Protein Kinase C Pathway Is Involved in Transcriptional Regulation of C-Reactive Protein Synthesis in Human Hepatocytes

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Objective—C-Reactive protein (CRP) is the prototype acute phase protein and a cardiovascular risk factor. Interleukin-1β (IL-1β) and IL-6 stimulate CRP synthesis in hepatocytes. We searched for additional pathways regulating CRP expression.

Methods and Results—Primary human hepatocytes (PHHs) were treated with IL-1β, IL-6, and protein kinase C (PKC) activator phorbol 12,13-dibutyrate (PDBu). CRP was analyzed by quantitative RT-PCR and ELISA. PDBu significantly induced CRP transcription by 21.0±9.24-fold and protein release by 2.9±0.5-fold. Transcriptional regulation was studied in detail in hepatoma G2 (HepG2) cells stably transfected with the 1-kb CRP promoter (HepG2–ABEK14 cells). In these cells, PDBu significantly induced CRP transcription by 5.39±0.66-fold. Competitive inhibition with bisindolylmaleimide derivative LY333531 abolished PDBu-mediated promoter activation. Competitive inhibition with IκB kinase inhibitor I229 also inhibited PDBu effects. Importantly, IL-8 significantly induced CRP release in PHHs by 58.67±19.1-fold, which was blockable by LY333531.

Conclusions—This study describes a novel PKC-dependent transcriptional regulation of CRP gene expression, which, in analogy to the classical IL-1β and IL-6 pathways, is operational in hepatocytes only. It also identifies IL-8 as a potential physiological PKC activator. HepG2–ABEK14 cells may be useful for high throughput screening to identify inhibitors of CRP synthesis for the prevention of cardiovascular disease. (Arterioscler Thromb Vasc Biol. 2005;25:186-192.)

Key Words: atherosclerosis ■ C-reactive protein ■ drug development ■ gene expression ■ protein kinase C
strongly activates the conventional PKC pathway, resulting in phosphorylation of Ser 105 within the activation domain of C/EBPβ, enhancing its transcriptional activity.28 Despite the importance of this signaling cascade, involvement of the PKC pathway in regulation of CRP expression has never been investigated.

Here we identify the PKC pathway as a novel pathway in the regulation of CRP expression. Furthermore, we show that stable transfection of hepatoma G2 (HepG2) cells with the 1-kb CRP promoter–luciferase construct provides a reporter cell line strongly inducible by IL-1β, IL-6, or PDBu. This cell line may be useful for high throughput screening (HTS) to identify inhibitors of CRP synthesis in hepatocytes for the prevention of cardiovascular disease.

Materials and Methods

Cell Culture

Human hepatoma cell line HepG2 (DSMZ) was grown in Iscove’s medium (GIBCO) supplemented with 10% FCS and 1% penicillin/streptomycin (GIBCO). PHHs were obtained from Cytomet GmbH, aortic smooth muscle cells (AoSMCs) from PromoCell GmbH, and human umbilical vein endothelial cells (HUVECs) from Clonetics.

Stable Transfection of HepG2 Cells With p1000CRP/Luciferase

A fragment of human CRP promoter (~1005 to +12 bp) was cloned from human genomic DNA. Forward primer: 5'-ATGGTACCAGTA-AGATTGACAGACAGTGTGGAG-3'; reverse primer: 5'-ATCTC-TGAGGCTAGAATGTCAGGAGTGG-3'. The polymerase chain reaction (PCR) fragment was digested with Kpn-Xhol restriction enzymes, cloned into pGL3-BasicVector (Promega) and sequenced. Cells were cotransfected with the p1000/CRP/luciferase construct and pcDNA 3.1 using Lipofectamine 2000 (FL2000; Invitrogen). Standard medium containing 1 mg/mL G418 (Invitrogen) was added. Clones were isolated after 18 days; 1 of the clones (HepG2–ABEK14) with IL-6–inducible p1000/CRP/luciferase reporter activity was used for further experiments. Batch to batch differences were tested with a second stably transfected cell line.

Transient Transfection of HepG2 Cells With NF-κB–Luciferase

Transfection was performed using LF2000 (Invitrogen). Cells were incubated with the transfection mixture (0.1 µg of reporter plasmid DNA and 2.5 µL of LF2000 in 500 µL medium) for 5 hours. Transfection efficiency was ~20% to 30% as assessed by green fluorescent protein–expressing plasmid (pEGFP; Clontech). One day after transfection, cells were treated with the cytokines or PDBu. Bright-Glo Luciferase Assays (Promega) were performed and measured by luminometer (Lumistar). Data were normalized to total protein.

