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降血脂藥物 statin 誘導環氧化脢之作用機轉,細胞種類及動物種類上的差異及在臨床治療上的意義

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計畫主持人：黃國晉

計畫參與人員：林琬琬、陳瑞菁

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HMG–CoA reductase inhibitors upregulate heme oxygenase-1 expression in murine RAW264.7 macrophages via ERK, p38 MAPK and protein kinase G pathways

Jui-Ching Chen[a], Kuo-Chin Huang[b], Wan-Wan Lin[a,*]

[a]Department of Pharmacology, College of Medicine, National Taiwan University, Taipei, Taiwan
[b]Department of Family Medicine of National Taiwan University Hospital, Taipei, Taiwan

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Abstract

Heme oxygenase-1 (HO-1) is the rate-limiting enzyme in heme catabolism, which confers cytoprotection against oxidative injury and provides a vital function in maintaining tissue homeostasis. HMG–CoA reductase inhibitors (statins) possess several anti-inflammatory mechanisms and may be beneficial in the treatment of inflammatory diseases. Our previous study has shown that statins can inhibit iNOS gene expression in murine RAW264.7 macrophages. In this study, we showed that lovastatin, fluvastatin, atorvastatin, simvastatin, mevastatin and pravastatin are able to upregulate the mRNA expression of HO-1 gene. This effect of lovastatin was attenuated by farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP), a protein kinase G (PKG) inhibitor (KT5823), a soluble guanylyl cyclase inhibitor (ODQ), a p38 MAPK inhibitor (SB203580), and MEK inhibitors (U0126 and PD98059), but not by inhibitors of protein kinase C (PKC), protein kinase A (PKA), c-*jun* N-terminal kinase (JNK) and Rho kinase. Consistent with this notion, our previous study has reported the ability of statins to activate ERK and p38 MAPK in RAW264.7 macrophages. Here we further found the participation of cyclic guanosine monophosphate (cGMP)/PKG pathway for ERK activation in cells stimulated with statin and the ability of statin to induce AP-1 activity, which is an essential transcription factor in the regulation of HO-1 gene expression. In addition, a Ras inhibitor (manumycin A) treatment also caused a marked induction of HO-1 mRNA followed by a corresponding increase in HO-1 protein; instead, inhibition of Rho activity by toxin B only led to a transient and weak induction of HO-1. The involvement of signal pathways in manumycin A-induced HO-1 gene expression was associated with p38 MAPK, JNK and ERK activation. Taken together, these results demonstrate for the first time that statins might activate PKG to elicit activations of ERK and p38 MAPK pathways and finally induce HO-1 gene expression, which provides a novel anti-inflammatory mechanism in the therapeutic validity.

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Keywords: HMG–CoA reductase inhibitor; HO-1; p38 MAPK; ERK; cGMP; PKG; Isoprenoid; RAW264.7 macrophages

1. Introduction

Heme oxygenase (HO) is the rate-limiting enzyme in the oxidative degradation of heme into bilirubin, iron, and carbon monoxide (CO). While HO-2 and HO-3 are constitutively expressed, HO-1 is the inducible form. HO-1 is expressed with low level under basal conditions and can be highly induced in response to various agents causing oxidative stress including hyperthermia, UV irradiation [3], hydrogen peroxide [3], heavy metals [3], inflammatory cytokines [4], endotoxin [5], hypoxia [6], hyperoxia [7], and nitric oxide (NO) [8,9]. HO-1 induction provides cytopro-

Abbreviations: AP-1, Activator protein 1; 8BrcGMP, 8-Bromo-cyclic guanosine monophosphate; dbcgmp, Dibutyryl 3′,5′-cyclic guanosine monophosphate; CO, Carbon monoxide; ERK, Extracellular signal-regulated kinase; FPP, Farnesyl pyrophosphate; GGPP, Geranylgeranyl pyrophosphate; HMG–CoA, 3-Hydroxy-3-methylglutaryl–coenzyme A; HO-1, Heme oxygenase-1; iNOS, Inducible nitric oxide synthase; JNK, c-*jun* N-terminal kinase; LPS, Lipopolysaccharide; MAPK, Mitogen-activated protein kinase; MEK, Mitogen-activated protein/ERK kinase; NO, Nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one; PDE, Phosphodiesterase; PKA, Protein kinase A; PKC, Protein kinase C; PKG, Protein kinase G; RT-PCR, Reverse transcription-polymerase chain reaction; sGC, Soluble guanylyl cyclase; SNP, Sodium nitroprusside.

* Corresponding author. Tel.: +886 2 23123456x8315; fax: +886 2 23915297.
E-mail address: wwl@ha.mc.ntu.edu.tw (W.-W. Lin).

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tection against oxidative stress and apoptosis and preserves cellular homeostasis [1,2]. This action is demonstrated not only in cultured cell systems [10] but also in in vivo studies [11,12]. Although the mediators and mechanisms by which HO-1 provides protection are not clear and depend on cell types and stimuli, accumulating lines of evidence point the important role of CO [13,14]. A low concentration of CO can exert protection through a soluble guanylyl cyclase (sGC) and cyclic guanosine monophosphate (cGMP) pathway [15].

