Phosphorylation and Acetylation of Histone H3 and Autoregulation by Early Growth Response 1 Mediate Interleukin 1β Induction of Early Growth Response 1 Transcription

Bo Wang, Jinbiao Chen, Fernando S. Santiago, Mary Janes, Mary M. Kavurma, Beng H. Chong, John E. Pimanda, Levon M. Khachigian

Objective—The transcription factor early growth response (EGR)-1 has been implicated as a key vascular phenotypic switch through its control of inducible transcription. EGR-1 autoregulation, and histone modification in the EGR-1 promoter, represent key mechanisms in EGR-1 control, but have not been explored.

Methods and Results—We demonstrate that EGR-1 regulates its own transcription and that this involves histone H3 phosphorylation and acetylation. EGR-1 transactivates its promoter in smooth muscle cells exposed to interleukin (IL) 1β through a novel cis-acting element (−211/−203). PD98059, which inhibits mitogen-activated protein kinase kinase/extracellular regulated kinase (MEK/ERK) attenuates IL-1β-inducible phosphorylation of extracellular signal–regulated kinase 1/2 and mitogen and stress–activated protein kinases 1/2; and reduces levels of phosphorylated and acetylated histone H3. Histone deacetylase inhibition enhances EGR-1 transcription in response to cytokine. Conversely, suppression of histone modification with mitogen and stress–activated protein kinase 1/2 short interfering RNA, or the histone H3 acetyltransferase inhibitor Garcinol, inhibits IL-1β-inducible EGR-1 transcription. EGR-1 interacts with the acetyltransferase p300. Acetylated H3 and phosphorylated H3 are enriched at the promoter of EGR-1; and EGR-1 is enriched at the promoters of tissue factor and plasminogen activator inhibitor 1 in response to IL-1β, and attenuated by PD98059, Garcinol, and mitogen and stress–activated protein kinase 1/2 short interfering RNA.

Conclusion—IL-1β induction of EGR-1 transcription involves histone H3 phosphorylation, acetylation, and autoregulation by EGR-1. (Arterioscler Thromb Vasc Biol. 2010;30:536-545.)

Key Words: histone modification  ■  immediate-early gene  ■  transcription

E
ternal growth response 1 (human EGR-1 or mouse EGR-1, also known as NGFI-A, Zif268, Krox24, and Tis8) is an inducible zinc finger transcription factor that plays a critical role in controlling cell growth, proliferation, differentiation, and apoptosis.1-5 EGR-1 belongs to a family of zinc finger transcription factors that also includes EGR-2, EGR-3, EGR-4, EGR-α, and the Wilm tumor gene product.6 EGR-1 contains a highly conserved DNA-binding domain (three zinc finger domains of the C2H2 subtype), an activation domain, a repression domain, and a nuclear localization signal.7,8 Carman and Monroe9 identified an activation domain between amino acids 174 and 270, a serine/threonine/proline-rich region that is critical for its activity. Mutants lacking this domain perturb wild-type EGR-1 function.9 EGR-1 regulates the transcription of target genes by binding to GC-rich consensus DNA elements present in the regulatory regions in vascular pathophysiologically relevant genes, such as insulin-like growth factor II, platelet-derived growth factor A and B chains, tissue factor, fibroblast growth factor 2, transforming growth factor β1, urokinase plasminogen activator, and plasminogen activator inhibitor (PAI) 1.5,7,10-13 EGR-1 also regulates the expression of its corepressor nerve growth factor I-A binding protein-2.11 Functional cis-acting elements within the human EGR-1 promoter have been identified in different cell types and include serum-response elements (SREs) and sites for Elk1, Ets, AP-1, and nuclear factor (NF)-κB binding sites.14,15 EGR-1 is induced by a wide range of stimuli, including growth factors, cytokines, ionizing radiation, UV light, and mechanical injury.16-19 Increasing evidence indicates that EGR-1 activation may function as a key switch in many pathological processes, including cancers and cardiovascular diseases. EGR-1 has been indicated in atherosclerosis, intimal thickening after acute vascular injury, ischemia, angiogene-
Figure 1. Early growth response (EGR) 1 transactivates its own transcription via a novel functional EGR-1 element within its promoter. A, The EGR-1 promoter sequence was retrieved from the University of Calif., Santa Cruz genome browser. Consensus elements are as follows: nuclear factor-κB, GGGRNNYYY; Elk1, MMGGAW; serum-response element, CCWWWGG; EGR-1, WTGCGTGGGCGK. B, Human aortic smooth muscle cells (HASMCs) grown to 70% to 80% confluence were transfected with EGR-1 expression vector (pCB6–EGR-1) or empty vector (pCB6), and EGR-1 promoter-reporter construct containing wild type or mutant putative EGR-1 binding site. The cells were incubated for a further 24 hours in serum-free medium before determination of relative luciferase activity, as described in the “Methods” section. Western blotting 24 hours after transfection confirmed that pCB6–EGR-1, but not the empty vector (pCB6\*), increased EGR-1 levels in the HASMCs. Increased EGR-1 immunoreactivity represents total EGR-1, which includes both endogenous and exogenous proteins. C, Rat aortic smooth muscle cells (RASMCs) were transfected with empty plasmid or wild-type EGR-1
EGR-1 is overexpressed in esophageal cancer and plays a key role in mediating and maintaining growth-related oncogene/CXC chemokine receptor 2 proliferative signaling. Primary human prostate carcinomas express high levels of EGR-1, and several EGR-1 target genes (e.g., insulin-like growth factor II, transforming growth factor β1, and platelet-derived growth factor A-chain) have been implicated in prostate tumorigenesis. EGR-1 knockdown inhibits prostate cancer cell proliferation and tumor development in transgenic adenocarcinoma mouse prostate mice. Furthermore, an indispensable role for EGR-1 has been demonstrated in breast cancer cell proliferation, migration, and invasion in nude mice using DNAzymes. This is due, in part, to EGR-1 regulation of angiogenesis.

