Induction of C-reactive protein by cytokines in human hepatoma cell lines is potentiated by caffeine

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Induction of C-reactive protein (CRP) by conditioned medium from lipopolysaccharide-stimulated human monocytes in two human hepatoma-cell lines, Hep 3B and NPLC/PRF/5, was potentiated 3–6-fold by the methylxanthine caffeine. The induction observed in the presence of conditioned medium plus caffeine was as much as 180-fold, comparable with that seen after many stimuli *in vivo*. This potentiation was accompanied by an increase in the levels of CRP mRNA. By contrast, no potentiating effect on CRP induction by conditioned medium was found when we tested theophylline, forskolin, 8-bromo cyclic AMP or two Ca^{2+} ionophores, namely ionomycin and A23187. None of the above compounds, including caffeine, when tested alone, had any detectable effect on the synthesis and secretion of CRP. Our previous study [Ganapathi, May, Schultz, Brabenec, Weinstein, Sehgal & Kushner (1988) *Biochem. Biophys. Res. Commun.* **157**, 271–277], employing defined cytokines, had shown that induction of CRP in Hep 3B cells requires IL(interleukin)-6 plus IL-1, whereas, in the NPLC/PRF/5 cell line, IL-6 alone is effective. Caffeine similarly potentiated induction of CRP by these defined cytokine signals in these two cell lines. Changes in synthesis of other acute-phase proteins, including serum amyloid A (SAA), α_1 -proteinase inhibitor, α_1 -antichymotrypsin and albumin, induced by conditioned medium or, in some cases, by IL-6 and/or IL-1 α , were only minimally affected by caffeine. Thus these results indicate that the mechanism by which caffeine potentiates CRP induction by cytokines appears to be independent of increases in intracellular concentrations of the two second messengers, cyclic AMP and Ca^{2+} ; the precise nature of this mechanism is unclear at the present time. Our results also indicate that the intracellular mechanisms by which cytokines regulate synthesis of CRP may differ from those regulating synthesis of some other acute-phase proteins. The differential response of CRP and SAA to caffeine is of particular interest, since induction of both of these two major acute-phase proteins can be accomplished by identical extracellular signals.

INTRODUCTION

Alteration in the concentration of a group of plasma proteins, the acute-phase proteins, represents an important biochemical response to tissue injury or infection [1]. Within the first few hours after an inflammatory stimulus, the hepatic rate of synthesis of a number of plasma proteins increases, whereas that of some others, most notably albumin, decreases. C-reactive protein (CRP) and serum amyloid A (SAA) are the two major human acute-phase proteins whose concentrations in the plasma may increase as much as several-thousand-fold.

The synthesis of acute-phase proteins by the liver is regulated by cytokines, which can be produced by a variety of cells, including monocytes/macrophages, fibroblasts, endothelial and epithelial cells [2–10]. The defined cytokines which have been implicated in the acute-phase response are interleukin-6 (IL-6), interleukin-1 (IL-1), tumour-necrosis factor α , interferon γ and leukaemia-inhibitory factor [4,6–8,11–21]. It has recently been demonstrated that IL-6 plays a central role in the induction of CRP and SAA in human primary hepatocyte cultures [12,13] and in human hepatoma-cell lines [14,15].

In contrast with the extensive studies which have been carried out to define extrahepatic signals regulating acute-phase-protein synthesis, there are only two reports [22,23] evaluating intracellular signal-transducing mechanisms. Data were presented in one study suggesting that induction of rat and human

fibrinogen and some other acute-phase proteins by cytokines involves protein kinase C as an intermediate, whereas in the other study it was found that protein kinase C did not directly mediate the action of IL-6 or IL-1, but rather appeared to exert a modulating effect. Our present series of studies were undertaken to further explore the signal-transduction mechanisms by which cytokines regulate the synthesis of acute-phase proteins. In the present study we evaluated the possible role of two known second-messenger systems, namely cyclic AMP and Ca^{2+} , in the induction of CRP by determining the effect of two methylxanthines, two agents that lead to increased intracellular cyclic AMP levels and two Ca^{2+} ionophores on cytokine-induced changes in CRP synthesis. In addition, the effect of caffeine on induction of several other acute-phase proteins was evaluated.

