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Vascular smooth muscle cell activation by C-reactive protein

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Abstract

Objective: C-reactive protein (CRP) is an important cardiovascular risk factor. Although the role of CRP has been implicated in atherogenesis, its direct effects on vascular cells are poorly defined. **Methods:** We investigated the responses to CRP in vascular smooth muscle cells (VSMC). **Results:** The present study shows that CRP induces parallel activation of the redox-responsive transcription factors NF-kappa B (NF-κB) and AP-1 and increases the activity of the MAP kinases (MAPKs), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38MAPK, in VSMC. C-reactive protein increased the expression of early response genes, c-fos and c-jun and inflammatory genes, monocyte chemoattractant peptide (MCP-1) and interleukin-6 (IL-6). When VSMC were incubated with CRP, the inducible nitric oxide synthase (iNOS) promoter was activated. CRP alone was a weak inducer of NO production in VSMC as measured by determining nitrite levels, and interferon-γ alone was totally ineffective, whereas CRP plus interferon-γ was a powerful stimulus. This synergy for NO production corresponded to the results of iNOS mRNA expression analyzed by Northern blotting. The NF-κB activation caused by CRP was inhibited by 15-deoxy-12,14-prostaglandin J2 and the PPARγ activators, rosiglitazone and pioglitazone. Fluvastatin and cerivastatin also reduced the activation of NF-κB by CRP. **Conclusions:** CRP causes NF-κB activation which could lead to the induction of MCP-1, IL-6, and iNOS gene expression. CRP also activates the MAPK→c-Fos/cJun→AP-1 pathway. Thus, CRP may play a role in atherogenesis by activating VSMC.

Keywords: Gene expression; Infection/inflammation; Nitric oxide; Signal transduction; Smooth muscle

1. Introduction

Inflammation is a key mechanism associated with the formation of atherosclerotic lesions [1]. Many epidemiological studies have shown that C-reactive protein (CRP), which is a marker of inflammatory disease, is an important risk factor for atherosclerosis. The presence of CRP appears to predict a poor prognosis for patients with unstable angina, acute myocardial infarction, or acute stroke [2–5]. Thus, the CRP level may reflect the degree of an underlying inflammatory response. However, the mechanisms underlying this association are not completely clear. One possibility is that CRP directly participates in amplifying the response, thus leading to further tissue damage. C-reactive protein upregulates the expression of adhesion molecules on endothelial cells [6,7] and mediates LDL uptake by macrophages [8]. The direct effects of CRP on those cells further strengthen its role in the pathogenesis of vascular inflammation and atherosclerosis. Thus, the present study assessed the effects of CRP on vascular smooth muscle cells (VSMC). We investigated whether CRP delivers signals that induce activation of the redox-responsive transcription factors NF-kappa B (NF-κB) and AP-1, since CRP may contribute to atherosclerosis through redox-sensitive signaling events. We also examined the ability of CRP to activate the mitogen-activated protein (MAP) kinases, extracellular signal-regulated kinase (ERK), Jun N-terminal kinase and p38 MAP kinase. Inflammatory processes represent a hallmark of atherosclerosis and involve the release of inflammatory cytokines and chemokines with subsequent activation of distinct signaling cascades. Therefore, we investigated whether CRP induces the expression of mRNA for monocyte chemoattractant peptide (MCP-1) and interleukin-6 (IL-6)

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and whether induced gene expression and activated signaling pathways are functionally related. We also investigated whether CRP induces nitric oxide (NO) formation by VSMC with emphasis on the activation of NF-κB and the inducible NO synthase (iNOS) promoter followed by iNOS gene expression.

2. Methods

2.1. Cell culture and RNA extraction

We digested thoracic aortae from male Wistar rats using elastase and collagenase, then isolated VSMC [9] were cultured in DMEM containing 10% heat-inactivated FBS, 100 μg/ml piperacillin and 100 μg/ml streptomycin. The experiments were performed using VSMC after 10–15 passages. In the experiments, the cells were grown to confluence in 24-well plates or 9-cm dishes and then made quiescent by incubation with serum-free media for 24 h. Then the cells were incubated under experimental conditions. Total RNA was extracted from confluent VSMC using guanidinium isothiocyanate as described [10].

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85-23, revised 1985).

2.2. NF-κB and AP-1 activation

Cells were stably transfected with a *cis*-reporter plasmid containing the luciferase reporter gene linked to five repeats of NF-κB binding sites (pNFκB-Luc: Stratagene) as described [11], then NF-κB activation was examined in several selected clones. Luciferase activity was measured using a luciferase assay kit (Stratagene). Similarly, VSMC were stably transfected with a *cis*-reporter plasmid containing the luciferase reporter gene joined to seven AP-1 sites (pAP1-Luc: Stratagene) to study AP-1 activation [12].

The levels of NF-κB and AP-1 proteins in nuclear extracts from VSMC were also analyzed by electrophoretic mobility shift assay (EMSA). Nuclear proteins from these cells were isolated according to the method of Schreiber et al. [13], and were subjected to EMSA using ^{32}P-labeled NF-κB and AP-1 double-strand oligonucleotide (NF-κB: 5′-AGT TGA GGG GAC TTT CCC AGG C-3′, AP-1: 5′-CGC TTG ATG ACT CAG CCG GAA-3′, Santa Cruz Biotechnology). Nuclear proteins were incubated with oligonucleotide for 30 min, subjected to polyacrylamide gel electrophoresis, and autoradiographed.