Inhibitors

Stock solutions were prepared in DMSO (10 mmol/L). Cells were pretreated with inhibitors (final DMSO concentration 0.2%) for 1 hour before stimulation with cytokines or PDBu. Control cells were treated with identical media, including DMSO without cytokines. IκB kinase complex inhibitor I229 inhibits the IκB kinase complex (IKKα and IKKβ) with an in vitro IC₅₀ of 3.0 mmol/L.29 Specificity of 1229 against 14 different serine/threonine and tyrosine kinases (percent inhibition at 10 µmol/L and 5.0 µmol/L ATP [in vitro kinase reaction]) is summarized in the Table (n=5). The bisindolylmaleimide derivative LY333531 (Calbiochem) inhibits the PKCβI (in vitro IC₅₀=4.7 nmol/L) and PKCβII (IC₅₀=5.9 nmol/L) isozymes30,31 60- to 70-fold more selectively than PKCα and other PKC isoforms.31

Quantitative Real-Time PCR

Total RNA was isolated from PHHs, HUVECs, and AoSMCs using RNeasy Kit (Qiagen). Purity was assessed using capillary electrophoresis Caliper Laboratory Chip system (Agilent 2100 Bioanalyzer). Real-time quantitative PCR was performed using QuantiTect SYBR-Green Probe RT-PCR Kit (Qiagen). Each sample was assayed in triplicate. For relative quantification, the ΔCt method (ratio = 2ΔΔCt) was used with GAPDH as a control. For copy number determination, a calibration curve was obtained using serial dilutions of a linearized GAPDH cDNA with the GAPDH primer pair forward: 5'-GAAGAGGTGAAGGTCGGAGTC-3' and reverse: 5'-GAAGATGTTGATGGGATTTC-3'. A statistical analysis of RT-PCR data were performed using Q-gene software. Human CRP primer pair 1 (product size 133 bp; forward 5'-ACTCTCCTATGTTATCCCTCAAG-3'; reverse: 5'-CTCATTTGTTGTCTTGGT-3'). Human CRP primer pair 2 (product size 440 bp; forward 5'-TCTGATGCCAACAGAGAGACA-3'; reverse: 5'-AACATCTCGGATGATGGTTC-3'). Primer pair 3 was designed to distinguish between mRNA and genomic DNA because forward and reverse primers are specific for the different CRP exons (expected product size 196 bp for mRNA and 481 bp for genomic DNA): forward 5'-TCTCATGCTTGTGCCAGAC-3'; reverse: 5'-TCTATGTCTTGTCTTGGT-3'.

End Point PCR Product Analysis

Quality and size of PCR end point product were determined using capillary electrophoresis Caliper Laboratory Chip system (Agilent 2100 Bioanalyzer).

Immunostaining

HepG2 cells were fixed in 4% formaldehyde and permeabilized with 0.5% Triton X-100 (Sigma). Non-specific binding was blocked with Super Block (Pierce). Cells were stained with polyclonal rabbit anti-PKCβI (Santa Cruz Biotechnology) and goat anti-rabbit IgG (Alexa Fluor 594). Microscopy and photoimaging were assessed with Nikon Eclipse TE300 microscope (×60).

CRP ELISA

Concentration of CRP in the supernatant of PHHs and other cells was measured by CRP ELISA (IMUCLONE CRP (high sensitivity) ELISA; American Diagnostica) according to manufacturer recommendations. Samples were diluted 1:10 and measured in triplicates. Experiments were repeated 3× and statistical analysis performed as indicated below.

Statistical Analysis

Statistical analysis was performed using SigmaStat version 2.0 software. Results are presented as mean±SD. Tests included 1-way ANOVA, Tukey Test, and Student t test. A P<0.05 was considered statistically significant (*). A P<0.001 was considered highly significant (**).

Results

PDBu Induces CRP Transcription and Protein Synthesis in PHHs

PHHs were treated with IL-1β, IL-6 (10 ng/mL), and PDBu (100 nmol/L; Figure 1). CRP mRNA was measured by
quantitative RT-PCR (Figure 1A), whereas CRP release was assessed by ELISA from cell supernatants (Figure 1B). The figure shows that PDBu significantly stimulates CRP transcription by 21.0/9.24-fold and protein release by 2.9/0.5-fold.