EXPERIMENTAL

Materials

The human hepatoma-cell line Hep 3B was generously given by Dr. G. J. Darlington, Baylor College of Medicine, Houston, TX, U.S.A., and the human hepatoma-cell line NPLC/RPF/5 was obtained from Dr. B. B. Knowles, Wistar Institute, Philadelphia, PA, U.S.A. RPMI-1640 was obtained from Mediatech–Fisher Scientific Co., Springfield, NJ, U.S.A., and fetal-bovine serum was from Gibco Laboratories, Grand Island,

Abbreviations used: CRP, C-reactive protein; SAA, serum amyloid A; IL-6, interleukin-6; IL-1, interleukin-1; PMA, phorbol 12-myristate 13-acetate ('TPA'); USP, United States Pharmacopeia; rIL, recombinant interleukin.

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NY, U.S.A. Tobramycin sulphate injection USP (80 mg/2 ml) and human insulin injection USP (recombinant-DNA origin), were from Eli Lilly, Indianapolis, IN, U.S.A.; dexamethasone sodium phosphate injection USP (4 mg/ml) was from Natcon Corp., Shirley, NY, U.S.A.; lipopolysaccharide from *Escherichia coli* 0.55:B5 was from Sigma Chemical Co., St. Louis, MO, U.S.A. Ficoll-Hypaque was purchased from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. Sheep anti-(human CRP) and goat anti-(human albumin) antiserum were from Cappel, Cochranville, PA, U.S.A.; rabbit anti-(human SAA) antiserum was from Calbiochem-Behring, La Jolla, CA, U.S.A.; and goat anti-(human α_1 -proteinase inhibitor) and goat anti-(human α_1 -antichymotrypsin) antisera were from Atlantic Antibodies, Scarborough, ME, U.S.A. Caffeine, theophylline and 8-bromo cyclic AMP were purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A.; forskolin, ionophore A23187 and ionomycin were obtained from Calbiochem-Behring, La Jolla, CA, U.S.A.; fura2-AM (acetomethoxy ester) was from Molecular Probes, Eugene, OR, U.S.A. Recombinant human IL-6 (rIL-6) derived from Chinese-hamster ovary cells was from Genzyme, Boston, MA, U.S.A., and *E. coli* derived recombinant human IL-1 α (rIL-1 α) was generously given by Dr. P. Lomedico (Roche Institute, Nutley, NJ, U.S.A.). L-[³⁵S]Methionine and [γ -³²P]ATP were obtained from du Pont-New England Nuclear, Boston, MA, U.S.A. Cyclic AMP assay kit was from Amersham Corporation, Arlington Heights, IL, U.S.A. Polynucleotide kinase and S1 nuclease were obtained from Boehringer Mannheim, Indianapolis, IN, U.S.A.

Preparation of conditioned medium

Monocyte-derived conditioned medium was prepared from lipopolysaccharide stimulated peripheral-blood mononuclear cells, essentially as described previously [7].

Cell culture and induction of acute-phase proteins

The human hepatoma-cell lines Hep 3B and NPLC/PRF/5 were maintained as described previously [7]. The hepatoma cells were incubated in 35 or 60 mm-diameter dishes, in the absence or presence of additives (cytokines and/or pharmacological agents), in RPMI-1640 containing 1 μ M-dexamethasone, insulin (0.02 unit/ml) and tobramycin (50 μ g/ml) at 37 °C in a humidified atmosphere of CO₂/air (1:19) for 18–24 h. In experiments carried out to study the effect of caffeine on the synthesis of acute-phase proteins by conditioned medium, cells were grown in 60 mm dishes and incubated in a final volume of 2.5 ml of medium, whereas in later experiments, carried out using IL-6 and IL-1 α as the extracellular signals, cells were grown in 35 mm dishes and incubated in a final volume of 1 ml. After the 18–24 h incubation period the medium was harvested, cells were washed twice with Hanks Balanced Salt Solution and labelled with 0.1 mCi of L-[³⁵S]methionine (1 mCi/mmol)/ml in either 2.0 ml (for 60 mm dishes) or 1.0 ml (for 35 mm dishes) of methionine-deficient RPMI-1640. Cells were then incubated at 37 °C for an additional 4 h and the medium harvested at the end of the labelling period.