2.3. MAPK assay

To create stable reporter cell lines with which to evaluate MAPK activation, we used a *trans*-reporting system with GAL4 fusion transactivators as pathway-specific sensors as described [12]. These systems use a fusion transactivator plasmid that consists of the DNA binding domain of the yeast GAL4 (residues 1–147) protein and the activation domain of Elk-1, c-Jun, or Chop. The clones that gave the best response when transfected with positive control vectors for each reporting system were then analyzed.

VSMC lysates (15 mg per lane) were subjected to 12% SDS–PAGE and transferred to PVD membranes (Bio-Rad) which were incubated with rabbit polyclonal antibodies against phospho ERK1/2 and nonphosphorylated ERK1/2 (Cell Signaling Technology). Antibody binding was detected using donkey anti-rabbit IgG horseradish peroxidase and the ECL Plus system (Amersham).

2.4. Analysis of mRNA expression

We investigated MCP-1 and iNOS mRNA expression using standard Northern blotting procedures as described [14]. Probes were obtained by reverse transcription-polymerase chain reaction (RT-PCR) with specific primers for MCP-1 and iNOS, then labeled with [α-^{32}P]dCTP by random priming. The labeled probes were applied to Northern blotting to analyze mRNA expression. Expression of the mRNA for MCP-1, IL-6, c-fos, and c-jun was also analyzed by RT-PCR as described [14].

2.5. Production of NO and iNOS promoter analysis

We studied the iNOS promoter function as described [11], using rat VSMC stably transfected with a construct containing a 1.7-kb fragment of the iNOS promoter that was cloned in front of a reporter gene encoding the secreted form of human placental alkaline phosphatase (SEAP). We measured levels of SEAP activity released into the cell culture medium using a sensitive chemiluminescent assay. Nitrite accumulation, which is an indicator of NO synthesis, was measured in the culture medium of confluent VSMC [15].

2.6. Statistical analysis

Data are presented as means±S.E.M. Multiple comparisons were evaluated by ANOVA followed by Fisher's protected least significant difference test. Student's unpaired *t*-test compared results between two experiments. A value of $P<0.05$ was considered statistically significant.

2.7. Materials

Recombinant human CRP and highly purified CRP from human serum were purchased from Calbiochem. All reagents were endotoxin-free according to the limulus test (from Sigma; sensitivity, 0.06 U/ml). The purity of CRP

preparations was also confirmed by SDS–PAGE (a single band on silver stained and overloaded gels). MAPK inhibitors, PD98059, SB203580, and SP60125 were obtained from Biomol. For inhibitory experiments, VSMC were incubated for 1 h with various peroxisome proliferator-activated receptor (PPAR)-agonists, including rosiglitazone (Glaxo), pioglitazone (Takeda), 15-deoxy-12,14-prostaglandin J2 (15d-PGJ2; Calbiochem); the PPAR agonists fenofibrate and Wy 14649 (Sigma); the hydroxymethylglutaryl coenzyme A antagonist (statin) cerivastatin or fluvastatin at the indicated concentrations. Thereafter, the cells were incubated with CRP for 4 h.

3. Results

3.1. NF-κB and AP-1 activation

We examined the ability of CRP to induce NF-κB-mediated reporter gene expression in VSMC. The results showed that CRP dose-dependently activated NF-κB-mediated gene transcription. NF-κB-dependent transactivation increased 40-fold relative to unstimulated levels in CRP-treated VSMC at a concentration of 0.1 mg/ml (Fig. 1A). EMSA, performed using NF-κB double stranded oligonucleotide as a probe, also confirmed the NF-κB activation