PDBu Stimulates CRP Promoter Activity in HepG2–ABEK14 Cells

In search for an appropriate cell culture model to study CRP synthesis in high throughput applications, HepG2 cells were stably transfected with p1000CRP/luciferase. The clones were treated with IL-1β, IL-6, and PDBu, and 1 of the clones (HepG2–ABEK14) was chosen for further analyses. Whereas IL-1β and IL-6 increased promoter activity up to 5-fold only, synergistic activation was seen for IL-1β/IL-6 (10 ng/mL each) in combination with an up to 28-fold increase. B, PDBu induces CRP promoter in HepG2–ABEK14 cells. Cells were stimulated with IL-1β (10 ng/mL), IL-6 (10 ng/mL), PDBu (100 nmol/L), and their combination. PDBu induced highly significant activation of the CRP promoter (5.39/0.66-fold). In contrast to PHHs (Figure 1A), combined IL-1β/IL-6 or IL-1β/IL-6/PDBu treatment shows superinduction of CRP promoter in HepG2–ABEK14 cells. C, Dose-response curve. Effect of increasing concentrations of PDBu (0 to 800 nmol/L) combined with fixed concentrations of IL-1β and IL-6 (10 ng/mL).

Figure 2. PKC activation induces CRP transcription in HepG2–ABEK14 cells. A, HepG2–ABEK14 cells. Activation of CRP luciferase reporter system by IL-1β and IL-6. Whereas IL-1β and IL-6 increased promoter activity up to 5-fold only, synergistic activation was seen for IL-1β/IL-6 (10 ng/mL each) in combination with an up to 28-fold increase. B, PDBu induces CRP promoter in HepG2–ABEK14 cells. Cells were stimulated with IL-1β (10 ng/mL), IL-6 (10 ng/mL), PDBu (100 nmol/L), and their combination. PDBu induced highly significant activation of the CRP promoter (5.39/0.66-fold). In contrast to PHHs (Figure 1A), combined IL-1β/IL-6 or IL-1β/IL-6/PDBu treatment shows superinduction of CRP promoter in HepG2–ABEK14 cells. C, Dose-response curve. Effect of increasing concentrations of PDBu (0 to 800 nmol/L) combined with fixed concentrations of IL-1β and IL-6 (10 ng/mL).
PDBu Activates PKCβII in HepG2–ABEK14 Cells

To confirm that phorbol ester treatment indeed engages PKC, PKCβII translocation was measured on PDBu treatment in HepG2 cells. PKCβII indeed translocated to the nuclear membrane 60 minutes after stimulation with PDBu (100 nmol/L; Figure 5A). During longer treatment (24 hours), we observed a marked decrease of PKC protein content, which is common phenomenon related to PKC activation.

LY333531 dose-dependently inhibited CRP promoter activation by PDBu. However, IL-1β/IL-6–mediated activation was not inhibited by LY333531, indicating independency of the pathways (Figure 5B) in HepG2–ABEK14 cells.

Figure 3. PDBu NF-κB–dependently activates PKCβII in HepG2–ABEK14 cells. A, Immunofluorescent staining, PDBu (100 nmol/L) induced PKCβII translocation to the nuclear membrane 60 minutes after stimulation. n indicates nucleus; c, cytoplasm. Arrows indicate PKCβII. B, LY333531 and I229 dose-dependently inhibit PDBu-mediated stimulation of CRP luciferase reporter in HepG2–ABEK14 cells. LY333531 has no effect on IL-1β/IL-6–mediated CRP promoter activation. C, NF-κB reporter analysis. HepG2 cells were transiently transfected with a NF-κB–luciferase reporter. IL-1β and PDBu but not IL-6 induce NF-κB, PKC inhibitor abolishes PDBu but not IL-1β effects, whereas I229 abolishes IL-1β and partly reduces PDBu effects. **Highly significant inhibition compared with control.

Figure 4. IL-8 PKC-dependently induces CRP protein release in PHHs. PHHs were treated with IL-8 (10 ng/mL). CRP concentration in cell supernatants was measured by ELISA. IL-8 significantly induced CRP protein release by 58.675±19.1-fold, and release was highly significantly reduced by LY333531 treatment down to 12.36±5.39-fold.