Analysis of acute-phase proteins secreted into medium

In initial experiments carried out with conditioned medium as the extracellular signal, newly synthesized radiolabelled CRP, α_1 -proteinase inhibitor and albumin were assayed in medium collected at the end of the 4 h labelling period by immunoprecipitation with specific antibodies as previously described [7]. Accumulation of SAA secreted into the medium, collected either at the end of the 18–24 h incubation period or after the 4 h labelling period, was determined by Western-blot analysis [7]. Accumu-

lation of α_1 -antichymotrypsin was determined in medium collected at the end of the 18–24 h incubation period by electroimmunoassay with specific antibodies [24].

In experiments using IL-6 and IL-1 α as the extracellular signals, CRP was analysed as described above, with the exception that the volume of the radiolabelled sample used for the assay was reduced to 0.8 ml rather than 1.4 ml. Analysis of SAA and α_1 -proteinase inhibitor was carried out by immunoprecipitation of the radiolabelled medium with specific rabbit anti-(human protein) antibodies, followed by absorption of the antigen-antibody complex to formaldehyde-fixed, heat-killed *Staphylococcus aureus* cells bearing Protein A (IgGSorb; Enzyme Center, Malden, MA, U.S.A.), SDS/PAGE, fluorography and autoradiography. The immunoprecipitation and washing procedure for SAA was essentially as described previously [15]. For immunoprecipitation of α_1 -proteinase inhibitor, 20 μ l of the sample was incubated with 1.5 μ l of antibody, employing the same conditions as those used for SAA, with the exception that no SDS was employed in the Protein A dilution buffer and wash buffer, and the concentration of sodium deoxycholate was increased to 1% in both of these buffers.

Analysis of CRP mRNA

CRP mRNA was analysed essentially as described previously [7]. Control S1-nuclease analysis was carried out with human glyceraldehyde 3-phosphate dehydrogenase cDNA pH1c GAP3 [25] (generously given by Dr. X-H. Sun and Dr. R. Wu, Cornell University, Ithaca, NY, U.S.A.), digested with endonuclease *Xba*I and 5'-end-labelled with [γ -³²P]ATP.

Fig. 1. Effect of different pharmacological agents on induction of CRP by conditioned medium

Hep 3B cells were incubated in the absence (control) or presence of 20% conditioned medium (CM), or conditioned medium plus additives for 18–24 h and labelled with L-[³⁵S]methionine (0.1 mCi/ml) for 4 h. Radiolabelled CRP secreted into the culture medium was analysed by immunoprecipitation followed by fractionation of the immunoprecipitate on SDS/polyacrylamide gels as described in the Experimental section. Fold induction corresponds to the ratio of radioactivity (c.p.m.) in treated cultures to that in control cultures. The pharmacological compounds were analysed at various doses: caffeine and theophylline, 0.5–4.0 mM; forskolin, 50–200 μ M; 8-bromo cyclic AMP, 0.25–2 mM; ionomycin, 16 and 32 ng/ml; and ionophore A23187, 0.1 and 0.2 μ M. Only one representative dose is shown in the figure since similar results were obtained with different doses for all the compounds except caffeine, the dose response of which is shown in Fig. 3. Values correspond to the averages for two or more independent experiments carried out in duplicate.

Determination of intracellular concentrations of cyclic AMP and Ca^{2+}

Hep 3B cells were incubated in RPMI-1640 in the absence or presence of 1 μM -dexamethasone plus insulin (0.02 unit/ml) and the inducers in a total volume of 0.5 ml for 30 min in 12-well tissue-culture dishes. The medium was then removed, and the cells were washed once with normal saline (0.9% NaCl), and incubated in 0.5 ml of 0.1 M-HCl at room temperature for 2 h. The HCl extract was then harvested, neutralized with 0.1 vol. of 1.0 M-NaOH and stored at -70°C . To the cells in the tissue-culture dishes was then added 0.25 ml of 0.1 M-NaOH. The dishes were tightly wrapped in Parafilm and stored at room temperature for 4 days to extract proteins. Cyclic AMP was assayed in the neutralized HCl extracts by using the cyclic AMP assay kit (Amersham Corp.), essentially as described by the manufacturer. The concentration of cyclic AMP in the samples was determined by comparison with a linear standard curve and expressed as μg of cyclic AMP/mg of protein. Protein concentration was estimated with the BCA Pierce microassay kit. For determination of cytosolic Ca^{2+} , cells were incubated for 18 h in RPMI-1640 in the presence or absence of conditioned medium. Cells were processed and loaded with fura2, and cytosolic Ca^{2+} was determined after the addition of 16 ng of ionomycin/ml essentially as described in [26].