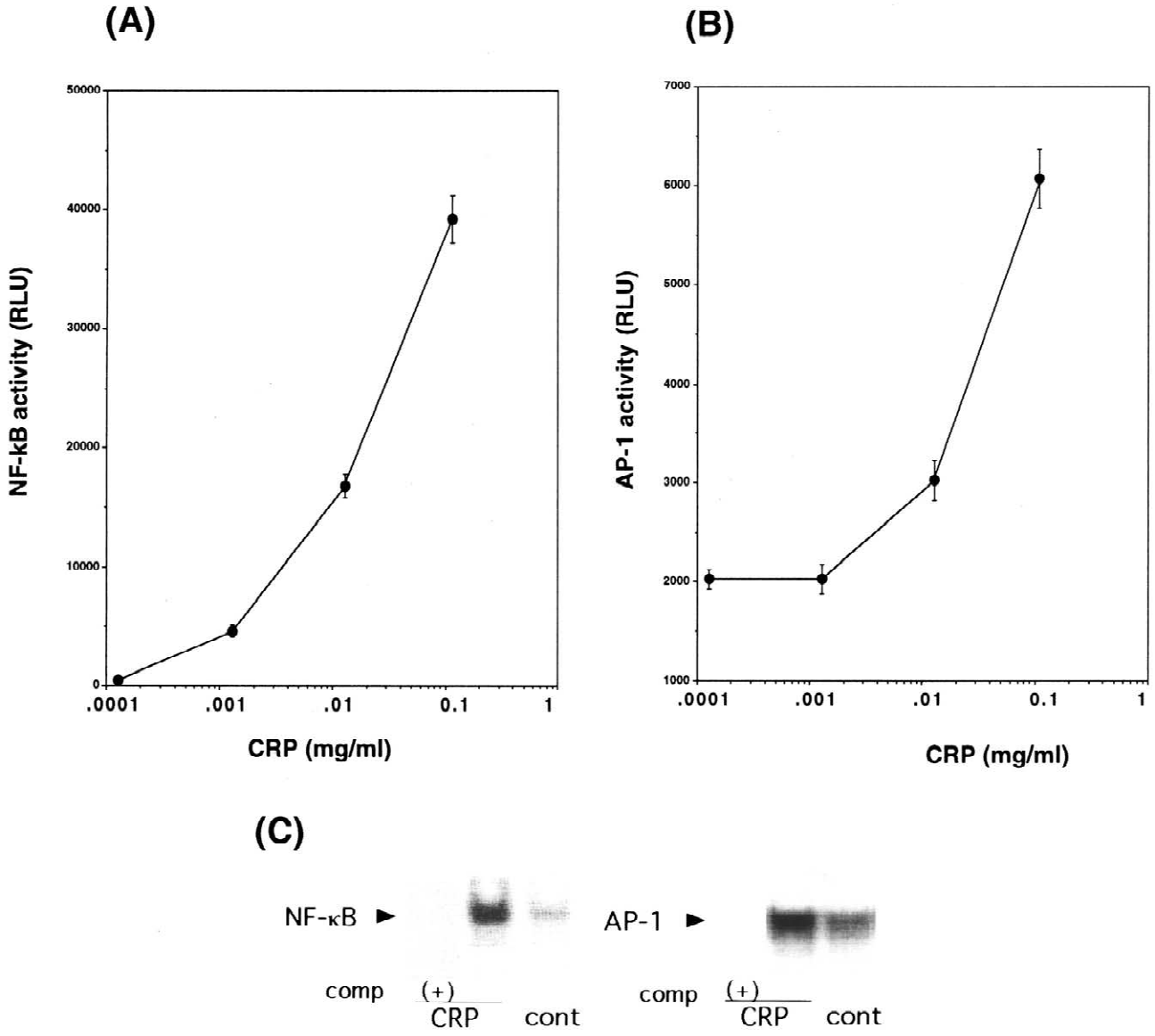

Fig. 1. (A) Effects of CRP on NF-κB-dependent transcriptional activity. Quiescent VSMC (transfected with pNFκB-Luc) were incubated with various concentrations of CRP. (B) Effects of CRP on AP-1-dependent transcriptional activity. Quiescent VSMC (transfected with pAP-1-Luc) were incubated with various concentrations of CRP. After 3 h, cells were lysed, and luciferase activities were measured. Data are mean±S.E.M. of triplicate observations. (C) VSMC were treated with or without CRP (0.1 mg/ml) for 2.5 h. The nuclear protein was extracted and subjected to EMSA using NF-kB or AP-1 double-strand oligonucleotide without or with 100-fold excess unlabeled probe as a competitor.

by CRP in those cells. As shown in Fig. 1C, CRP (0.1 mg/ml) caused a distinct shifted band, whereas there was no distinct band in the control cells. The strong band elicited by CRP was completely eliminated in the presence of a 100-fold molar excess of unlabeled probe. We also found that CRP dose-dependently activated AP-1-mediated gene transcription and that AP-1-dependent transactivation increased 3.0-fold relative to unstimulated levels in VSMC incubated with CRP at a concentration of 0.1 μg/ml (Fig. 1B). EMSA also confirmed the AP-1 activation, the specificity of which was disappearance of a band in the presence of a 100-fold molar excess of unlabeled AP-1 probe (Fig. 1C).

3.2. MAPK activation

We next examined the effect of CRP on the activation of MAPK pathways. We evaluated activation of the transcription factors Elk-1, c-Jun, or Chop by a *trans*-reporting using GAL4 fusion transactivators as pathway-specific sensors. When activated by phosphorylation, these fusion proteins bind to the promoter and induce luciferase expression. Therefore, luciferase activity in stable cell lines reflects the activation status of the fusion transactivator and hence, the activation status of corresponding signal pathways. The results showed that CRP substantially increased Elk-1 activity. Elk-1 activity increased 10-fold relative to unstimulated levels in VSMC incubated with 0.1 mg/ml of CRP (Fig. 2A). The increase in Elk-1 activity induced by CRP appears to reflect ERK activation in VSMC. In fact, Elk-1 activity stimulated by CRP in this system was completely prevented by the MAPK kinase (MEK) inhibitor PD98059 (inset in Fig. 2A). Measurements of ERK activity by Western blot showed that CRP caused a dose-dependent activation of ERK1/2 activity (Fig. 2B). Reprobing of the Western blot with an antibody against nonphosphorylated ERK1/2 was used to control equal protein loading (Fig. 2B). CRP also significantly increased c-Jun as well as Chop activity. These activities in VSMC were increased by CRP (0.1 mg/ml) 3.1- and 4.8-fold, respectively, relative to unstimulated levels (Fig. 2C). c-Jun or Chop activity stimulated by CRP was prevented by the specific inhibitor, SP600125 or SB203580, respectively (inset in Fig. 2C).