Although EGR-1 plays a key role in many pathological processes, it is unknown whether EGR-1 regulates its own transcription. Moreover, information on the role of chromatin remodeling and histone modification in the control of EGR-1 transcription is lacking. Herein, we provide novel insights on the role of EGR-1 and histone H3 modifications in the transcriptional regulation of the human EGR-1 gene.

**Methods**

Primary human aortic smooth muscle cells (HASMCs) and rat aortic smooth muscle cells were purchased from the American Type Culture Collection and cultured at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air in Waymouth medium containing 10% fetal bovine serum. SMCs grown to 90% confluence were starved of serum for 24 hours and then treated with 10 ng/mL of interleukin (IL) 1β for 30 minutes before the assessment of relative luciferase activity. Determination of EGR-1 expression was performed with 32 phosphorlated oligo EGR-1-225/-187 or messenger EGR-1-225/-187 units, and increasing amounts of EGR-1-glutathione S-transferase protein, 4 and 8 μg. The antibody used (sc-110; Santa Cruz Biotechnology) targets native EGR-1. E, Twenty-four hours after starvation, HASMCs were exposed to 10 ng/mL of IL-1β for 30 minutes. Conventional chromatin immunoprecipitation (ChIP) (right) and real-time ChIP (lower) were performed with EGR-1 antibodies or IgG, as described in the “Methods” section. Quantitation of EGR-1 enriched at its promoter incorporates an IgG control, which was subtracted from the EGR-1 value before quantitative comparison. The asterisk indicates *P<0.05.

**Results**

**EGR-1 Regulates Transcription of Its Own Gene**

EGR-1 is implicated as a key regulatory switch in a wide variety of vascular disorders through its control of inducible transcription. Although the EGR-1 promoter has previously been studied, it is unknown whether EGR-1 can control its own transcription. Moreover, the critical role of histone modification in EGR-1 transcription is completely unknown. Bioinformatic analysis of the proximal region of the EGR-1 promoter shows the existence of numerous conserved putative cis-acting elements, including SREs, and motifs for Ets, Elk1, and NF-κB. Interestingly, the EGR-1 promoter contains a putative atypical binding motif (5'-GGCGGGCCG-3') for EGR-1 located at -211/-203 (Figure 1A). We used the proinflammatory cytokine IL-1β as a model agonist of EGR-1 to gain valuable insights into the EGR-1 transcription in HASMCs. Quantitative real-time RT-PCR showed both a time- and dose-dependent induction in EGR-1 messenger RNA (mRNA) expression in HASMCs exposed to IL-1β (Supplemental Figure 1A and 1B). EGR-1 was induced by as little as 2.5 ng/mL of IL-1β (Supplemental Figure 1A) and peaked at 30 minutes in cells exposed to 10 ng/mL of IL-1β (Supplemental Figure 1B). Immunofluorescence staining revealed that IL-1β triggered the rapid accumulation of EGR-1 in HASMC nuclei within 0.5 to 1.0 hours (Supplemental Figure 1C), which correlates with the time course of IL-1β-inducible EGR-1 expression (Supplemental Figure 1B).

Overexpression of EGR-1 in HASMCs transfected with a luciferase reporter construct driven by 450 base pairs of the human EGR-1 promoter spanning the putative EGR-1 binding motif significantly increased luciferase activity (Figure 1B, upper). When the -206CGCG motif in the promoter was mutated to -206AAA, induction by EGR-1 of the EGR-1 promoter was abolished (Figure 1B, lower). Western blotting...
confirmed that pCB6–EGR-1, but not the empty vector (pCB6*), increased EGR-1 levels in the HASMCs (Figure 1B, lower, inset). Luciferase activity was induced by IL-1β in SMCs transfected with wild-type pGL3–450EGR-1-luc (Figure 1C). IL-1β responsiveness of the EGR-1 promoter was attenuated in cells transfected with the EGR-1 mutant promoter-reporter construct (Figure 1C). Mutation of SREs in the promoter also abolished IL-1β inducibility (Figure 1C). In contrast, mutation of the NF-κB element had no effect on both basal expression and inducibility of EGR-1. Electrophoretic mobility shift analysis using a double-stranded oligonucleotide spanning the putative EGR-1 binding site (Oligo EGR-1-225/-187) and recombinant EGR-1 protein revealed the formation of an EGR-1/DNA complex, which was supershifted by EGR-1 antibodies (Figure 1D). In contrast, the –208CGG–206 to –208AAA–206 mutation in Oligo EGR-1-225/-187 failed to support the complex formation with recombinant EGR-1 protein (Figure 1D). ChIP analysis, using conventional PCR, confirmed that IL-1β induced the interaction of EGR-1 with its own promoter (Figure 1E, top right). Moreover, real-time ChIP revealed the enrichment of EGR-1 protein at the EGR-1 promoter in HASMCs exposed to IL-1β (Figure 1E, lower). Therefore, by using a variety of approaches, these results demonstrate that IL-1β–inducible EGR-1 binds to, and transactivates, its own promoter.