Fig. 2. Effect of caffeine on induction of synthesis (a and b) and mRNA levels (c) of CRP by conditioned medium

Hep 3B (a and c) and NPLC/PRF/5 (b) cells were incubated in the absence (lane 1) or presence of 20% conditioned medium (lane 2), 20% conditioned medium plus 2 mM-caffeine (lane 3). Newly synthesized radiolabelled CRP secreted into the culture medium was analysed as described in Fig. 1. Total cellular RNA was isolated at the end of the labelling period and CRP mRNA was determined by S1-nuclease analysis as described in the Experimental section. The S1-nuclease-protected fragments were fractionated on a 5%-polyacrylamide gel and the gel autoradiographed to reveal the radiolabelled band. For control hybridization, S1-nuclease analysis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, a constitutively expressed liver gene, was carried out. The similar intensities of the hybridization signals for GAPDH mRNA in all three lanes indicates that an equal amount of total cellular RNA was used for analysis of each sample.

RESULTS

We first examined the effect of the following agents on the induction of CRP in Hep 3B cells: (a) the methylxanthines caffeine and theophylline, which are known to inhibit cyclic AMP phosphodiesterase; (b) forskolin, which leads to activation of adenylate cyclase; (c) 8-bromo cyclic AMP, the lipophilic analogue of cyclic AMP; and (d) the Ca^{2+} ionophores ionomycin and A23187. Caffeine led to significant potentiation of CRP induction in the presence of conditioned medium, whereas none of the other agents we tested affected CRP induction (Fig. 1). Induction of CRP by conditioned medium was potentiated 4.8 ± 1.0 -fold by 2 mM-caffeine. Since conditioned medium normally increases synthesis of CRP by about 10–30-fold, the total increase in CRP synthesis in the presence of conditioned medium plus caffeine ranged between 40- and 180-fold above that seen in control cultures. Although no visible band was observed on the autoradiograms in control cultures, the radioactivity in the CRP band was usually higher than that detected

Fig. 3. Dose-response curve for the effect of caffeine on the induction of acute-phase proteins and on synthesis of total proteins (TP) by conditioned medium (CM)

Hep 3B cells were incubated in the presence of 20% conditioned medium and various doses of caffeine. The acute-phase proteins were analysed as described in the Experimental section, and synthesis of total proteins (TP) secreted into the culture medium was determined by trichloroacetic acid precipitation. Fold induction (above that elicited by the conditioned medium alone) corresponds to the ratio of radioactivity in cells treated with 20% conditioned medium plus various doses of caffeine to that in cells treated with 20% conditioned medium alone. Values for CRP are averages $\pm$ S.D. for three independent experiments carried out in duplicate. Values for SAA, α_1 -proteinase inhibitor ($\alpha_1\text{PI}$) and TP are averages for duplicate samples. The bands at the top correspond to the intensity of L-[^{35}S]methionine-labelled CRP on the autoradiogram. C, control.

in background slices cut immediately above or below the CRP band or randomly within the gel, allowing us to estimate fold increases above control. In the absence of conditioned medium, none of these compounds had any detectable effect on synthesis of CRP (results not shown).

Figs. 2(a) and 2(b) illustrate the potentiating effect of caffeine on CRP induction by conditioned medium in Hep 3B and NPLC/PRF/5 cells respectively. The degree of potentiation observed in both cell lines was comparable. Caffeine similarly enhanced by 3–4-fold the accumulation of CRP mRNA induced by conditioned medium in Hep 3B cells (Fig. 2c). The potentiating effect of caffeine on CRP induction by conditioned medium was found to be dose-dependent in Hep 3B cells; a linear response was observed between 0.5 and 2.0 mM-caffeine (Fig. 3).