3.3. Expression of mRNA

We examined the effect of CRP on the mRNA levels of MCP-1, IL-6, c-Fos, and c-Jun in VSMC by RT-PCR. The results showed that CRP substantially increased MCP-1 and IL-6 mRNA levels and upregulated c-fos and c-jun gene expression (Fig. 3A). Control experiments demonstrated equivalent amounts of GAPDH mRNA.

We further characterized the ability of CRP to induce MCP-1 gene expression using signaling pathway inhibitors. Northern blotting revealed a basal level of MCP-1 mRNA in untreated VSMC. Following exposure to CRP, MCP-1 mRNA substantially increased within 2 h, and remained elevated for at least 24 h (data not shown). The effects of the antioxidant, *N*-acetylcysteine (NAC, 10 mmol/l) and pyrrolidine dithiocarbamate (PDTC, 50 μmol/l) or of the MAPK inhibitors, PD98059 (30 μmol/l) and SB203580 (10 μmol/l), on CRP-induced increases in MCP-1 mRNA were evaluated in VSMC. The CRP-induced MCP-1 mRNA levels were inhibited by PDTC and NAC (Fig. 3A). Fig. 3B shows that CRP-elicited MCP-1 mRNA expression was significantly suppressed by PD98059, but it was not affected by SB203580 (Fig. 3B).

3.4. NO production

We examined whether CRP stimulates NO production in VSMC. Unstimulated VSMC did not produce detectable levels of nitrite, whereas CRP slightly increased nitrite accumulation in the culture medium at higher concentrations (Fig. 4A). Alone, interferon-γ (IFN) at concentrations up to 1000 U/ml did not induce detectable levels of nitrite (data not shown). However, CRP-induced nitrite release was obviously potentiated by IFN. Nitrite accumulation increased in the presence of IFN as a function of the CRP concentration (0.001 to 0.1 μg/ml) (Fig. 4A).

To rule out the possible involvement of endotoxin, the effect of polymyxin B on nitrite accumulation induced by CRP or LPS was compared. While polymyxin B had little effect on CRP-induced nitrite production, LPS-induced nitrite production was dose-dependently attenuated by polymyxin B (Fig. 4B).

The expression of iNOS mRNA was minimal in unstimulated VSMC, according to Northern blot analysis. Unlike IFN, which is totally ineffective and CRP, which weakly induces iNOS mRNA, a combination of these two factors powerfully inducted iNOS mRNA (Fig. 4C).

CRP activated the iNOS promoter in a concentration-dependent fashion in VSMC, whereas IFN alone did not affect the iNOS promoter activity but enhanced CRP-induced activity. The amount of iNOS promoter activity increased in VSMC incubated with CRP alone or with CRP plus IFN 3.1- and 4.4-fold, respectively, relative to unstimulated levels (data not shown). The CRP/IFN-induced iNOS promoter activity and nitrite production was potently reduced by NAC (10 mmol/l) and by PDTC (50 μmol/l) (Fig. 4D).

3.5. Modulation of CRP effects on NF-κB activation by PPAR activators and statins

We examined the effects of several PPAR activators and statins on subsequent NF-κB activation by CRP (Fig. 5). The NF-κB activation caused by CRP was inhibited by 15d-PGJ2 (10 μmol/l) and the PPARγ activators rosiglitazone (20 μmol/l) and pioglitazone (20 μmol/l) were also significantly inhibitory. However, the PPARα ac-