IL-1β Stimulates EGR-1 Expression in HASMCs via the MEK-ERK1/2 Pathway

To identify the signaling pathway(s) mediating IL-1β induction of EGR-1, we first evaluated the influence of IL-1β on levels of phosphorylated extracellular signal–regulated kinase (ERK) 1/2, phosphorylated p38, and phosphorylated c-Jun NH2-terminal kinases 1/2 (JNK1/2) in this cell type. IL-1β activated ERK1/2 within 15 minutes (Supplemental Figure IIA and B). The cytokine increased levels of phosphorylated p38, albeit less impressively (Supplemental Figure IIA), and had no effect on levels of phosphorylated JNK1/2 or phosphorylated c-Jun (Supplemental Figure IIA). Levels of phosphorylated c-Jun were also unaffected (Supplemental Figure IIA). Because Elk1 is immediately downstream of ERK1/2, we assessed the effect of IL-1β on Elk1 phosphorylation in the HASMCs. Levels of phosphorylated Elk1 in nuclear extracts increased within 15 minutes (Supplemental Figure IIC). As expected, IL-1β also increased p65 levels in the nucleus (Supplemental Figure IIC). These results indicate that ERK1/2-Elk1, p38, and NF-κB pathways are activated in HASMCs in response to IL-1β.

Next, HASMCs were treated with 10 ng/mL of IL-1β in the absence or presence of various concentrations of PD98059 (MEK-ERK1/2 inhibitor), BAY 11 to 7085, and Oridonin (2 NF-κB inhibitors) or SB202190 (a p38 inhibitor); then, the EGR-1 mRNA levels were examined. Quantitative real-time RT-PCR analysis revealed that PD98059, 5 to
10 μmol/L, inhibits IL-1β–inducible EGR-1 mRNA expression in a dose-dependent manner (Figure 2A, column 2 versus column 3; and Figure 4), whereas SB202190, 5 to 10 μmol/L, stimulates EGR-1 mRNA expression (Figure 2A, column 2 versus columns 5 and 6). In contrast, BAY 11 to 7085 and Oridonin had no effect on IL-1β–inducible EGR-1 expression (Figure 2A, column 2 versus columns 5 and 6 or 7 and 8). PD98059 completely inhibits IL-1β–inducible EGR-1 protein expression (Figure 2B, left), whereas SB202190 stimulates EGR-1 protein expression (Figure 2B, right). This demonstrates the negative regulatory role of p38 and the positive regulatory role of ERK1/2 in IL-1β–inducible EGR-1 expression. These results indicate that, in HASMCs, IL-1β activates EGR-1 via the MEK-ERK1/2-Elk1 pathway.

**Influence of IL-1β–Inducible Histone H3 Modifications on EGR-1 Transcription**

Although chromatin remodeling is a key process in transcriptional regulation, surprisingly there is nothing known about the role of histone modifications in the control of EGR-1 transcription. Mitogen and stress–activated protein kinases (MSK1/2) mediate the phosphorylation of histone H3. Having demonstrated that PD98059 inhibits IL-1β–inducible ERK1/2 activation (Figure 2B, left) and EGR-1 expression in HASMCs (Figure 2A and B), we next investigated the effect of MEK-ERK1/2 inhibition on levels of phosphorylated MSK1 and MSK2 in HASMCs treated with IL-1β. MSK1 and MSK2 were phosphorylated within 30 minutes of exposure to IL-1β (Figure 3A). PD98059 suppressed MSK1 and MSK2 phosphorylation in a dose-dependent manner (Figure 3A). H3 phosphorylation and acetylation also increased in response to IL-1β, as did ribosomal S6 kinase phosphorylation; each was reduced by MEK-ERK1/2 inhibition (Figure 3A), demonstrating the importance of ERK1/2-MSK1/2 signaling in IL-1β–mediated histone H3 phosphorylation and acetylation. To establish a causal relationship between histone H3 modification and EGR-1 transcription, we then determined the levels of phosphorylated, acetylated, and methylated H3 in HASMCs treated with IL-1β over time. Western blot analysis indicated a rapid and transient induction in both phosphorylated and acetylated H3 (Supplemental Figure 3A and B) but not methylated H3 (Supplemental Figure II A). We further explored the role of H3 acetylation in IL-1β–mediated EGR-1 transcription in HASMCs using
the histone deacetylase inhibitor, suberoylanilide hydroxamic acid (SAHA).\textsuperscript{33} SAHA caused a dramatic increase in the levels of acetylated H3 (Figure 3B). Interestingly, it also increased the levels of phosphorylated H3 (Figure 3B). Real-time RT-PCR revealed that EGR-1 expression induced by IL-1β was further increased by SAHA (Figure 3B). This indicates the permissive role of H3 acetylation in IL-1β–inducible EGR-1 expression.