Statins are inhibitors of the 3-hydroxy-3-methyl-glutaryl–coenzyme A (HMG–CoA) reductase and are widely used as lipid-lowering agents [16]. Besides the therapeutic use in hyperlipidemia, the anti-inflammatory and immunomodulatory benefits of statins have been recently reported in many aspects, although mechanisms are not yet completely defined [17]. Most identified anti-inflammatory benefits of statins rely on the reduction of cellular levels of mevalonate, the direct product of HMG–CoA reductase, and mevalonate-derived isoprenoids, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), which are involved in post-translational modification of several small G proteins, such as Rho, Rac, Cdc42, and Ras [18,19].

Since the understanding and evaluation of the pharmacological effects of statins are increasing and accelerating their clinical importance and validity, in this study we intended to identify the action of statins on HO-1 gene expression in murine RAW264.7 macrophages. Using this cell type we previously have demonstrated the abilities of statins to block inducible nitric oxide synthase (iNOS) induction caused by lipopolysaccharide (LPS) and interferon-γ [20]. Intriguingly in the present study we demonstrated that statins are capable of inducing HO-1 gene transcription in murine RAW264.7 macrophages and elucidated the mechanisms involved.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY). Rabbit polyclonal antibodies specific for HO-1, β-actin, ERK, JNK and p38 mitogen activated protein kinase (MAPK) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specific to the phosphorylated ERK, JNK and p38 MAPK were purchased from Cell Signaling Technology (Beverly, MA). The ECL detection agents were purchased from Amersham Biosciences (Piscataway, NJ). Toxin B from *Clostridium difficile* was obtained from Calbiochem (San Diego, CA). The Ras inhibitor manumycin A, lovastatin, phenol-extracted LPS (L8274) from *E. coli*, farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP), KT5720, KT5823, Y27632, 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ), anisomycin, 8-bromo-cyclic guanosine monophosphate (8BrcGMP), dibutyryl 3′,5′-cyclic guanosine monophosphate (dBcGMP) and other chemicals were purchased from Sigma Aldrich (St. Louis, MO). GF109203X, SB203580, Ro 31-8220, U0126, PD98059 and mevastatin were purchased from Calbiochem (San Diego, CA). SP600125 was obtained from Tocris Cookson (Ellisville, MO). Atorvastatin, fluvastatin, pravastatin and simvastatin were respectively provided by Pflizer Inc. (NY), Novartis (Basel, Switzerland), Sankyo Co., Ltd. (Tokyo, Japan) and Merck and Co., Inc. (NJ). The AP-1 luciferase construct was provided by Dr. G. Hageman (Flanders Interuniversity Institute for Biotechnology and University of Gent, Gent, Belgium). All materials for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad (Hercules, CA).

2.2. Cell culture

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

2.3. Protein preparation and Western blotting

After stimulation, cells were rinsed twice with ice-cold PBS and 100 μl of cell lysis buffer (20 mM Tris–HCl, pH7.5, 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 to each plate. Protein was denatured in SDS, electrophoresed on 10% SDS/polyacrylamide gel, and transferred to nitrocellulose membrane. Nonspecific binding was blocked with TBST (50 mM Tris–HCl, pH7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% non-fat milk for 1 h at room temperature. After incubation with the appropriate first antibodies, membranes were washed three times with TBST. The secondary antibody was incubated for 1 h. Following 3 washes with TBST, the protein bands were detected with the ECL reagent.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

To amplify HO-1 mRNA, the specific primers for RT-PCR analysis were synthesized. Macrophages treated with indicated agents were homogenized with 1 ml of RNAzol B reagent (Gibco) and total RNA was extracted by acid guanidinium thiocyanate-phenol-chloroform extraction. RT was performed using StrataScript RT-PCR Kit and 10 μg of total RNA was reverse transcribed to cDNA following the manufacturer's recommended procedures. RT-generated cDNA encoding HO-1 and β-actin genes were amplified using PCR. The oligonucleotide primers used correspond to the mouse HO-1 (5′-GAG AAT GCT GAG TTC ATG-3′

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and 5′-ATG TTG AGC AGG AAG GC-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 in a final volume of 50 μl containing: Taq DNA polymerase buffer, all four dNTPs, oligonucleotide primers, Taq DNA polymerase, and RT products. After an initial denaturation for 2 min at 94 °C, 35 cycles of amplification (94 °C for 45 s, 65 °C for 45 s, and 72 °C for 2 min) were performed followed by a 10-min extension at 72 °C. PCR products were analyzed on 2% agarose gel. The mRNA of β-actin served as an internal control for sample loading and mRNA integrity.

2.5. Transfection and AP-1-luciferase assays

Using electroporation (280 V, 1070 μF, 30 ms time constant), cells (2×10^7 cells/cuvette) were cotransfected with 1 μg of AP-1 promoter construct and 1 μg β-galactosidase expression vector (pCR3lacZ; Pharmacia, Sweden). After electroporation, cells were cultured in 24-well plate at 2×10^6 cells/well. After 24-h incubation, cells were incubated with the indicated concentrations of agents. After another 24-h incubation, the media were removed and the cells were washed once with cold PBS. To prepare lysates, 100 μl of reporter lysis buffer (Promega) was added to each well and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 30 s. Aliquots of cell lysates (5 μl) containing equal amounts of protein (10–20 μg) were placed into the wells of an opaque, black 96-well microplate. An equal volume of luciferase substrate (Promega) was added to all samples and the luminescence was measured in 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 in control group without statin treatment.