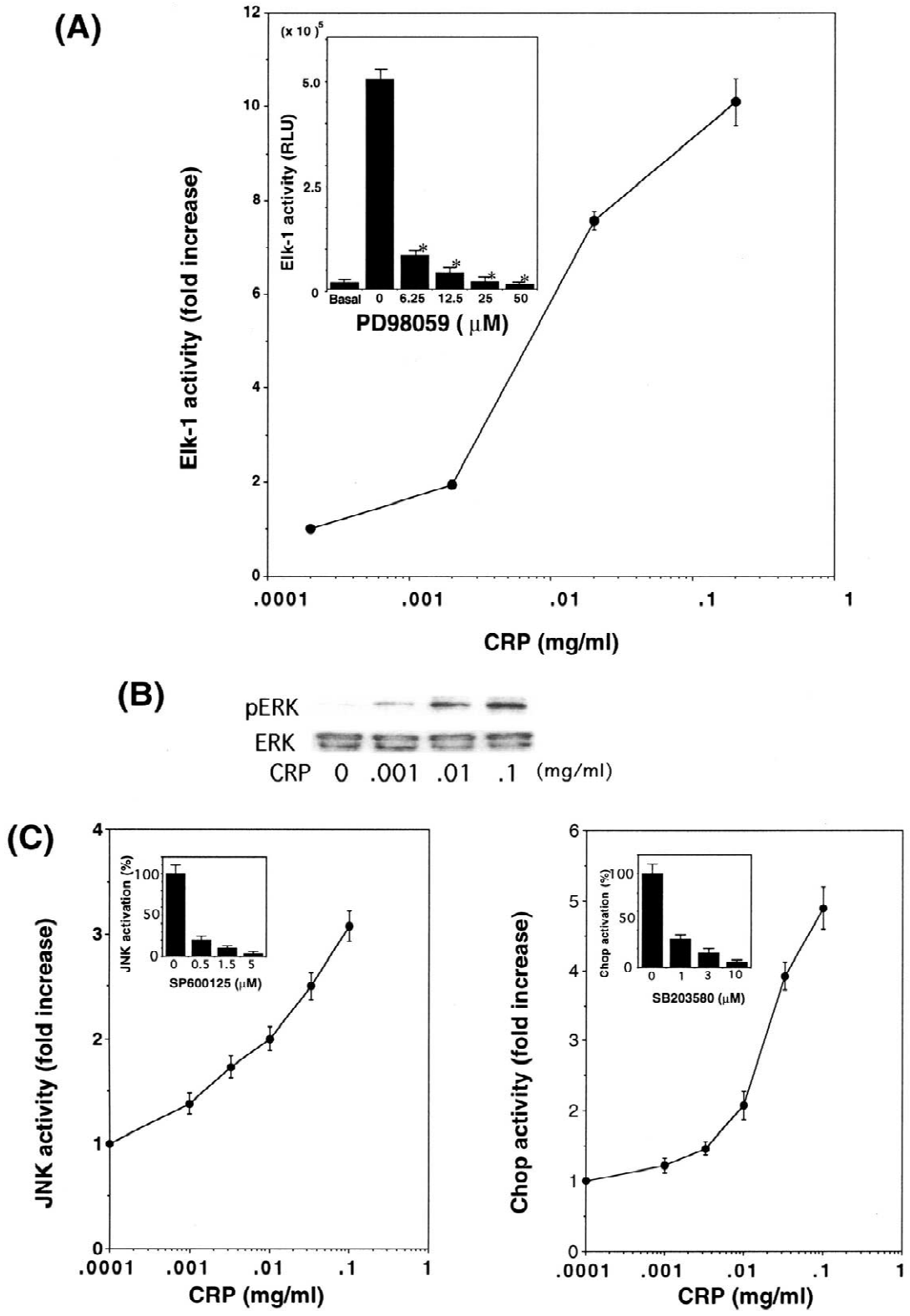

Fig. 2. (A) Effects of CRP-mediated activation of Elk-1. Quiescent VSMC (transfected with pFR-Luc/pFA2-Elk1) were stimulated with various concentrations of CRP. After 4 h, cells were lysed, and luciferase activities were measured. Inset: effects of PD98059 on CRP-mediated Elk-1 activation. Quiescent VSMC (transfected with pFR-Luc/pFA2-Elk1) were incubated with MEK inhibitor PD98059 at various concentrations for 30 min and then stimulated with CRP (0.1 mg/ml). (B) Activation of ERK1/2 by CRP. VSMC were stimulated with CRP for 30 min. The activity of ERK1/2 was assayed with a phospho-specific anti-ERK1/2 antibody. Equal protein loading was ascertained by immunoblotting with antibody against nonphosphorylated ERK1/2. (C) Effects of CRP on cJun and Chop activity. Quiescent VSMC (transfected with pFR-Luc/pFA2-cJun, or pFR-Luc/pFA-Chop) were incubated with various concentrations of CRP for 4 h, then cells were lysed and luciferase activities were measured. Inset: effects of SP600125 or SB203580 on CRP-mediated JNK or Chop activation, respectively. Data are means±S.E.M. of triplicate observations. *$P<0.01$ compared with control.