To further explore the role of histone acetylation and phosphorylation in IL-1β–inducible EGR-1 transcription, we treated HASMCs with Garcinol, an inhibitor of H3 acetyltransferases,\textsuperscript{34} or transfected the cells with short interfering RNA (siRNA) targeting MSK1 and MSK2. H3 phosphorylation and acetylation induced by IL-1β were reduced by MSK1 and MSK2 siRNA (Figure 4A). H3 acetylation was inhibited by Garcinol, with less effect on H3 phosphorylation (Figure 4A). Real-time RT-PCR revealed that IL-1β–inducible EGR-1 expression is indeed reliant on H3 acetylation and phosphorylation (Figure 4B). Co-IP analysis revealed the formation of an IL-1β–inducible complex between EGR-1 and the histone acetyltransferase p300 in HASMC extracts (Figure 4C, upper). EGR-1 and p300 also interact as recombinant proteins (Figure 4C, lower). Real-time ChIP showed the enrichment of p300 at the EGR-1 promoter in response to IL-1β stimulation (Figure 4D), whereas transient transfection analysis demonstrated that p300 stimulates EGR-1 promoter activity through the EGR-1 binding site (Figure 4E). Moreover, both phosphorylated and acetylated H3 are enriched, together with EGR-1 itself, at the EGR-1 promoter in response to IL-1β stimulation (Figure 4F). Figure 3A and Supplemental Figure IIIA and Supplemental Figure IVA indicate that although IL-1β affects change in H3 acetylation and phosphorylation,
there is no change in total H3 levels in cells exposed to the cytokine. We used an antibody against total H3 (nonacetylated and nonphosphorylated forms) as a control in the ChIP assay to show that H3 acetylation and phosphorylation are increased at the EGR-1 promoter in response to IL-1β. Figure 4F demonstrates that total H3 occupancy of the EGR-1 promoter does not differ between untreated and IL-1β-treated samples. Taken together, these data indicate that H3
acetylation and phosphorylation, and EGR-1, are increased at the EGR-1 promoter in response to IL-1β.

**EGR-1 Is Enriched at the Promoters of EGR-1–Dependent Genes in an MSK- and Histone H3 Acetyltransferase–Dependent Manner**

To determine whether IL-1β–inducible EGR-1 functionally regulates transcription of inflammatory mediators, we treated HASMCs with a course of IL-1β, and the mRNA levels of tissue factor and PAI-1 were examined. Quantitative real-time RT-PCR showed a time-dependent induction in both TF and PAI-1 in HASMCs exposed to IL-1β (Supplemental Figure IV), consistent with the time-course induction of EGR-1 expression (Supplemental Figure IA). Both conventional and real-time ChIP revealed the enrichment of EGR-1 at the promoter region of TF and PAI-1 in response to IL-1β (Figure 5A and B), which is suppressed by PD98059, Garcinol, and MSK1/2 siRNA (Figure 5C). EGR-1 siRNA blocked IL-1β–inducible EGR-1, PAI-1, and tissue factor expression (Figure 5D). Thus, EGR-1 induced by IL-1β binds to and induces TF and PAI-1 expression, in an MEK/ERK, MSK, and H3 acetyltransferase–dependent manner (Figure 6).

**Discussion**

The proinflammatory cytokine IL-1β has long been implicated in the pathogenesis of cardiovascular disease. IL-1β and its receptor are expressed in atheromatous tissue; IL-1β activates macrophages, stimulates SMC proliferation, and upregulates endothelial adhesion molecule expression. The zinc finger transcription factor EGR-1 is a “master regulator” in that it controls the expression of many pathophysiologically relevant genes. EGR-1–dependent genes have been strongly implicated in the initiation and progression of atherosclerosis and postangioplasty restenosis. Understanding the mechanisms regulating the inducible expression of EGR-1 in vascular cells by IL-1β would provide an important backdrop for future interventional approaches targeting this transcription factor.

To our knowledge, this study has revealed, for the first time, that EGR-1 regulates its own transcription and provides important clues on the role of chromatin remodeling and histone modification in EGR-1 transcription. We show that IL-1β controls EGR-1 expression via the MEK-ERK1/2 and MSK pathway. EGR-1 binds to and transactivates its own gene, and IL-1β induction of EGR-1 transcription involves histone H3 phosphorylation and acetylation. In contrast, p38 is a negative regulator of EGR-1, because SB202190, a selective p38 inhibitor, dose dependently enhances IL-1β–inducible EGR-1 transcription in HASMCs. The opposing influence of different mitogen-activated protein kinases on EGR-1 transcription demonstrates the tight control of this transcription factor at the level of signaling and transcription.

