RESEARCH COMMUNICATION

Okadaic acid, an inhibitor of protein phosphatases 1 and 2A, inhibits induction of acute-phase proteins by interleukin-6 alone or in combination with interleukin-1 in human hepatoma cell lines

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Okadaic acid (OA), a specific inhibitor of protein phosphatases 1 and 2A, inhibited in a dose-dependent manner (5–20 nM) the induction of C-reactive protein (CRP), serum amyloid A (SAA) and fibrinogen by interleukin-6 (IL-6) plus interleukin-1 (IL-1), and of fibrinogen by IL-6 alone, in Hep 3B cells. Induction of α_1 -proteinase inhibitor (α_1 -PI) by IL-6 plus IL-1 or IL-6 alone was not significantly affected by OA up to concentrations of 20 nM, above which concentration OA was toxic in Hep 3B cells. OA also inhibited the induction of CRP, fibrinogen and α_1 -PI by IL-6 in the NPLC/PRF/5 cell line, albeit at a higher concentration (80 nM). These results suggest that the signal transduction mechanisms regulating induction of acute-phase proteins by IL-6, either alone or in combination with IL-1, are mediated by activation of protein phosphatases 1 and/or 2A.

INTRODUCTION

The normal physiological homeostasis of the organism is perturbed in response to inflammatory stimuli, leading to alterations in a wide variety of biochemical processes. These changes are collectively referred to as the 'acute-phase response'; they represent the organism's first line of defence and precede the more specific changes of the immune response [1–3]. The term 'acute-phase response' is often used in a more restrictive sense to refer to alterations in the plasma concentrations of a group of proteins of hepatic origin, the acute-phase proteins. The concentrations of most acute-phase proteins (positive acute-phase proteins) increase, whereas the concentrations of a few others (negative acute-phase proteins), e.g. albumin and transferrin in humans, decrease. C-reactive protein (CRP) and serum amyloid A (SAA) are the two major human acute-phase proteins; their plasma concentrations may increase by 1000-fold or more following an inflammatory stimulus.

Interleukin-6 (IL-6) is the major cytokine so far characterized that is capable of regulating synthesis of almost all acute-phase proteins [4–8]. IL-6 exhibits pleiotropic biological functions and is produced by a variety of cell types, including monocytes/macrophages, fibroblasts, endothelial cells and epithelial cells [8–11]. Other cytokines, including interleukin-1 (IL-1), tumour necrosis factor α , interferon γ , transforming growth factor β , leukaemia inhibitory factor, interleukin-11 and oncostatin M, also modulate synthesis of acute-phase proteins [12–18]. These cytokines can act independently or, as is often the case, interact with each other and other extracellular signals, such as hormones, resulting in the production of specific patterns of acute-phase proteins [4,6,14–22]. We previously showed that the induction of CRP and SAA in two human hepatoma cell lines, Hep 3B and NPLC/PRF/5, requires different extracellular signals [6]. In NPLC/PRF/5 cells, IL-6 alone was capable of inducing synthesis of these two acute-phase proteins as well as of fibrinogen and α_1 -proteinase inhibitor (α_1 -PI). In contrast, in the Hep 3B

cell line IL-6 alone was capable of inducing fibrinogen, α_1 -PI and some other acute-phase proteins, whereas induction of CRP and SAA required co-operative interaction between IL-6 and IL-1.