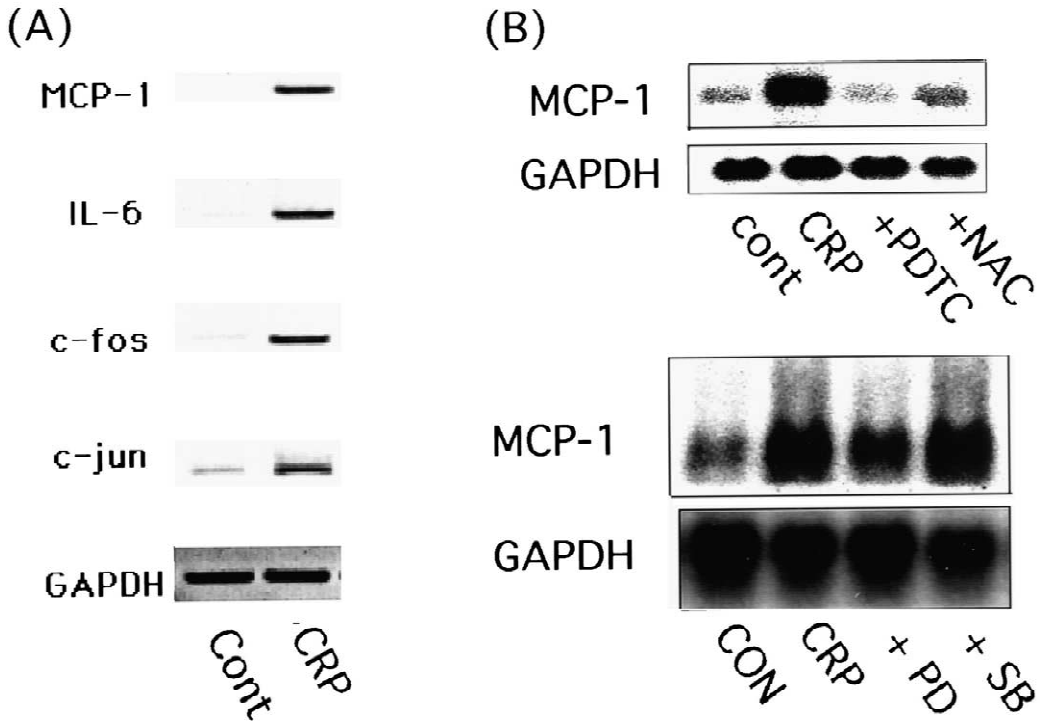

Fig. 3. (A) Effects of CRP on MCP-1, IL-6, c-fos, or c-jun mRNA. VSMC were incubated with CRP (0.1 mg/ml), then total RNA prepared from the harvested cells was assayed by RT-PCR using gene-specific primers. Results for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA are shown for comparison. Incubation periods were 6 h for MCP-1 and IL-6, and 1 h for c-fos and c-jun. (B) Effects of antioxidant and MAP kinase inhibitors on CRP-induced changes in MCP-1 mRNA levels. Cells were incubated with CRP (0.1 mg/ml) in the absence or presence of NAC (10 mmol/l), PDTC (50 μmol/l), PD98059 (30 μmol/l), or SB203580 (10 μmol/l) for 6 h. Total RNA was prepared from the cells, harvested and Northern blotted using MCP-1-specific probes followed by a GAPDH-specific probe.

tivators fenofibrate (100 μmol/l) and Wy 14649 (100 μmol/l) did not affect NF-κB activation. Fluvastatin (5 μmol/l) and cerivastatin (1 μmol/l) reduced the activation of NF-κB by CRP.

4. Discussion

Biological effects of CRP on VSMC may be involved in atherogenesis. The present study demonstrated that CRP potently stimulates NF-κB activation. The transcription factor NF-κB has an important function in the regulation of many genes involved in the inflammatory and proliferative responses of cells, and recent studies indicate that NF-κB is involved in the pathogenesis of atherosclerosis [16–18]. We found that CRP-induced MCP-1 and iNOS gene expression, which was inhibited by NAC and PDTC. Those results suggest that the induction of those genes is at least partly mediated by NF-κB activation. We also demonstrated that CRP stimulates AP-1 activation and that CRP is a potent MAPK stimulator. Since activation of ERK leads to c-fos gene expression, the inducibility of CRP of ERK activation may be functionally related to the c-fos gene. We found that CRP also stimulated JNK and p38 MAPK, which might be responsible for the induction of c-jun gene expression. Being the major components of the AP-1 transcription factor, activated c-fos and c-jun can subsequently activate the transcription of several genes controlling cellular growth. This could be the mechanism of CRP-stimulated VSMC. Thus, one of the signaling pathways by which CRP activates VSMC appears to be via NF-κB activation, while another is MAP kinases→c-Fos/c-Jun→AP-1 pathway.

MCP-1, which is chemotactic for monocytes both in vitro and in vivo, has been detected in atherosclerotic lesions from both humans and experimental animals but not in normal arteries, suggesting that it plays a significant role in the pathogenesis of atherosclerosis [19,20]. Here, we found that NAC inhibited the induction of MCP-1 gene expression by CRP in VSMC, suggesting that CRP regulates MCP-1 gene expression through redox-sensitive transcriptional mechanisms. The finding that MCP-1 gene expression is induced in VSMC in response to agents that potently induce NF-κB activation suggests that MCP-1 gene expression induced by CRP is at least partly NF-κB dependent. The MAPKs are a family of serine/threonine protein kinases that are activated as early responses to a variety of stimuli involved in cellular growth, transformation, and differentiation. CRP obviously activated ERK and significantly activated JNK and p38 MAPK in VSMC. Use of the MAPK kinase (MEK) inhibitor PD98059 demonstrated that CRP induced MCP-1 gene expression at