Chromatin structure and remodeling can have a profound influence on gene transcription; however, this influence has been unexplored because of the EGR-1 promoter until the present study. DNA methylation and histone modifications can lead to recruitment of protein complexes that regulate transcription. Our findings demonstrate that histone H3 modifications, acetylation, and phosphorylation, in particular, are associated with IL-1β–induced EGR-1 transcription in HASMCs. This study investigated the role of histone modification in the EGR-1 promoter, with a focus on H3 (rather than H4), given that H3 is the most extensively modified of the 5 known histones (H1–H5) and that it builds on prior work showing that EGR-1 transcription can be repressed by histone demethylase lysine demethylase 5b, which demethylates lysine 4 of H3. IL-1β–inducible phosphorylation and acetylation of histone H3 are attenuated by PD98059, consistent with the stimulatory effect of IL-1β on phosphorylated ERK1/2, phosphorylated MSK1/2, and EGR-1 mRNA. IL-1β
causes a rapid and transient induction in acetylated and phosphorylated histone H3, in line with the time course of IL-1β-induced EGR-1 mRNA expression. Furthermore, histone deacetylase inhibition enhances EGR-1 transcription in HASMCs treated with IL-1β, clearly indicating a role for histone acetylation in EGR-1 expression. Conversely, suppression of histone modification reduced cytokine-inducible EGR-1 transcription. Although IL-1β–inducible EGR-1 expression involves H3 acetylation and phosphorylation, Figure 3B and Figures 4A and B do not suggest absolute specificity of H3 acetylation on the EGR-1 gene. SAHA, in the absence of cytokine, caused a dramatic increase in acetylated H3 and EGR-1 mRNA levels (Figure 3B). SAHA potentiated IL-1β–inducible EGR-1 expression (Figure 3B, lower); however, SAHA caused little or no change in the total levels of H3 acetylation (Figure 3B, upper). There are several possible explanations for this. First, it is possible that acetylated H3 was totally recovered from many gene loci, not just those of EGR-1. Second, there may be the involvement of transfection effects, additional to cis-action, induced by H3 acetylation. Alternatively, saturating acetylated H3 levels induced by SAHA would render further increases difficult to detect. Nevertheless, acetylated H3 and phosphorylated H3 were markedly enriched at the EGR-1 promoter by real-time ChIP, further confirming the role of histone modifications in EGR-1 transcription. Histone H3 acetyltransferase and MSK1/2, as well as MEK-ERK1/2, are needed for EGR-1 occupancy of the PAI-1 and TF promoters. EGR-1 physically and functionally interacts with p300 and interacts with its own promoter. Thus, these findings demonstrate that histone H3 modifications are crucial for the transcriptional activation of EGR-1 in cells exposed to IL-1β, and that EGR-1 controls transcription of its own gene. EGR-1 control of EGR-1 transcription suggests the existence of a “self-amplification” cascade in injured arteries, involving IL-1β–driven chromatin remodeling, EGR-1 and EGR-1–dependent gene expression, a shift to a synthetic cellular and proinflammatory phenotype, and clinical manifestation of vascular disease.

Acknowledgments
We thank Sudha Rao, PhD (John Curtin School of Medical Research, Australia National University, Canberra) for her helpful advice.

Sources of Funding
This study was supported by grants from the National Health and Medical Research Council and the National Heart Foundation of Australia. Dr Khachigian is an Australia Fellow of the NHMRC.

Disclosures
None.

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Arterioscler Thromb Vasc Biol. 2010;30:536-545; originally published online December 17, 2009;
doi: 10.1161/ATVBAHA.109.193821

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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A

Relative expression of EGR-1 (qRT-PCR)

- Untreated
- 2.5 ng/ml
- 5 ng/ml
- 10 ng/ml
- 20 ng/ml

IL-1β, 30 min

B

Relative expression of EGR-1 (qRT-PCR)

- Untreated
- 15 min
- 30 min
- 1 h
- 2 h
- 4 h

IL-1β (10 ng/ml)

C

10 ng/ml IL-1β (h)

- 0
- 0.5
- 1
- 2
- 4

EGR-1

DAPI

Wang et al., Supplementary Figs. 1A,B,C
C

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Wang et al., Supplementary Fig. 2C
Figure A: Time course of protein expression after IL-1β treatment
- IL-1β (10 ng/ml), h
- UT, 0.25, 0.5, 1, 2, 4 h
- Phospho-H3 (Ser10)
- Acetyl-H3 (Lys23)
- dM-H3 (Lys4)
- Histone H3
- β-Actin

Figure B: IL-1β (10 ng/ml) effect on cell morphology
- Phospho-H3
- DAPI
- 0, 0.5 h
Wang et al., Supplementary Fig. 4
Phosphorylation and Acetylation of Histone H3 and Autoregulation by EGR-1 Mediate IL-1beta

Induction of EGR-1 Transcription

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Materials and Methods

Generation of promoter reporter constructs. To evaluate the contribution of transcription factor binding sites in the human EGR-1 promoter, the 506 bp Not I-Xho I fragment of the human EGR-1 promoter was amplified by PCR and cloned in pGL3 Basic plasmid (Promega) using the following primers, -450 forward: 5’-ATA TAA GGT ACC CAG CTG CGA C-3’, +56 reverse: 5’-ATA TAA CTC GAG GAA GCT GGC TG-3’. The murine Egr-1 expression vector (pCB6-Egr-1) was a gift from Dr Vikas Sukhatme (Beth Israel Deaconess Hospital, Boston). Sequence identity of constructs was confirmed by automated DNA sequencing. pRL-null was purchased from Promega.