2.6. 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 less than 0.05 is considered statistically significant.

3. Results

3.1. Statins transcriptionally induce HO-1 gene expression in murine RAW264.7 macrophages

Murine RAW264.7 macrophages were chosen to investigate the signal pathways of statin in HO-1 expression, an anti-inflammatory gene. Treatment with lovastatin, fluvastatin and simvastatin (each at 30 μM) induced HO-1 protein expression. At basal state, a weak immunoreactivity of HO-1 protein was detected. The stimulating action of 30 μM lovastatin and fluvastatin displayed the time-dependency, occurring after 3 h exposure, peaking at 12 h and maintaining for up to 24 h (Fig. 1a). The HO-1 expression induced by simvastatin also occurred after 3 h exposure but peaked at 6 h and then disappeared. Next concentration-dependency of this action of six statins was examined. Cells were incubated with indicated concen-

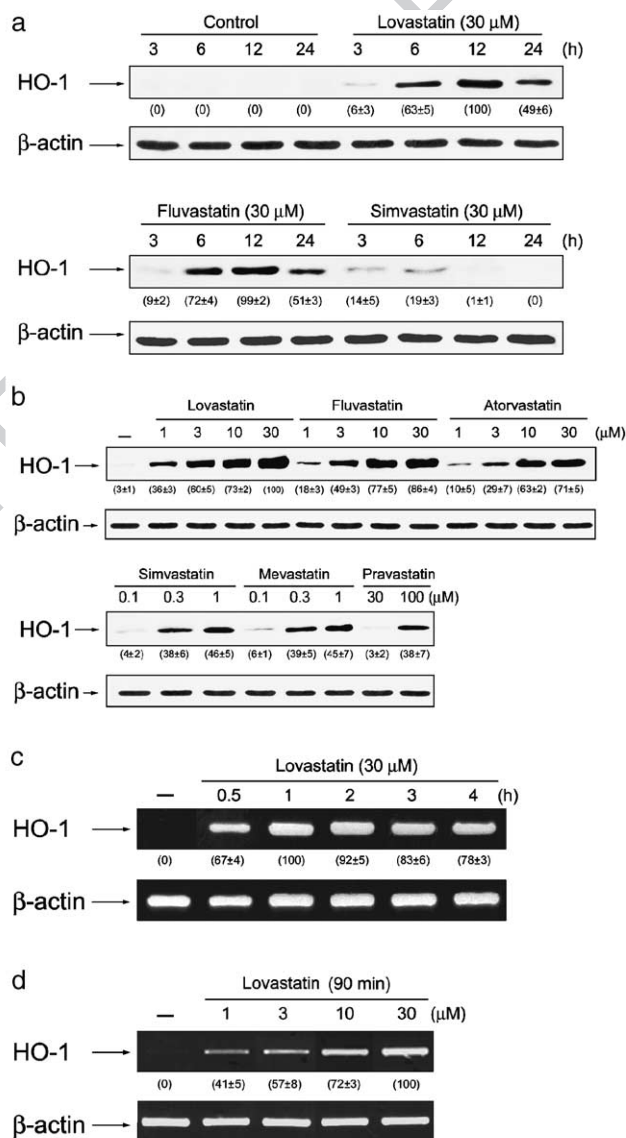

Fig. 1. Time- and dose-dependent effects of statins on the gene transcription of HO-1. (a, c) Cells were treated with statins at concentrations indicated for different periods. (b, d) Cells were treated with different concentrations of statins for 6 h (b) or 90 min (d). After stimulation, cell lysate or RNA was prepared respectively for determining HO-1 and β-actin proteins with immunoblotting (a, b) or for determining mRNA levels with RT-PCR (c, d). The β-actin level was considered as an internal control. Data on HO-1 protein and mRNA levels were measured by densitometry, normalized to the level of β-actin, and calculated as percentages of the maximal response of lovastatin (30 μM). Traces shown are representative of three separate experiments and the mean ± S.E.M. was shown in parentheses.

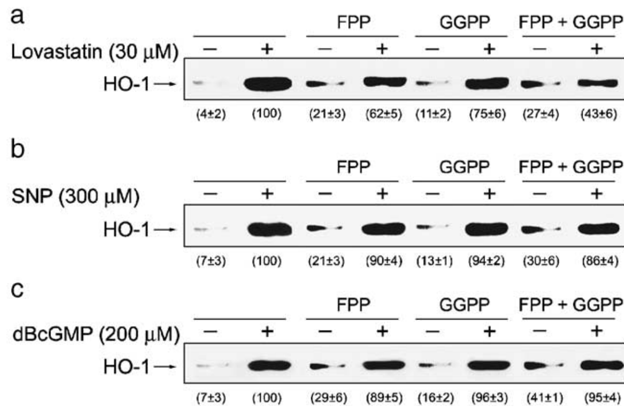

Fig. 2. HO-1 induction by statins was reversed by GGPP and FPP. GGPP (30 μM) and/or FPP (30 μM) was pretreated for 30 min prior to the incubation of lovastatin (30 μM, a), SNP (300 μM, b), or dBcGMP (200 μM, c) for 6 h. The protein levels of HO-1 were measured in the cell lysates by Western blot and calculated as percentages of the control response of each stimulus. The results are representative of three separate experiments and the mean ± S.E.M. was shown in parentheses.