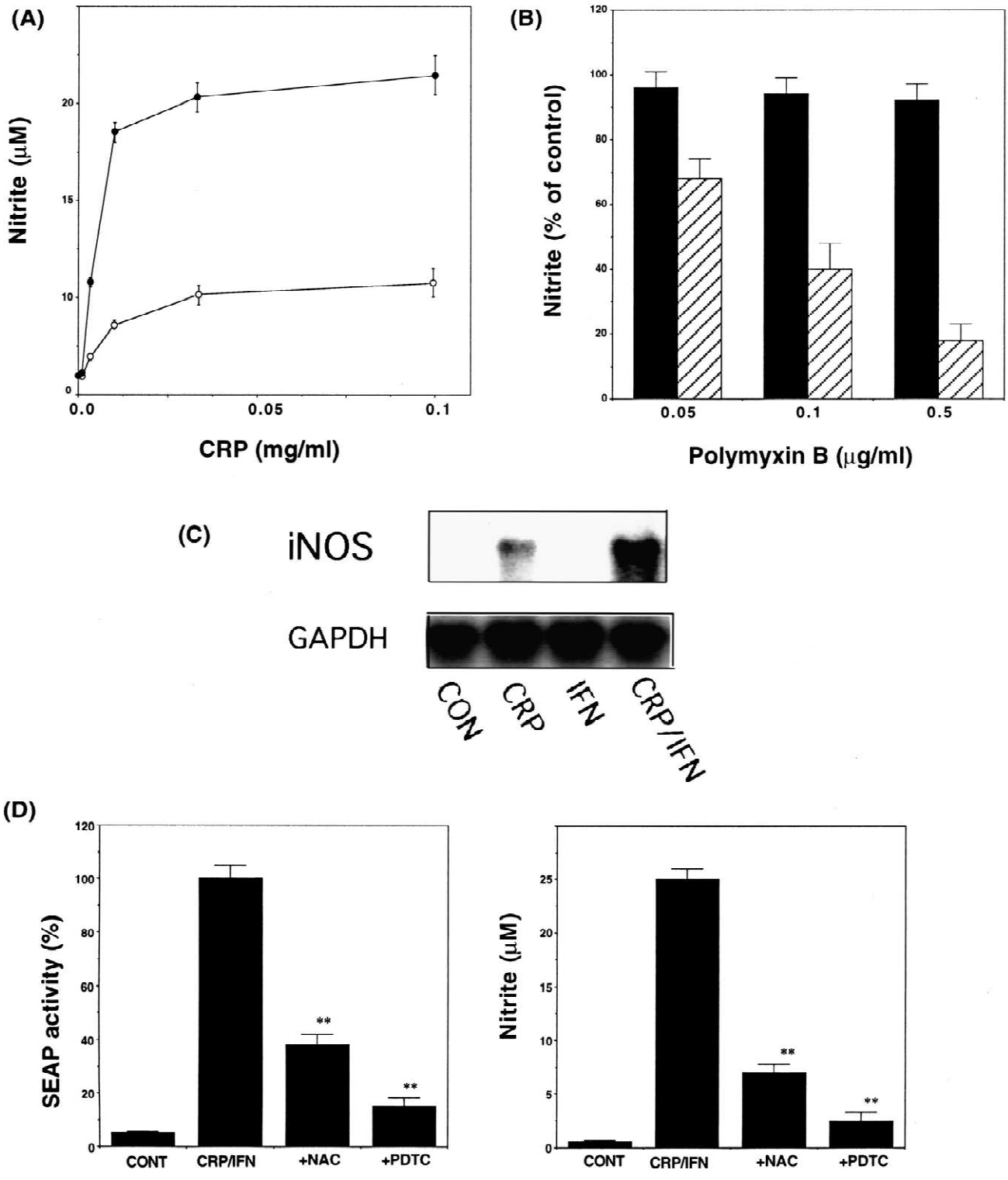

Fig. 4. (A) CRP and IFN synergize to increase nitrite synthesis in VSMC. After an incubation with CRP (0.001–0.1 mg/ml, open circles) alone or in combination with IFN (100 U/ml, closed circles) for 24 h, nitrite accumulation in the medium was measured. (B) Effect of polymyxin B on nitrite accumulation induced by CRP or LPS in VSMC. After an incubation with CRP (0.1 mg/ml, black bars) or LPS (30 μg/ml, hatched bars) in the presence of polymyxin B (0.05–0.5 μg/ml) for 24 h, nitrite accumulation in the medium was measured. (C) CRP and IFN synergistically induced mRNA for iNOS in VSMC. Total RNA was prepared from untreated cells (CONT), and from those incubated with IFN (100 U/ml) alone, CRP (0.1 mg/ml) alone, or both (CRP/IFN) for 16 h. Levels of iNOS mRNA were evaluated by Northern blotting using an iNOS-specific probe followed by a GAPDH-specific probe. (D) Effects of PDTC and NAC on CRP/IFN-induced nitrite production and iNOS promoter activity. Nitrite production and iNOS promoter/SEAP reporter activity activity in the culture medium was measured in the absence and presence of PDTC (50 μmol/l) or NAC (10 mmol/l) in VSMC stimulated with CRP/IFN for 24 h. Data are means±S.E.M. of four experiments. $^{**}P<0.01$ compared with control.

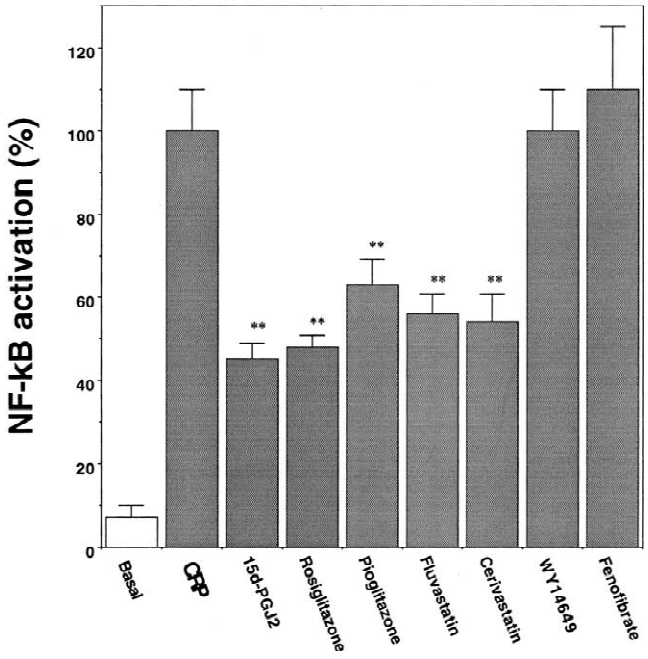

Fig. 5. Effects of PPAR activators and statins on NF-κB activation by CRP. VSMC (transfected with pNFκB-Luc) were incubated with PPARγ agonist (15d-PGJ2, rosiglitazone, and pioglitazone), PPARα agonists (Wy 14649 and fenofibrate), and statins (fluvastatin and cerivastatin) for 1 h and then with CRP for 4 h. Cells were lysed and luciferase activities were measured. Data are means±S.E.M. of triplicate observations. $^{**}P<0.01$ compared with control (CRP only).