Site-directed mutagenesis. Site-directed mutation of putative transcription factor binding sites was carried out using QuickChange II Site-Directed Mutagenesis Kit (Stratagene) as the manufacturer’s instruction. The following primers were utilized to generate site-directed mutants, SRE1mt forward: 5’-GCT TCC TTC CCA TGG GTG GCC ATG TAC GTC ACG-3’, SRE1mt reverse: 5’-CGT GAC GTA CAT GGC CAC CCA TGG GAA GCA GGA AGC-3’; SRE2mt forward: 5’-GGC CGG TCC TGC CAT GGG AGG GCT TCC TGC-3’, SRE2mt reverse: 5’-GCA GGA AGC CCT CCC ATG GCA GGA CCG GCC-3’; EGR-1mt forward: 5’-CCT GGG ATG CGG GCC AGC GAA AAA GCG CGG GCC CTA GG-3’, EGR-1mt reverse: 5’-CCT AGG GCC CGC GCT TTC GCC CGC ATC CCA GG-3’;
NF-kappaBmt forward: 5’-GGG GCA ACG CGG GCC TTC CGG AGC TGC GC-3’, NF-kappaBmt reverse: 5’-GCG CAG CTC CGG AAG GCC CGC GTT GCC CC-3’. All mutants were confirmed by automated DNA sequencing.

**Transient transfection and luciferase assays.** Human and rat aortic SMCs grown to 70-80% confluence in 6-well plate in Waymouth medium containing 10% FBS were transiently transfected with 0.5 µg reporter plasmid, 1 µg pCB6-Egr-1 plasmid, 1 µg pCMVβ-p300 and 0.1 µg pRL-null plasmid using FuGENE6 (Roche) as manufacturer’s instruction (ratio of DNA:FuGENE6 is 1:3). The total amount of transfected DNA was kept constant using the corresponding empty vector. 24 h after co-transfection, cells were incubated in serum-free medium for another 24 h, and/or treated with 10 ng/mL IL-1beta for the indicated times, the relative luciferase activity was measured by Dual-Luciferase Reporter Assay system (Promega) using a TD-20/20 luminometer (Turner Designs) and with Firefly luciferase data normalized to Renilla.

**Real-time quantitative reverse transcription-PCR.** Total RNA was prepared from HASMC treated with 10 ng/mL IL-1beta for the indicated times using TRIzol (Invitrogen) according to the manufacturer’s instructions and quantified by UV absorbance at 260 nm in a GeneQuant Pro spectrophotometer (Amersham Biosciences). cDNA was synthesized using 2 µg total RNA and 25 µg/mL oligo (dT)12-18 primer using the SuperScript II reverse transcriptase (Invitrogen). The target primers for amplifying *EGR-1* were forward primer: 5’-AGC AGC ACC TTC AAC CCT CA-3’ and reverse primer: 5’-CAG CAC CTT CTC GTT GTT CAG A-3’. For amplifying *PAI-1*, forward primer: 5’-GTG ATT CCA TCA ATG CAT GGG TTA-3’ and reverse primer: 5’-TTG GAA CCG TGA TTT CCA CAG A-3’. For amplifying *TF*, forward primer: 5’-TCC CCA GAG TTC AAC CCT TAC C-3’ and reverse primer: 5’-TGA CCA CAA ATA CCA CAG CTC C-3’. Each 20 µL of reaction mixture
for real-time RT-PCR contained 1 mmol/L MgCl₂, 0.15 μmol/L of each primer and 1 μL of SYBR Green PCR Master Mix (Applied Biosystems). Real-time quantitative RT-PCR analysis was carried out with Corbett Research RG-6000 (Corbett Life Science, Australia). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control to standardize and to test the RNA integrity with sequence for forward primer: 5’-GAA GGC TGG GGC TCA TTT-3’ and for reverse primer: 5’-CAG GAG GCA TTG CTG ATG AT-3’⁴. All experiments for real-time RT-PCR were performed in triplicate and data was analysed using the comparative Ct method⁵. Results are shown as fold induction of mRNA.

**Immunocytofluorescence.** To detect the expression and subcellular distribution of EGR-1, phospho-p44/42 MAPK, and phospho-H3 in HASMC treated with 10 ng/mL IL-1beta, the cells were cultured on coverslips, fixed and permeabilized with methanol and 4% paraformaldehyde, and incubated with 1:400 dilution of rabbit monoclonal antibody to EGR-1 (Cell Signalling Technology) or 1:200 dilution of rabbit monoclonal antibody to phospho-p44/42 MAPK, or 1:100 dilution of rabbit polyclonal antibody to phospho-histone H3 (Cell Signalling Technology) in blocking buffer at 4°C overnight. After incubation with primary antibody, cells were washed five times in PBS (pH 7.4) and incubated with 1:100 dilution of FITC-conjugated goat anti-rabbit antibody (Zymed) for 2 h at room temperature. After five-time washes in PBS, nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma). Fluorescence was observed under 600x on an inverted microscope (Olympus Digital DP70).