PKC-Mediated Transcriptional Regulation of CRP Promoter Is NF-κB Dependent

IKK inhibitor I229 abolished stimulatory effects of IL-1β/IL-6 and PDBu on regulation of the CRP reporter (Figure 3B), indicating NF-κB involvement in regulation of the CRP promoter. This was further supported by NF-κB reporter analyses in HepG2 cells transiently transfected with NF-κB–luciferase reporter (Figure 3C). IL-1β and PDBu induced IKK and, subsequently, NF-κB activation. IL-1β and PDBu induced NF-κB–luciferase reporter equally strong, whereas IL-6 had no effect (Figure 3C). Inhibition of the IKK complex with I229 completely blocked the effect of IL-1β and partially the effect of PDBu. In analogy to the observation on CRP promoter activation (Figure 3B), LY333531 dose-dependently inhibited the effects of PDBu on NF-κB promoter, whereas IL-1β/IL-6–mediated activation was not inhibited by LY333531. Competition experiments with LY333531 revealed that PDBu-induced NF-κB activation is totally PKC dependent but only partially IKK dependent, thus indicating potential involvement of additional kinases in PKC-mediated NF-κB stimulation. Neither LY333531 nor I229 had an effect on basal luciferase activity on its own (data not shown).

IL-8 PKC-Dependently Induces CRP Protein Release in PHHs

In search for a physiological PKC activator, PHHs were treated with IL-8 (10 ng/mL). CRP concentration in cell supernatants was measured by ELISA. IL-8 significantly induced CRP protein release by 58.675±19.1-fold, and release was highly significantly reduced by LY333531 treatment down to 12.36±5.39-fold. Toxic effects were excluded by measurement of lactat dehydrogenase release (Figure 4).

Regulation of CRP Promoter in Other Cell Types

To investigate whether the stimulatory effect of PDBu is restricted to PHHs and HepG2–ABEK14 cells, we also tested AoSMCs and HUVECs. Cells were treated with IL-1β, IL-6, or PDBu (Figure 5A through 5C). In PHHs, CRP mRNA levels measured by quantitative RT-PCR showed, as expected, a strong regulation of CRP mRNA after IL-6 stimulation and a weaker although still highly
significant regulation after IL-1β and PDBu treatment. However, in contrast to HepG2–ABEK14 cells, no synergistic enhancement was observed for either double treatment with IL-1β and IL-6 nor for triple treatment with IL-1β, IL-6, and PDBu in PHHs (Figure 1). This may be because of the fact that in PHHs, IL-6 alone elicits maximum possible stimulation or that PDBu induces IL-6 receptor shedding in some cells. In AoSMCs and HUVECs, no significant regulation of CRP mRNA was observed for IL-1β, IL-6, or PDBu treatment. Assessment of copy numbers per 1000 nontreated cells showed that CRP expression in PHHs is several orders of magnitude higher than CRP expression in vascular cells. Furthermore, we were not able to detect any CRP release from AoSMCs and HUVECs by ELISA (data not shown).

**Discussion**

In addition to its role in innate immunity, CRP is an important cardiovascular risk factor and may well be a target for cardiovascular therapy. Three strategies are feasible: (1) inhibition of CRP-mediated complement activation; (2) blockage of CRP receptors; and (3) inhibition of CRP synthesis. The third of these strategies seems to be the most appropriate because the proatherogenic effects of CRP obviously involve a 1 mechanism. To address this goal, an appropriate cellular model with well-defined signaling pathways controlling CRP expression is absolutely required.

Whereas IL-1β and IL-6 (signaling via the Janus kinase/STAT pathway, C/EBP, and NF-κB) are considered the major mediators of CRP synthesis, other pathways are poorly investigated. The PKC pathway identified in this report may be of potential importance for CRP regulation in vivo because stimulation of some receptor subclasses (eg, the receptors for thrombin or IL-8) induce PKC activation. Thrombin, IL-8, and angiotensin II are possible in vivo candidates for PKC-mediated CRP induction. Their receptors are present on hepatocytes, and they are generated during sepsis and inflammatory diseases, in parallel with elevated CRP plasma levels. IL-8 has also been shown to be intimately associated with atherosclerosis. Importantly, activation of PKC is known to result in the phosphorylation of Ser 105 within the activation domain of C/EBPβ, thereby enhancing its transcriptional activity. This may provide a transcriptional link explaining the effect of CRP induction via PDBu. Thus, the major result of this report is the first description of an involvement of the PKC pathway in CRP gene expression. The latter is true for 3 hepatocytic cell types: PHHs and HepG2–ABEK14 cells.