The effect of caffeine on induction of CRP by defined cytokines was also examined. Caffeine was found to potentiate the pre-

viously reported induction of CRP by rIL-6 plus rIL-1 α in Hep 3B cells (Fig. 4a, lanes 4 and 5) and by both partially purified (Fig. 4b, lanes 2 and 3) and recombinant preparations of IL-6 (Fig. 4b, lanes 5 and 6) in NPLC/PRF/5 cells. In the Hep 3B cell line, in which CRP does not respond to IL-6 or IL-1 alone, addition of caffeine to either rIL-6 alone (Fig. 4a, lanes 2 and 3) or IL-1 alone (result not shown) did not alter the basal level of CRP synthesis.

Although the exact mechanisms by which caffeine exerts its biological effects are not fully understood, some of the actions of caffeine are thought to be mediated by increases in intracellular concentrations of cyclic AMP or Ca²⁺ [27,28]. Our finding, that other agents that are also known to lead to increased concentrations of these two second messengers, including forskolin and ionomycin, did not potentiate CRP induction, suggested that the effect of caffeine we observed was independent of either mechanism. To confirm the validity of this conclusion, we tested the abilities of forskolin and ionomycin to cause increases in the concentrations of cyclic AMP and Ca²⁺ respectively in our system. In the absence or presence of conditioned medium, forskolin at concentrations of 50 and 100 μ M led to between 100- and 200-fold increases in intracellular levels of cyclic AMP (Table 1). Ionomycin increased intracellular concentrations of Ca²⁺ from 82.5 to 154.6 nM for Hep 3B cells that had been incubated in RPMI-1640 for 18 h and from 115 to 176 nM for Hep 3B cells that had been treated with conditioned medium for 18 h. In addition, we determined whether conditioned medium or caffeine, alone or in combination with each other, led to increase in cyclic AMP levels. None of these stimuli led to any significant increases in the concentration of cyclic AMP (Table 1).

Fig. 4. Effect of caffeine on induction of CRP by IL-6 or by IL-6 plus IL-1 α in Hep 3B and NPLC/PRF/5 cells

(a) Hep 3B cells were incubated in the absence (lane 1) or presence of rIL-6 (lane 2), rIL-6 plus caffeine (lane 3), rIL-6 plus rIL-1 α (lane 4), rIL-6 plus rIL-1 α plus caffeine (lane 5); (b) NPLC/PRF/5 cells were incubated in the absence (lanes 1 and 4) or presence of purified IL-6 (lane 2), purified IL-6 + caffeine (lane 3), rIL-6 (lane 5), rIL-6 plus caffeine (lane 6) and CRP analysed as described in the legend to Fig. 1. Lanes 1–3 correspond to one representative experiment and lanes 4–6 to another. The doses employed were: rIL-6, 25 ng/ml; rIL-1 α , 0.04 ng/ml; purified IL-6, 50 ng/ml; caffeine, 2 mM.

Table 1. Effect of conditioned medium, caffeine and forskolin on cyclic AMP levels in Hep 3B cells

Values correspond to the average of two separate samples assayed in duplicate. The values in parentheses correspond to the ratio of cyclic AMP in treated cells to that in control cells.

Treatment	[Cyclic AMP] (μ g/mg of protein)	
	Expt. 1	Expt. 2
Control	2.21	1.32
Conditioned medium (20%)	1.87 (0.85)	1.99 (1.5)
Caffeine (2 mM)	1.84 (0.83)	0.840 (0.63)
Conditioned medium (20%) + caffeine (2 mM)	2.38 (1.08)	1.32 (1)
Forskolin (50 μ M)	368.4 (167)	176.7 (133)
Conditioned medium (20%) + forskolin (50 μ M)	345 (156.6)	141.6 (107.3)
Forskolin (100 μ M)	481 (218)	190 (143.9)
Conditioned medium (20%) + forskolin (100 μ M)	423 (191.9)	242 (184.7)

Fig. 5. Effect of caffeine on synthesis and secretion of other acute-phase proteins in the presence of conditioned medium in Hep 3B cells

Cells were incubated in the absence (lane 1) or presence of 20% conditioned medium (lane 2), 20% conditioned medium plus 2 mM-caffeine (lane 3) as described in the Experimental section. α_1 -Proteinase inhibitor (α_1 PI) and albumin (ALB) secreted into the culture medium were analysed by immunoprecipitation of radiolabelled medium, followed by SDS/PAGE, fluorography and autoradiography. SAA was analysed by Western-blot analysis and α_1 -antichymotrypsin (ACT) by rocket immunoelectrophoresis as described in the Experimental section.