201 trations of statins for 6 h and all of them were compared 202 and showed different potencies (Fig. 1b). Lovastatin, 203 fluvastatin and approximately atorvastatin induced com- 204 parable extents of HO-1 protein induction within similar 205 concentration range of 1–30 μM. Pravastatin did not 206 stimulate HO-1 protein expression until 100 μM. Due to 207 moderate cell toxicity for simvastatin and mevastatin with 208 10 μM (data not shown), we used lower concentrations 209 (0.1–1 μM) of both agents. Results indicated that HO-1 210 induction by 0.3 μM simvastatin and mevastatin was 211 comparable to that of 1 μM lovastatin and maximal 212 response was almost achieved around 1 μM.

213 Since HO-1 is an inducible gene product, we determined 214 whether the effect of statin results from increased gene 215 transcription. Using RT-PCR analysis, we found that HO-1 216 mRNA was time- and concentration-dependently increased 217 by 30 μM of lovastatin (Fig. 1c,d). The incubation period as 218 short as 30 min was sufficient for lovastatin (30 μM) to 219 increase HO-1 mRNA level, which was further increased 220 and maintained for at least for 4 h.

221 3.2. HO-1 stimulation by statin is dependent on protein 222 prenylation

223 To further identify the product of HMG–CoA reductase 224 reaction necessary for the effect of statins, we incubated 225 cells with FPP and/or GGPP in the presence of lovastatin. 226 FPP and GGPP are involved in farnesylation and geranyl- 227 geranylation of small G proteins, respectively. Fig. 2a 228 showed that FPP (30 μM) as well as GGPP (30 μM) 229 partially reversed the effect of lovastatin on HO-1 induction 230 and simultaneous presence of FPP and GGPP led to an 231 additive inhibition on HO-1 protein expression. These 232 observations suggest that the effect of statins on HO-1 233 expression is ascribed to the reduction of protein isopreny- 234 lation, which negatively regulates HO-1 gene expression.

As mentioned previously, HO-1 was a cGMP-inducible 235 protein [8] and sodium nitroprusside (SNP) could induce 236 HO-1 protein expression in murine RAW264.7 macro- 237 phages [9]. To examine whether protein prenylation is 238 involved in the sGC/cGMP-mediated HO-1 induction, we 239 pretreated murine RAW264.7 macrophages with FPP and/or 240 GGPP (each at 30 μM) for 30 min. We found that both 241 isoprenoids did not reverse SNP or dBcGMP-induced HO-1 242 protein levels (Fig. 2b,c). 243

3.3. Ras and Rho inhibition are involved in the upregulation 244 of HO-1 expression 245

To further verify the involvement of reduced isopreny- 246 lation of signaling proteins in statin-induced HO-1 expres- 247 sion, we studied the effects of manumycin A. Manumycin A 248 is a potent and selective inhibitor of farnesyltransferase 249 (IC_{50} = 5 μM) compared to geranylgeranyltransferase 250 (IC_{50} = 180 μM) and acts as an inhibitor of Ras function 251 [21,22]. Our results showed that manumycin A (10 or 30 252 μM) caused HO-1 protein induction in a time- and 253 concentration-dependent manner (Fig. 3a,b). Likewise direct 254 inhibition of Rho family proteins with toxin B (400 pM) 255 caused HO-1 protein expression, while this action was slight 256 and transient (Fig. 3a). Because of cytotoxicity occurrence 257 at 100 μM manumycin A, we did not further determine the 258 HO-1 response of manumycin A at higher concentrations. 259

3.4. Signaling pathways of statin- and manumycin A- 260 induced HO-1 expression 261

Numbers of pathways have been implicated in trans- 262 mitting the extracellular signals to the nuclei for HO-1 263 gene expression. To investigate the signal transduction 264 pathway(s) involved in regulating HO-1 expression in 265 response to statin and manumycin A, we examined the 266

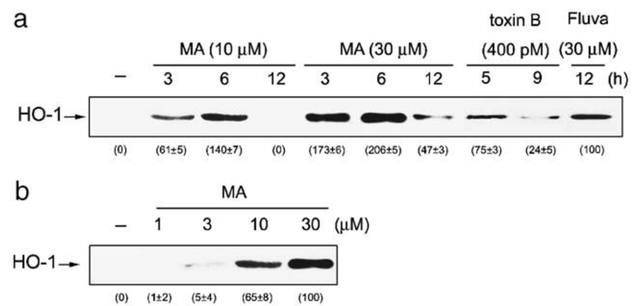

Fig. 3. Manumycin A and toxin B mimic statins' effect on HO-1 induction. (a) Murine RAW264.7 macrophages were treated with manumycin A (10 and 30 μM), toxin B (400 pM) or fluvastatin (30 μM) for the indicated time periods. (b) Murine RAW264.7 macrophages were treated with different concentrations of manumycin A for 6 h. Protein levels of HO-1 were measured in the cell lysates by Western blot and calculated as percentages of the response of fluvastatin (30 μM, a) or manumycin A (30 μM, b). The results are representative of three separate experiments and the mean ± S.E.M. was shown in parentheses.

