin). Even though our results showing potency order as lovastatin > fluvastatin > atorvastatin, we predict differences in lipid solubility, structure form, and metabolic factor might be the reasons for the issue of selectivity and potency. As such previous in vivo studies observed different actions among statins on inhibition of thromboxane production [69].

In conclusion, we demonstrated a novel and unique action of statins in stimulating COX-2 gene expression in murine macrophages. This action is resulting from the interference with Rho family proteins, leading to ERK and p38 MAPK activation. In turn COX-2 expression is up-regulated through integration of promoter elements for CRE and c/EBPβ activation.

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Legend

FIG. 1. Effects of lovastatin on LPS- and IFN-γ-induced PGE_2 release and COX-2 induction in RAW264.7 macrophages. After cells were treated with lovastatin, LPS and/or IFN-γ for 24 h, culture medium was collected for PGE_2 measurement with ELISA kit (A), and cell lysates were harvested for immunoblotting with COX-2 antibody (B). Data in (A) represent the mean ± S.E.M. from at least three independent experiments. *$p<0.05$ as compared with control responses of LPS and IFN-γ. The trace shown in (B) is a representative of three separate experiments.

FIG. 2. Statins stimulate COX-2 gene transcription, COX-2 induction and PGE_2 formation. Cells were treated with statins at the indicated concentrations for 24 h (A-C), different periods (D and E) or 8 h (F). Culture medium was collected for PGE_2 measurement with ELISA kit (A), cell lysates were analyzed by immunobloting with COX-2 antibody (B-D), and total mRNA was prepared and analyzed by RT-PCR (E) or real-time PCR (F) with COX-2 specific primers. The amount of β-actin was also measured as an internal control. Data in (A) represent the mean ± S.E.M. from at least

three independent experiments. *p<0.05, indicates the significant stimulation. The results shown in (B)-(F) are representative of three separate experiments.

FIG. 3. Lovastatin increases COX-2 promoter activity. As described in “Methods”, the luciferase activity derived from COX-2 activation was normalized to the transfection efficiency with β-gal-lacZ, and was expressed as percentage of control activity in cells treated with vehicle. The data represent the mean ± S.E.M. from at least 3 independent experiments performed in duplicate. *p<0.05, indicating the significant activation of wild type COX-2 luciferase activity (A) or reduction of promoter activity in each deletion or mutant experiment as compared with the wild-type luciferase activity (B and C). Dots shown in (C) identify bases changed from the wild-type sequence. The corresponding wild-type sequences are presented under “Methods”.

FIG. 4. Effects of mevalonate and isoprenoids on the stimulating action of statins. (A), RAW264.7 macrophages stimulated with lovastatin, fluvastatin or LPS were incubated in the presence of mevalonate, FPP or GGPP for 24 h. PGE_2 accumulation in culture

medium was measured; values were normalized to percentages of net PGE_2 accumulation by each stimulus in the absence of mevalonate, FPP or GGPP. (B), cells were treated with lovastatin, LPS and different concentrations of FPP, GGPP or mevalonate. COX-2 level was determined by immunobloting and quantified by densitometry. The results are representative of three separate experiments. (C), wild-type COX-2 reporter assay as previously mentioned was performed in the presence of mevalonate, FPP, GGPP and/or lovastatin. Data in (A) and (C) represent the mean ± S.E.M. from at least 3 independent experiments performed in duplicate. *$p<0.05$ indicates the significant reduction of statin response in the presence of mevalonate, FPP or GGPP.

FIG. 5. Effects of manumycin A, FTI, GGTI and toxin B on COX-2 induction and PGE_2 production. After 24 h treatment with manumycin A, FTI, GGTI, toxin B and/or LPS, PGE_2 production and COX-2 protein expression were measured. Data represent the mean ± S.E.M. from at least 3 independent experiments performed in duplicate. *$p<0.05$ indicates the significant stimulation.

FIG. 6. Effects of protein kinase inhibitors on statin- and LPS-induced PGE_2 release. Cells were pretreated with herbimycin (3 μM), PP2 (1 μM), Ro31-8220 (1 μM), GF109203X (1 μM), PD98059 (30 μM), SB203580 (3 μM), Y27632 (30 μM) or KT5720 (1 μM) for 30 min, then stimulated with lovastatin, fluvastatin or LPS for 24 h. PGE_2 accumulation in culture medium was measured; values were normalized to percentages of net PGE_2 accumulation by each stimuli in the absence of inhibitors. Data represent the mean ± S.E.M. from three or four independent experiments. *$P<0.05$ as compared to the control response without inhibitor treatment.

FIG. 7. Statins activate p38 MAPK and ERK. In (A), cells were treated with lovastatin or LPS for different periods, and immunocomplex kinase assay using MBP as a substrate for p38 MAPK was then performed. In (B), cell lysates prepared from cells following lovastatin or pravastatin stimulation for different periods were immunoblotted with antibody specific for total or phosphorylated ERK. Results are representative of three independent experiments.