Fig. 6. Effect of caffeine on induction of SAA and α_1 -proteinase inhibitor (α PI) by rIL-6 or the combination of rIL-6 plus rIL-1 α in Hep 3B (a and b) and NPLC/PRF/5 cells (c)

(a) Control culture (lane 1), culture treated with rIL-6 plus rIL-1 α (lane 2), rIL-6 plus rIL-1 α plus 2 mM-caffeine (lane 3); (b) control culture (lane 1), culture treated with rIL-6 (lane 2), rIL-6 plus caffeine (lane 3), rIL-6 plus rIL-1 α (lane 4), rIL-6 plus rIL-1 α plus 2 mM-caffeine (lane 5); (c) control culture (lane 1), rIL-6 (lane 2), rIL-6 plus caffeine (lane 3). SAA and α_1 -proteinase inhibitor (α PI) were analysed by immunoprecipitation and SDS/polyacrylamide gels as described in the Experimental section. Doses employed were: rIL-6, 25 ng/ml; rIL-1 α , 0.04 ng/ml; caffeine, 2 mM.

We also evaluated the ability of caffeine to modify the response of several other acute-phase proteins to conditioned medium. Induction of SAA, α_1 -proteinase inhibitor and α_1 -antichymotrypsin synthesis by conditioned medium was only minimally augmented by caffeine (Fig. 3 and Fig. 5, lanes 1–3). In addition, synthesis of the negative acute-phase protein, albumin, in the presence of conditioned medium plus caffeine was greater than in the presence of conditioned medium alone. These increases correlated with the increase observed in synthesis of total proteins secreted into the culture medium (Fig. 3). The increased synthesis of total proteins and of the acute-phase proteins we studied ranged from none to no more than 70% in multiple experiments, substantially lower than the effects on CRP synthesis we observed. Furthermore, caffeine only minimally increased induction of SAA by the combination of rIL-6 plus rIL-1 α in Hep 3B cells (Fig. 6a, lanes 4 and 5). Similarly, the induction of α_1 -proteinase inhibitor by either rIL-6 in Hep 3B (Fig. 6b, lanes 2 and 3) and NPLC/PRF/5 cells (Fig. 6c), or by the combination of rIL-6 plus rIL-1 α in Hep 3B cells (Fig. 6b, lanes 2 and 3) was only minimally altered. Caffeine did not affect synthesis of either of these two acute-phase proteins in Hep 3B cells in the presence of IL-1, which by itself does not lead to induction of SAA or α_1 -proteinase inhibitor (results not shown).

DISCUSSION

The salient finding of the present study is that the methylxanthine caffeine potentiates induction of CRP by cytokines 3–6-fold. The mechanism by which caffeine modifies the induction of CRP by cytokines is unclear, but seems to be

independent of increases in the intracellular concentrations of the two second messengers cyclic AMP and Ca^{2+} , which also do not seem to directly mediate the induction of CRP by cytokines alone. This conclusion is based on several lines of experimental evidence: (1) neither caffeine, conditioned medium acting alone, or the two in combination led to an increase in intracellular levels of cyclic AMP; (2) other methylxanthines, namely theophylline and 3-isobutyl-1-methylxanthine (results not shown), which are more potent inhibitors of cyclic AMP phosphodiesterase, did not mimic the potentiating effect of caffeine on CRP induction; (3) neither forskolin, which we found capable of increasing cyclic AMP levels in Hep 3B cells, nor 8-bromo cyclic AMP potentiated CRP induction by conditioned medium; (4) the Ca^{2+} ionophores A23187 and ionomycin did not mimic the effects of caffeine, although ionomycin was found capable of increasing intracellular Ca^{2+} levels; and (5) none of the above compounds acting alone influenced CRP synthesis. The degree of CRP induction we observed in the presence of both caffeine and cytokines was as much as 180-fold greater than that seen in control cultures. This degree of induction approximates responses seen *in vivo* in many inflammatory states.