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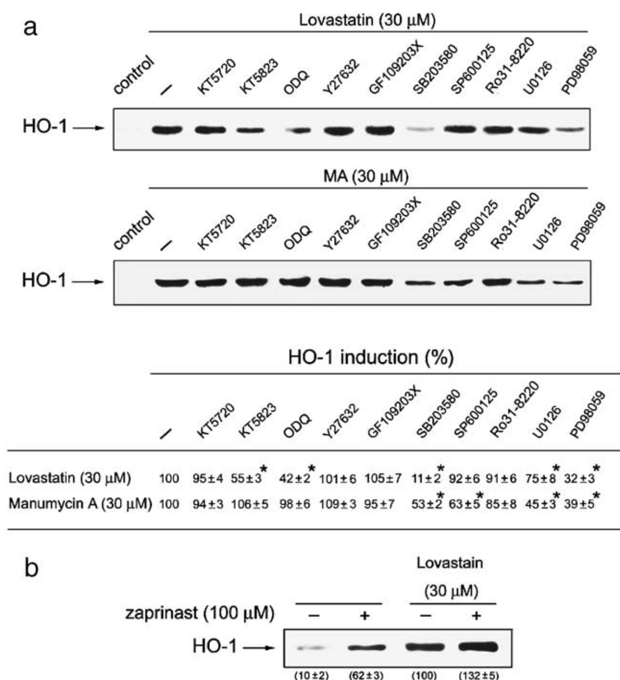

HO-1 induction (%)	/	KT5720	KT5823	ODQ	Y27632	GF109203X	SB203580	SP600125	Ro31-8220	U0126	PD98059
Lovastatin (30 μM)	100	95±4	55±3*	42±2*	101±6	105±7	11±2*	92±6	91±6	75±8*	32±3*
Manumycin A (30 μM)	100	94±3	106±5	98±6	109±3	95±7	53±2*	63±5*	85±8	45±3*	39±5*

Fig. 4. Effects of protein kinase inhibitors on lovastatin- and manumycin A-induced HO-1 expression. Cells were pretreated with KT5720 (1 μM), KT5823 (3 μM), ODQ (1 μM), Y27632 (30 μM), GF109203X (3 μM), SB203580 (10 μM), SP600125 (10 μM), Ro 31-8220 (3 μM), U0126 (1 μM) or PD98059 (30 μM) for 30 min, then stimulated with lovastatin (30 μM) or manumycin A (30 μM) for 6 h. Protein levels of HO-1 were measured in the cell lysates by Western blot. Data on protein levels were measured by densitometry and calculated as percentages of the respective basal response of lovastatin or manumycin A (lane 2). Traces shown are representative of three separate experiments and the mean±S.E.M. was shown in a table. Asterisks are used to indicate the significance of these effects. In (b), zaprinast (100 μM) and lovastatin (30 μM) were treated as indicated for 6 h and HO-1 protein level was measured and quantified from three individual experiments.

267 effects of chemical inhibitors of signaling intermediates on
268 HO-1 protein levels. As shown in Fig. 4, we found that
269 treatment of cells with the PKG inhibitor (KT5823), the
270 sGC inhibitor (ODQ), two MEK inhibitors (PD98059 and
271 U0126), and the p38 MAPK inhibitor (SB203580) reduced
272 lovastatin-induced HO-1 induction. In contrast to the
273 inhibition on lovastatin response, KT5823 and ODQ failed
274 to change the response of manumycin A. Otherwise,
275 inhibitors of MAPKs, including U0126, PD98059,
276 SB203580 and the JNK inhibitor (SP600125), inhibited
277 manumycin A-induced HO-1 expression. KT5720 [a
278 protein kinase A (PKA) inhibitor], Y27632 (a Rho kinase
279 inhibitor), Ro 31-8220 and GF109203X [two protein
280 kinase C (PKC) inhibitors] did not have any effects. These
281 results suggest the participation of PKG, ERK and p38
282 MAPK, but not JNK, PKC, PKA, Rho kinase or PI3K, in
283 HO-1 expression in cells treated with statins, while the
284 action of manumycin A is dependent on ERK, JNK and
285 p38 MAPK. To further understand the involvement of
286 intracellular cGMP, we conducted experiments using
287 phosphodiesterase (PDE) inhibitor zaprinast. As shown in
288 Fig. 4b, zaprinast (100 μM) is able to induce HO-1 and
289 enhance the response of lovastatin.

3.5. *Manumycin A mediates phosphorylation of p38 MAPK, JNK and ERK* 290 291

292 Since in previous study conducted in the same cell line
293 we have demonstrated that statin is able to cause ERK and
294 p38 MAPK activation [23], we attempted to further confirm
295 the crucial roles of three MAPKs in HO-1 expression by
296 manumycin A. As shown in Fig. 5, treatment of murine
297 RAW264.7 macrophages with 30 μM manumycin A
298 resulted in a time-dependent phosphorylation of p38
299 MAPK, JNK and ERK. Compared with the rapid onset of
300 anisomycin for these events seen at 5 min, manumycin A-
301 induced p38 MAPK phosphorylation occurred after 60 min
302 of incubation and then declined to basal level at 180 min
303 (Fig. 5a). Furthermore, immunoblotting to reflect JNK
304 activation indicated a delayed but significant response after
305 120 min of stimulation and the response continued until 180
306 min (Fig. 5b). In contrast to the delayed action on p38
307 MAPK and JNK, the manumycin A-induced increase in
308 ERK phosphorylation began at 5 min, peaked at 60–120