A further investigation in HepG2–ABEK14 cells revealed that NF-κB activation is involved in PKC-mediated transcriptional regulation of CRP synthesis. This is in line with observations from other cells, in which NF-κB activation involves upstream PKC activation. In HepG2 cells transiently transfected with the NF-κB reporter, PDBu and IL-1β induced NF-κB equally strong. Competition experiments with LY333531 revealed that PDBu-induced NF-κB activation is totally PKC dependent, whereas PDBu-induced NF-κB activation is only partially blocked by the IKK inhibitors, indicating potential involvement of additional signal transducers. As expected, IL-1β/IL-6–mediated induction of CRP also involves NF-κB, but this is a PKC-independent process. Altogether, these data show that effects of PKC activation on CRP promoter are partially mediated by NF-κB on the 1 hand and another unknown mechanism on the other hand (eg, PKC-dependent activation of C/EBP as described previously by Trautwein et al). It is intriguing to speculate that activation of G-protein–coupled receptors (eg, the IL-8 receptor or angiotensin II receptor) known to stimulate phospholipase Cβ and diacylglycerol production and, subsequently, PKC activation may induce CRP expression through NF-κB and C/EBP transcription factors.

Whereas in PHHs, activation of the PKC pathway by PDBu results in induction of CRP on the transcriptional and protein level, there is no detectable protein induction by PDBu in HepG2–ABEK14 cells. The latter is not surprising because endogenous CRP synthesis in HepG2 cells is known not to be intact. However, hepatoma cell lines, and in particular HepG2 cells, display some major experimental advantages: (1) They are widely available and very easy to grow over several months; (2) In contrast to other hepatoma cell lines (eg, Hep3B cells), they do not require S2 conditions because they are hepatitis negative; and (3) Stable transfection of these cells with the 1-kb CRP promoter coupled to luciferase results in a cell line (HepG2–ABEK14) that responds to IL-1β and IL-6 (and PDBu). The cell line is stable, very easy to grow, and provides a simple readout (luciferase). Developing an effective transcriptional inhibitor of CRP synthesis for the prevention of cardiovascular disease is a multistep approach: (1) HTS with a cell culture model

**Figure 5.** IL-1β, IL-6, and PDBu strongly induce CRP gene expression in PHHs but not in primary vascular cells. A, Relative expression of the CRP gene in different cells. Fold regulation is calculated as described in Materials in Methods; n.d. indicates not detectable. B, End point PCR products show fragments of the right size. Capillary electrophoresis confirms that amplified DNA product has the predicted size: 213 bp (GAPDH, bottom) and 133 bp, 440 bp, 196 bp (CRP, pending on primer pairs, top panels). C, Copy numbers per 1000 cells in primary cells.
(relevant cells of hepatic origin, cheap, easy to culture over several months, simple readout); and (2) testing the “hits” in other models (ie, Hep3B, PHHs).

It is currently impossible to use PHHs for such HTS because they do not proliferate in culture, and to screen a drug library including hundreds of thousands of substances, billions of cells are required that need to be cultured over several weeks. Species differences in CRP biology argue against use of primary hepatocytes from other species. Therefore, as a side product of this study, the generation of HepG2–ABEK14 cells provides a cell line suitable for HTS to identify inhibitors of CRP synthesis.

To understand whether PKC-dependent CRP regulation is also critical in smooth muscle and endothelial cells, we performed analogous experiments with AoSMCs and HUVECs. We observed that IL-1ß and PDBu potently stimulate CRP mRNA expression and protein release in PHHs, although much weaker than IL-6 does. In AoSMCs and HUVECs, we detected extremely low amounts of CRP mRNA, and we did not observe any significant regulation in response to IL-1ß, IL-6, or PDBu. No detectable CRP protein secretion has been found as well. Thus, either CRP synthesis in these cells is not relevant, or the endogenous CRP promoter is controlled by other cell-specific factors. Although there is some evidence that CRP may be produced by inflamed kidneys, it is most likely that CRP, like many other genes, is mainly expressed in a tissue-dependent manner (ie, is a preferentially liver-specific gene). In this context, it is interesting to note that our CRP reporter construct showed no IL-1ß– or IL-6–dependent regulation in renal human embryonic kidney 293 cells (data not shown).

In summary, our report demonstrates: (1) the PKC pathway as a novel pathway of CRP synthesis; (2) NF-κB dependency of this pathway; (3) potential usefulness of HepG2–ABEK14 cells for HTS to identify transcriptional inhibitors of CRP synthesis; and (4) liver specificity of PKC-mediated CRP transcription.

Acknowledgments

We thank Aventis Pharma Deutschland GmbH, Deutsche Forschungsgemeinschaft (SFB 451).

References


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Arterioscler Thromb Vasc Biol. 2005;25:186-192; originally published online November 11, 2004;
doi: 10.1161/01.ATV.0000150041.81963.68

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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