least partly through the ERK-sensitive pathway. IL-6, which is a multifunctional cytokine that mediates B lymphocyte proliferation/induction of antibody synthesis and mediates the hepatic acute-phase response [21,22], may also play an important role as a VSMC growth factor in vessel walls through a paracrine mechanism. CRP could induce IL-6 mRNA in VSMC, which may in turn stimulate CRP production in liver in vivo. Atherosclerosis is now recognized as an inflammatory process. Our study showed that the expression of mRNAs for MCP-1 and IL-6 is induced in response to CRP, providing a molecular basis for this induction in VSMC.

Nitric oxide is an important regulator of vascular function. In normal arteries, NO produced by endothelial NOS probably plays an important anti-atherogenic role by causing vasorelaxation and inhibiting VSMC proliferation, platelet aggregation, and lipoprotein oxidation [23–25]. Recent studies also indicate that the inhibition of NO synthesis promotes atherosclerosis, whereas supplementation with L-arginine reduces atherogenesis [26]. However, contrasting evidence shows that the NO-derived oxidant peroxynitrite is actively involved in atherogenesis [27–29]. Peroxynitrite is a product of the reaction between NO and superoxide anions [30], and it is often formed at sites of stimulated NO overproduction [31]. In addition, an increase in iNOS mRNA expression at any level of stimulation may result in an increase in superoxide anions produced by the enzyme when L-arginine or selected cofactors are limited. High output production of NO, which may favor peroxynitrite formation, is generally associated with iNOS, and many of the inflammatory cytokines associated with atherosclerosis [32], in particular, interferon-γ, interleukin-1, and tumor necrosis factor (TNF)-α, might lead to the stimulated expression of iNOS. Thus, CRP may promote atherosclerosis somewhat by activating NF-κB and upregulating iNOS, thereby fostering local inflammation and oxidative stress. It has already been reported that CRP induces iNOS in macrophages or augments iNOS expression in cardiac myocytes [33–35]. Results of those studies and ourselves may provide a basis for the role of CRP in pathological conditions including atherosclerosis.

Epidemiological studies have suggested an interaction between CRP and statins [36]. Statins may reduce serum CRP levels and mortality in patients with low cholesterol, but high CRP concentrations. Furthermore, statins inhibit the NF-κB activation induced by angiotensin II and TNFα in vascular smooth muscle and in mononuclear cells [37] and reduce neointimal inflammation in a rabbit model of atherosclerosis by preventing NF-κB activation [38]. The present study showed that fluvastatin and cerivastatin partially inhibited the NF-κB activation caused by CRP in VSMC. Activators of PPAR may reduce the proinflammatory effects of cytokines on vascular cells and may have beneficial effects on the progression of atherosclerosis in animal models [39–42]. The present study showed that PPARγ agonists such as 15d-PGJ2 and thiazolidinediones inhibited the activation of NF-κB by CRP. However, PPARα activators, such as fenofibrate and Wy 14649, had no effects. These anti-inflammatory effects of PPAR activators may be explained, at least in part, by their inhibition of the nuclear factor-B, but other mechanisms remain to be elucidated.

The concentration of CRP required to produce half-maximal stimulation of NF-κB and AP-1 in VSMC was approximately 25 μg/ml. These values were rather higher than the normal plasma concentration range, but CRP appears to be active on VSMC even at lower concentrations and it may reach levels high enough to potently activate VSMC during the inflammatory process. It is, however, noted that in the rat the CRP concentration under normal conditions has been reported that between 300 and 600 μg/ml, which is much greater than has been described in any other species and exceeds even maximal acute phase levels of CRP in man [43]. Rat CRP closely resembled human CRP in its amino acid composition, in having five subunits per molecule and in its electron microscopic appearance as a pentameric annular disc. It differed, however, from all other mammalian CRP's characterized hitherto in being a glycoprotein bearing a single complex oligosaccharide on each polypeptide subunit. Furthermore one pair of its subunits per molecule was linked by a interchain disulphide bridges, whereas in

other animals the subunits of CRP are all non-covalently associated [43]. Thus, rat CRP may have different impact on cells, while it remains to be further elucidated whether human CRP has similar biological effect on rat cells.

Our findings support the hypothesis that CRP plays a direct role in the pathogenesis of inflammation/atherosclerosis and open the way to new pharmacological treatment strategies. Some important issues remain to be addressed. CRP activates signals such as inflammatory cytokines leading to NF-κB activation and the induction of inflammatory mediators, and also as growth factors leading to MAPK activation and cell growth. The means by which CRP elicits such a broad range of signals needs to be examined. In addition, whether CRP exert its effects via binding to receptors on VSMC awaits investigation. Elucidation of CRP receptor(s) and the resultant post-receptor signaling will provide new insights into the molecular mechanisms underlying CRP-mediated proinflammatory and pro-atherogenic responses in VSMC.

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