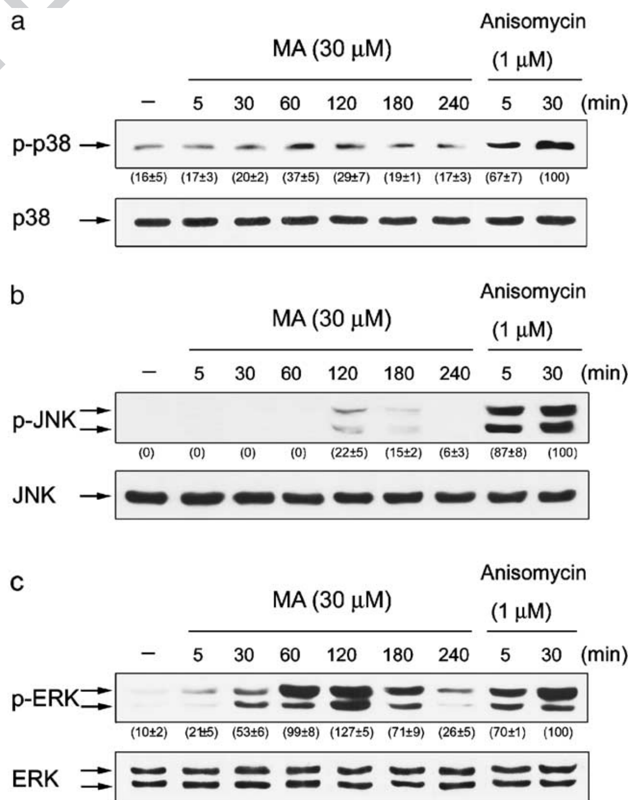

Fig. 5. Manumycin A activates p38 MAPK, JNK and ERK. Cell lysates prepared from cells following manumycin A (30 μM) or anisomycin (1 μM) stimulation for different periods were immunoblotted with antibody specific for total or phosphorylated p38, JNK and ERK. Data on protein levels were measured by densitometry and calculated as percentages of the 30-min response of anisomycin. The results are representative of three separate experiments.

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309 min and then declined at 240 min (Fig. 5c). The protein
310 levels of p38, JNK and ERK were not affected by
311 manumycin A treatment.

312 3.6. GC/cGMP pathway mediates statin-induced ERK 313 activation

314 Since p38 MAPK, ERK and PKG activation have been
315 implicated for lovastatin-induced HO-1 gene expression, we
316 used immunoblotting to verify the possible signaling
317 cascades underlying the action of statin. To test whether
318 cGMP pathway is upstream for p38 MAPK and ERK
319 activation, we treated murine RAW264.7 macrophages with
320 dBcGMP and 8BrcGMP, analogues of cGMP. Fig. 6a
321 showed the stimulating effects of dBcGMP (200 μM) on
322 ERK phosphorylation. dBcGMP treatment for a period as
323 short as 5 min was sufficient to activate ERK and this action
324 lasted at least for 4 h. In contrast, dBcGMP failed to alter the
325 phosphorylation of p38 MAPK and JNK (data not shown).
326 dBcGMP and 8BrcGMP, each at 200 μM, had the similar
327 efficacy to induce ERK activation at 30 min (Fig. 6b). Next
328 to further understanding the role of PKG, we examined the
329 effect of PKG inhibitor. Fig. 6b showed KT5823 incubation
330 indeed could block the ERK activation in response to
331 dBcGMP, 8BrcGMP and statin, but not to manumycin A.
332 These results suggest that cGMP/PKG-dependent signaling
333 pathway is involved in statin-induced ERK activation.

334 3.7. Statin induces AP-1 transactivation

335 Since AP-1 was shown as the major transcription factor
336 involved in HO-1 gene transcription [5,24–26], we

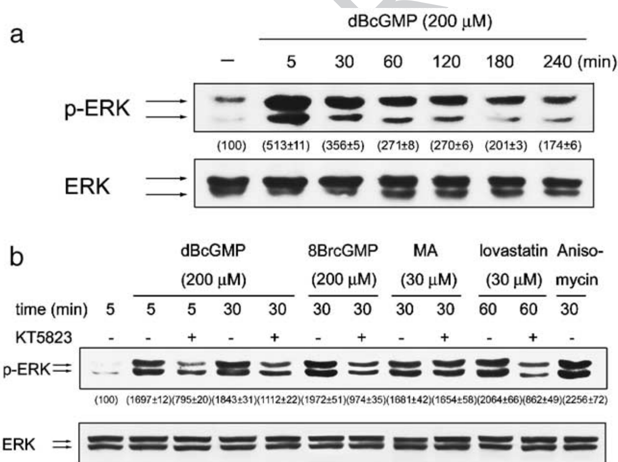

Fig. 6. cGMP/PKA pathway mediates ERK activation. Cells were stimulated with dBcGMP (200 μM) for different periods (a) or pretreated with KT5823 (3 μM) for 60 min, followed by the stimulation with dBcGMP (200 μM), 8BrcGMP (200 μM), lovastatin (30 μM) or manumycin A (30 μM) for 5, 30 or 60 min (b). Cell lysates were immunoblotted with antibody specific for total or phosphorylated ERK. Data on protein levels were measured by densitometry and calculated as percentages of the control response. Results are representative of three independent experiments and the mean ± S.E.M. was shown in parentheses.