Our studies also demonstrated that caffeine not only potentiated synthesis and secretion of CRP into culture medium, but also led to increased accumulation of CRP mRNA, at least in Hep 3B cells, indicating that caffeine acts at a pretranslational level and does not simply facilitate release of newly synthesized CRP from intracellular storage pools or decrease intracellular CRP degradation. However, it is not clear from these studies whether increased accumulation of CRP mRNA in the presence of caffeine results from increased stability of CRP mRNA or increased transcription of the CRP gene. One possible explanation for our findings is that caffeine might lead to induction of a protein(s) or covalent modification, e.g., phosphorylation/dephosphorylation, of a protein(s) that could affect either of these processes. Indeed, one of the effects of caffeine, that involving inhibition of DNA repair after u.v.- or chemically-induced damage to tumour cells, has been reported to involve synthesis of protein *de novo* [29]. Alternatively, caffeine could lead to modification of some step(s) in the signal-transduction pathway involved in cytokine-induced increase in synthesis of CRP.

In contrast with the potentiating effect of caffeine on induction of CRP by cytokines, there was only a minimal effect on induction of several other acute-phase proteins, including SAA, α_1 -proteinase inhibitor and α_1 -antichymotrypsin, comparable with the increase we observed in synthesis of total proteins secreted into the medium. Similarly, the decrease in albumin synthesis caused by conditioned medium was only minimally affected by caffeine. The modest increase in synthesis of total proteins and the other acute-phase proteins we studied was significantly lower than the marked increase that we observed for CRP synthesis. Thus these observations suggest that the intracellular mechanisms regulating synthesis of CRP likely differ from those regulating the other acute-phase proteins we evaluated.

Since different extracellular signals are involved in regulating synthesis of different acute-phase proteins, it is not surprising that intracellular mechanisms might also differ. However, the differential response of CRP and SAA to caffeine is of particular interest, since induction of both of these acute phase proteins has been shown to require identical extracellular signals in studies *in vitro*; induction of both these acute-phase proteins in the Hep 3B cell line requires the co-operative action of IL-6 plus IL-1 α , whereas in the NPLC/PRF/5 cell line, CRP and SAA are induced by IL-6 alone [15]. This observation suggests that caffeine is probably capable of activating a unique intracellular step involved in CRP induction which differs from that regulating

synthesis of SAA. Similar conclusions can be drawn for the differential effects of caffeine on induction of CRP and α_1 -proteinase inhibitor by IL-6 in the NPLC/PRF/5 cell line. The reported differential effect of dexamethasone on the induction of CRP and SAA in human primary hepatocyte cultures [12] further supports the view that different intracellular mechanisms may be involved in regulating synthesis of acute-phase proteins that respond to identical extracellular signals. Indeed, different transducing mechanisms have shown to be operative for the diverse actions of insulin in a single cell type [30].

Several studies have explored the possible role of the third defined second-messenger system, namely that which induces protein kinase C in acute-phase-protein induction. Evans *et al.* [22] reported that increased synthesis of rat and human fibrinogen can be achieved by the phorbol ester phorbol 12-myristate 13-acetate (PMA; 'TPA') and the diacylglycerol analogue 1-oleoyl-2-acetyl-glycerol, suggesting the activation of protein kinase C as an intermediate step in intracellular signalling pathways involved in the induction of these acute-phase proteins by cytokines. In contrast, studies reported by Baumann *et al.* [23] showed no direct involvement of protein kinase C in mediating the actions of IL-6 or IL-1 on acute-phase-protein production; rather, phorbol esters were shown capable of modifying the response of acute-phase proteins to the cytokines IL-6 or IL-1. A series of experiments carried out in our laboratory on the effect of PMA on induction of acute-phase-protein synthesis have revealed (results not shown) that PMA, in the presence of conditioned medium, inhibits induction of CRP, SAA and fibrinogen, but does not affect synthesis of total protein or α_1 -proteinase inhibitor in Hep 3B cells. Since different cell lines and different acute-phase proteins were examined in these three studies, it is difficult to define a precise role for protein kinase C in induction of acute-phase proteins. However, these data imply that it is possible that a particular intracellular signalling pathway may be capable of reciprocally regulating synthesis of different acute-phase proteins. In addition, the differential effect of caffeine and PMA on induction of CRP by cytokines in a single cell system (Hep 3B cells) suggests that synthesis of CRP within hepatocytes might involve bidirectional regulatory mechanisms.

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