**Western blot analysis.** Cells were rinsed three times with ice-cold PBS and scraped off the plate in RIPA buffer (150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, 25 mmol/L Tris-Cl (pH 7.5), 1% sodium deoxycholate, 1mmol/L Na₃VO₄, 20 μg/mL pepstatin, 5 μg/mL aprotinin and 1 mmol/L PMSF), pipetted up and down 8 times, and centrifuged for 10 min at 13,000g. Nuclear lysates were
prepared as previously described\(^6\). Protein concentrations of the lysates were determined using the BCA Protein Assay Kit (Pierce). 20-40 µg protein per sample was electrophoresed on 10% SDS-PAGE and electrophoretically transferred to PVDF membranes (Millipore) at 4°C for 1.5 h. Blots were incubated for 1 h with 5% non-fat dry milk or 5% BSA to block non-specific binding sites and then incubated with either 1:2,000 dilution of rabbit monoclonal antibody against Phospho-p44/42 MAPK or 1:2,000 dilution of mouse monoclonal antibody to p42 MAP kinase or 1:1,000 dilution of rabbit monoclonal antibody to p-p38 or 1:1,000 dilution of rabbit polyclonal antibody to p38 or 1:1,000 dilution of rabbit polyclonal antibody to phospho-MSK1 or 1:1000 dilution of mouse monoclonal antibody to phospho-Elk1 or 1:1000 dilution of rabbit polyclonal antibody to Elk1 or 1:1,000 dilution of rabbit monoclonal antibody to phospho-SAPK/JNK or 1:1,000 dilution of rabbit monoclonal antibody to SAPK/JNK or 1:1,000 dilution of monoclonal antibody to EGR1 (Cell Signaling Technology) or 0.5 µg/mL anti-phospho-MSK2/RSKB antibody (R&D Systems) or 1:2,000 dilution of rabbit polyclonal antibody to MSK2/RSKB (Abcam) or 1:1,000 dilution of rabbit polyclonal antibody to MSK1 or 1:500 dilution of mouse monoclonal antibody to p65 or 1:500 dilution of mouse monoclonal antibody to p-c-Jun (sc-822, Santa Cruz Biotechnology) or 1:1,000 dilution of rabbit monoclonal antibody to c-Jun (Abcam) or 1:2,000 dilution of rabbit monoclonal antibody to phospho-histone H3 (Ser10, Upstate) or 1:500 dilution of rabbit polyclonal antibody to acetyl-Histone H3 (Lys23, Cell Signaling) or 1:1,000 dilution of rabbit polyclonal antibody to di-Methyl-Histone H3 (Lys4, Cell Signaling Technology) or 1:1,000 dilution of rabbit polyclonal antibody to phospho-RSK or RSK (Cell Signaling Technology) or 2 µg/mL monoclonal antibodies to p300 (Upstate Biolabs) at 4°C overnight. Immunoreactivity was detected using peroxidase-conjugated antibody and visualized by enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Pierce). Blots were stripped and reprobing with antibody to beta-actin (Sigma).
siRNA. EGR-1 siRNA used in this study was SMARTpool L-006526-00-0010 (Dharmacon). MSK1 siRNA (s17691) and MSK2 siRNA (s17139) were obtained from Ambion. ON-TargetPlus Control Pool D-001810-10-20 (Dharmacon) was used as control siRNA.

**Phosphorylation and acetylation of histone H3.** HASMC (at 80% confluence) were transfected with 50 nmol/L MSK1 or MSK2 siRNA using FuGENE6 (Roche) for 24 h, followed by starvation in serum-free medium for 24 h. Cells were then treated with 10 μmol/L or 20 μmol/L Garcinol (BIOMOL) for 6 h prior to IL-1beta exposure. After preparation of total cellular RNA and protein samples, the levels of EGR-1 mRNA, phosphorylated-H3 and acetylated-H3 were determined by real-time RT-PCR and Western blot analysis.

**Electrophoretic mobility shift assay (EMSA).** EMSA was carried out as described previously. Briefly, 20 μL of reaction contained 1 or 2 μg of Egr-1-GST protein (mouse Egr-1 from pGEX-2T-murine Egr-1 and purified in-house using glutathione-agarose beads (Sigma), 1 μg poly(dI/dC)-poly(dI/dC) (Sigma), 1 μg salmon sperm DNA (Sigma), 10 mmol/L Tris-Cl, pH 7.5, 50 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 2.5% glycerol, 0.001% NP-40, 1 μg/μL BSA, and 150,000 cpm of 32P-labeled probe (EGR-1 EMSA1: 5’-CGC CTG GGA TGC GGG CGC GGG CGC CCC TAG GGT GCA-3’, and EGR-1 EMSA2: 5’-TGC ACC CTA GGG CCC GCG CCC GCG CCC GCA TCC CAG GCG-3’; EGR-1 EMSA-nt1: 5’-CGC CTG GGA TGC GGG CGA AAG CGC GGG CCC TAG GGT GCA-3’, AND EGR-1 EMSA-nt2: 5’- TGC ACC CTA GGG CCC GCG CTT TCG CCC GCA TCC CAG GCG-3’). DNA/protein complexes were resolved electrophoretically on 6% non-denaturing polyacrylamide gels. Gels were dried and exposed to X-ray film at -20°C.
Co-immunoprecipitation (Co-IP). Co-IP was performed with cell extracts as previously described using 50μg nuclear extract from IL-1beta-treated and untreated HASMC. To demonstrate an interaction between recombinant Egr-1 and p300, a fixed amount (0.6 μg) of purified recombinant p300 (Active Motif) was combined with 1 or 2 µg GST-Egr-1 and precipitated with glutathione-agarose beads (Sigma) then immunoblotted with p300 antibodies (sc-585, Santa Cruz Biotechnology). Alternatively, pull down was achieved with p300 antibodies and Protein A/G (GE Healthcare) prior to blotting with GST antibodies (GE Healthcare). Samples were loaded into 6% SDS-PAGE gels and transferred into PVDF membrane, and visualised by chemiluminescence.