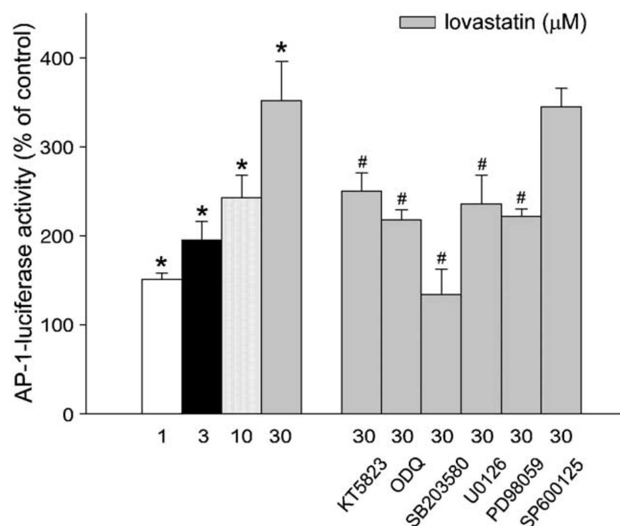

Fig. 7. AP-1 activation by lovastatin. Cells transfected with the AP-1 reporter gene and β-gal-lacZ plasmid were pretreated with different concentrations of lovastatin (left) or pretreated with each pharmacological inhibitor for 30 min (3 μM KT5328, 1 μM ODQ, 10 μM SB203580, 1 μM U0126, 30 μM PD98059, 10 μM SP600125) followed by lovastatin (30 μM) stimulation for 24 h. The luciferase activity derived from AP-1 activation was normalized to the transfection efficiency with β-gal-lacZ. The data represent the mean ± S.E.M. from at least 3 independent experiments. *$p < 0.05$, indicating the significant activation by lovastain. #$p < 0.05$, indicating the significant inhibition of lovastatin response.

explored the effect of lovastain on the transactivity of AP- 337
1. As assessed by transfection with reporter gene driven by 338
AP-1 binding, we found lovastatin within 1–30 μM was 339
able to increase AP-1 activation in a concentration-depend- 340
ent manner (Fig. 7). Furthermore, the stimulating effect of 341
lovastatin was inhibited by KT5823, ODQ, SB203580, 342
U0126 and PD98059, but not by SP600125. 343

4. Discussion 344

Accumulating evidence has indicated HO-1 functions as 345
a “therapeutic funnel”. Induction of HO-1 is suggested to 346
have cytoprotective effect against oxidative injury and have 347
the potent anti-inflammatory properties. Modulation of gene 348
transcription is the principal mechanism by which HO-1 is 349
regulated. Based on these results, induction of HO-1 is a 350
therapeutic strategy for treating inflammatory diseases. In 351
this aspect, animal studies and cell cultures have implicated 352
the anti-inflammatory benefits of HO-1 expression in 353
atherosclerosis, now considered as a kind of chronic 354
inflammatory process [27–31]. In order to investigate new 355
strategies to modify the pathophysiology of atherosclerosis, 356
we have tested whether HMG–CoA reductase inhibitors 357
could regulate the expression of HO-1 and have explored 358
the signal pathway involved in this regulation in RAW264.7 359
macrophages. In this study we unexpectedly found the 360
ability of statins to induce HO-1 expression in macrophages 361
and this action is mediated by impeding prenylation of small 362
G proteins, for example Ras protein in particular. Further- 363

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more, we suggest that p38 MAPK, ERK, PKG and AP-1 activation are required for statins-induced HO-1 regulation in murine RAW264.7 macrophages. However, in our preparation of this manuscript one recent study reported similar HO-1 induction by simvastatin in human and rat aortic smooth muscle cells [32].

Our observation that the inductive effect of statin was overcome by FPP and GGPP demonstrated the specificity of the HMG–CoA reductase inhibition. FPP and GGPP are metabolites of HMG–CoA reductase and substrates for protein isoprenylation, which takes place during C-terminus processing of small G proteins and is essential for their coupling with multiple effector systems to activate distinct physiological responses. Thus, reversal effect of FPP and GGPP on statin-elicited HO-1 induction suggests impeding small G protein functions are involved. Alternatively, we predict one or more small G proteins are exerting a negative role in HO-1 gene expression in basal condition of macrophages and this intracellular balanced environment is altered by statins. To clarify this notion, we determined whether inhibition of Ras isoprenylation by manumycin A, an inhibitor of Ras farnesyl transferase [21,22], could mimic HO-1 expression. As a result, manumycin A indeed induced HO-1 protein expression in a time- and concentration-dependent manner. Like the necessity of isoprenylation of Ras superfamily to achieve molecular function, Rho proteins acting as molecular switches to control cellular processes also require the attachment of geranylgeraniol, an isoprenoid intermediate of the cholesterol biosynthesis pathway. The fact that lovastatin blocks geranylgeraniol synthesis also prompts us to propose the notion that Rho proteins are possibly involve in the signaling regulation of HO-1 expression. We examined clostridial toxin B, which is a non-selective inhibitor of Rho proteins, Rho, Rac and Cdc42 [33,34] and observed a weak, transient but significant induction of HO-1. These results together suggest that interruption of the cellular activity of Ras in primary and Rho proteins, to a lesser extent, is involved to initiate stimulating signals for HO-1 protein expression in macrophages. Except Ras and Rho family proteins, whether additional G proteins are involved in the regulation of HO-1 gene expression needs future investigation.

In general, HO-1 gene expression is induced by stimuli that activate MAPKs [26,35]. Three major subgroups of the MAPK family identified to date include ERK, JNK and p38 MAPK. Depending on the stimuli specificity, contradictory results on the regulatory role of different MAPK pathways for HO-1 gene expression were observed. In this aspect recent mechanistic studies on HO-1 induction have pointed the critical intermediacy of the p38 MAPK cascade but not ERK in the regulation of HO-1 expression by TGF-β [4], hypoxia [6], cadmium [36], IL-10 [37], and $15dPGJ_2$ [38]. In contrast, both ERK and p38 MAPK pathways medicate HO-1 gene transcription by sodium arsenite [39–41] and NO [42]. Instead JNK mediates the induction of HO-1 gene expression by the glutathione depletor phorone [43]. On the other hand, HO-1 gene transcription after ischemia–reperfusion involves ERK, JNK, and p38 MAPK pathways [35]. In this study we found the effect of lovastatin on HO-1 induction is dependent on p38 MAPK and ERK, but not on JNK. These observations are in line with our previous study to show ERK and p38 MAPK activation by statin in RAW264.7 macrophages [23]. Nevertheless, activation of three MAPKs is involved in the action of manumycin A. The discrepancy between both stimuli is possibly resulting from the net outcome through the diverse effects of statins on multiple isoprenylated proteins.

Confirming previous findings showing intracellular second messenger cGMP as an intermediate to enhance HO-1 expression [8,9,42,44], the present investigation also observed such phenomena in murine RAW264.7 macrophages. We found dBcGMP, SNP (a direct sGC activator via NO release) and zaprinast (a PDE inhibitor) could induce HO-1 and ODQ (an inhibitor of sGC) could inhibit HO-1 response of lovastatin, suggesting the contribution of cGMP signaling in this event. In addition, our data provide new insight into the participation of PKG in this event, as in this study we observed PKG inhibitor could attenuate HO-1 induction in response to lovastatin. However, in contrast to lovastatin action, both sGC and PKG inhibitors did not prevent the action of manumycin A, suggesting that differential signaling pathways are exerted by both HO-1 inducers. Moreover, in this study we further provide new insight that PKG-mediated ERK signaling pathway plays a crucial role for HO-1 expression by statin. Even though PKG-dependent ERK activation was reported to participate in various cell functions [45,46], this signal cascade is for the first time shown in the present study to regulate HO-1 induction. In agreement with previous study detecting the ability of statins to stimulate cGMP formation in PC12 cells [47], our data with the use of pharmacological inhibitors point the essential role of cGMP/PKG in statin's action. To confirm this notion, experiment in the measurement of intracellular cGMP level was taken. Unfortunately we cannot detect significant cGMP change in cells following lovastatin incubation for different periods in macrophages (data not shown). Thus we speculate that, despite no increase of intracellular cGMP, the basal activity of PKG is sufficient to play a role in the modulation of HO-1 induction. In addition, the differential dependency of PKG pathway in statin- and manumycin A-mediated HO-1 response again strengthens the distinct action mechanisms underlying the HO-1 induction by these two inducers, despite some mechanisms, for example ERK, that might be in common.

A number of response elements in the mouse HO-1 promoter and 5′-flanking region have been identified. The mouse HO-1 gene contains two inducible enhancers, E1 and E2. E1 contains three stress response elements that encompass the consensus motifs for AP-1 proteins. Deletion and mutational analyses of regulatory element of HO-1 gene indicate that AP-1 binding site plays an important role in mediating HO-1 gene regulation and is a commonality in

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response to multiple agents in the activation mechanism of HO-1 [5–7,24–26,48]. Thus far, even though the response elements mediating cGMP-dependent transcriptional activation of HO-1 are poorly defined, one previous study has pointed the importance of AP-1 in cGMP-mediated HO-1 induction in rat hepatocytes [25]. Consistent with previous studies showing the ability of statins for AP-1 activity [49,50], our current data demonstrated that HMG–CoA reductase inhibitors indeed could induce AP-1 activation in macrophages, which, as previously indicated with strong evidence, leads to the induction of HO-1 gene transcription. Furthermore, pharmacological approaches coincidentally indicated the upstream signaling pathways of cGMP/PKG, ERK and p38 MAPK for AP-1 activation.

In conclusion, we are presenting novel data showing that HMG–CoA reductase inhibitors and manumycin A are able to induce HO-1 gene expression in murine RAW264.7 macrophages. This effect of statins is mediated through p38 MAPK, ERK, PKG pathways and involves AP-1 activation. Since it is conceivable that an ideal inducer of HO-1 activity being an appropriate therapeutic intervention, our data strongly support the protective effects of statins in the therapy of disorders associated with inflammation and oxidative injuries.

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