ChIP. Quantitative ChIP assays were performed on primary human vascular SMCs as detailed elsewhere. Briefly cells were treated with 0.4% formaldehyde and the cross-linked chromatin retrieved by nuclei isolation and lysis. The chromatin was sonicated to ~300 bp, pre-cleared with rabbit serum and immunoprecipitated with anti–Egr-1 (sc-110) from Santa Cruz Biotechnology, antiacetyl H3 (06-599), anti-phospho H3 (05-817) and anti-H3 (06-755) antibodies from Upstate (Lake Placid, NY). Enrichments were measured by real-time PCR using Sybr Green (Stratagene) as previously described. The levels of enrichment were normalized to that obtained with a control rabbit antibody or total input and were calculated as a fold increase over that measured at a control region, the LMO2 proximal promoter. The following primer pairs were used; EGR-1F: 5’-TTA TTT GGG CAG CAC CTT ATT TG-3’; EGR-1R: 5’-CTC CCT CCG CCT TCT TCC-3’; LMO2F: 5’-CCC AGC CTC TCT AAG CAA-3’, LMO2R: 5’-CAA TAC AAA CAG CAG CAT CAG-3’; PAI-1-330F: 5’-ACC CGG CAT GGC AGA CAG TCA ACC-3’, PAI-1+22R: 5’-GGC CCT GCA GCC AAA CAC AGC-3’; TF-205F: 5’-CAT CCC TTG CAG GGT CCC GGA GTT-3’, TF+2R: 5’-GGG GTG CGG GGA GCT CGC AGT C-3’.10
Statistical analysis. Data was analysed by ANOVA, and significant differences (where the P value<0.05) are indicated by asterisk. Experiments were performed in triplicate on at least two independent occasions, and studies (such as transient transfection analysis) subject to quantitative analysis were performed in triplicate.

Supplementary Figure Legends

Supplementary Fig. 1. IL-1beta stimulates EGR-1 expression and nuclear accumulation in HASMC. A, HASMC grown to 90% confluence were incubated in serum-free medium for 24 h then exposed to different concentrations of IL-1beta for 30 min. Total RNA was isolated and subjected to real-time RT-PCR using primers for EGR-1 as described in the Methods. B, HASMC grown to 90% confluence were incubated in serum-free medium for 24 h and exposed to 10 ng/mL IL-1beta for the indicated times. Total RNA was isolated and subjected to real-time RT-PCR. C, HASMC grown on coverslips were starved for 24 h in serum-free medium and treated with 10 ng/mL IL-1beta for 30 min, immunofluorescence staining was then performed at 0.5, 1, 2 and 4 h using EGR-1 antibodies as described in the Methods. *indicates P<0.05.

Supplementary Fig. 2. Pathways mediating IL-1beta-dependent EGR-1 expression in HASMC. A, HASMC were starved for 24 h and stimulated with 10 ng/mL IL-1beta for the indicated times. Whole cell lysates were prepared in RIPA buffer and separated by 10% SDS-PAGE. Western blot analysis was carried out using antibodies specific to either phospho-ERK1/2 or ERK1/2, phospho-p38 or p38, phospho-JNK1/2 or JNK1/2, phospho-c-Jun or c-Jun, as described in the Methods. B, HASMC grown on coverslips were starved for 24 h in serum-free medium and treated with 10 ng/mL IL-1beta for 30 min. Immunofluorescence staining was performed using phospho-ERK1/2 specific antibodies to determine the p-ERK1/2 immunoreactivity and its subcellular localization. C, HASMC
were starved for 24 h and exposed to 10 ng/mL IL-1beta for the indicated times. Nuclear lysates were prepared and separated by 10% SDS-PAGE. Western blot analysis was performed using antibodies specific to p-Elk1 or Elk1, or p65 as described in the Methods. Quantitative densitometric analysis of band intensity is also shown (right).

**Supplementary Fig. 3. IL-1beta mediated histone H3 modification.** A, HASMC were starved for 24 h and treated with 10 ng/mL IL-1beta for the indicated times. Whole cell lysates were prepared in RIPA buffer and separated on 10% SDS-PAGE. Western blot analysis was performed for phospho-H3, acetyl-H3, dM-H3, total H3 or beta-actin. B, HASMC grown on coverslips were starved for 24 h in serum-free medium and treated with 10 ng/mL IL-1beta for 30 min. Immunofluorescence staining was performed using phospho-H3 specific antibodies to determine phospho-H3 immunoreactivity and its subcellular localization.

**Supplementary Fig. 4. IL-1beta inducible TF and PAI-1 expression.** HASMC were incubated in serum-free medium for 24h and exposed to 10 ng/mL IL-1beta for the indicated times. Total RNA was isolated and subjected to real-time RT-PCR using primers for TF (upper) and PAI-1 (lower).

**References**


A

IL-1β (10 ng/ml), h

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- β-actin

Wang et al., Supplementary Fig. 2A
B

IL-1β (10 ng/ml), h

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pERK1/2

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