The signal transduction mechanisms by which IL-6 or other cytokines regulate synthesis of acute-phase proteins are poorly understood. It has been demonstrated that IL-6-stimulated signal transduction pathways involved in the regulation of some of its pleiotropic functions, including induction of acute-phase proteins, are not mediated by cyclic AMP, Ca^{2+} , phosphatidylinositol turnover or activation of protein kinase A or protein kinase C (PKC) [8,23–26]. IL-6, as well as other cytokines, has been shown to activate novel signal transduction mechanisms. The initial event in the IL-6 signal transduction cascade involves the interaction of IL-6 with its 80 kDa cell surface receptor, followed by association of the ligand–receptor complex with a membrane glycoprotein, gp 130 [23]. This IL-6–receptor–gp-130 signal transduction complex initiates IL-6 signal transduction by an as yet undefined mechanism. Characterization of some of the downstream events in IL-6 signal transduction pathways have revealed that IL-6 leads to tyrosine phosphorylation of a 160 kDa cellular protein [25,27] and of gp 130 [28] and to activation of an H-7-sensitive protein kinase, which is distinct from other defined protein kinases [25]. In addition, it has been demonstrated that the mechanism of the anti-proliferative effect of IL-6 in haematopoietic cell lines requires activation of two independent parallel pathways, one of which leads to a decrease in *c-myc* expression and the other to dephosphorylation of the retinoblastoma gene product [29]. In this study I demonstrate that okadaic acid (OA), a specific inhibitor of protein phosphatases 1 and 2A [30], inhibits induction of CRP, SAA and fibrinogen in Hep 3B cells and of CRP, fibrinogen and α_1 -PI in NPLC/PRF/5 cells, suggesting that IL-6-stimulated signal transduction pathways involved in the induction of acute-phase proteins are mediated by protein phosphatases 1 and/or 2A.

Abbreviations used: CRP, C-reactive protein; SAA, serum amyloid A; α_1 -PI, α_1 -proteinase inhibitor; IL-6, interleukin-6; IL-1, interleukin-1; OA, okadaic acid; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

EXPERIMENTAL

Materials

Escherichia coli-derived recombinant human IL-6 (specific activity 7×10^6 units/mg of protein) and IL-1 α (specific activity 3×10^6 units/mg of protein) were gifts from Dr. G. Wong, Genetics Institute, Boston, MA, U.S.A., and Dr. P. Lomedico, Roche Institute, Nutley, NJ, U.S.A., respectively. OA (0.5 mM in 10 % dimethyl sulphoxide) was obtained from Lifescience Technology (Gaithersburg, MD, U.S.A.), and was diluted in RPMI-1640. All other reagents and chemicals were obtained from sources previously described [6,26].

Cell culture

Hep 3B cells and NPLC/PRF/5 cells were maintained as described earlier [6,26]. Cells were cultured in RPMI-1640 supplemented with 10 % heat-inactivated fetal bovine serum in 35 mm-diam. dishes. On day 5 following subculture, media from confluent cells (approx. 1×10^6) were removed and cells were washed twice with Hanks balanced salt solution and incubated with IL-6 in the absence or presence of IL-1 α and/or OA in serum-free RPMI-1640 containing $1 \mu\text{M}$ -dexamethasone and 0.02 units of insulin/ml. The total volume of incubation medium was 1 ml. Cells were then labelled with 0.1 mCi of [^{35}S]methionine in methionine-deficient RPMI-1640 for 4 h as described [6,26].

Analysis of acute-phase proteins secreted into the culture medium

[^{35}S]Methionine-labelled acute-phase proteins secreted into the culture medium were analysed by immunoprecipitation, followed by SDS/PAGE and autoradiography as described previously [6,26]. Immunoprecipitation of fibrinogen was carried out with 50 or 25 μl of the sample, for Hep 3B and NPLC/PRF/5 cells respectively, and 3 μl of rabbit anti-(human fibrinogen) (Accurate Chemical and Scientific Corp., Westbury, NY, U.S.A.) in a total volume of 200 μl . All other conditions and procedures for fibrinogen were identical to those for SAA as previously described [6]. The relative amounts of these acute-phase proteins were determined by densitometric scans of the autoradiographic signals.

RESULTS AND DISCUSSION

Studies from our laboratory on the role of PKC in the induction of acute-phase proteins by cytokines demonstrated that activation of PKC by phorbol 12-myristate 13-acetate (PMA) led to inhibition of the induction of acute-phase proteins by IL-6 alone or in the presence of IL-1 α in Hep 3B cells [31]. These results suggested that dephosphorylation of a key protein(s), probably by activation of protein phosphatases, may be important in the signal transduction mechanisms regulating induction of acute-phase proteins by IL-6 alone or in combination with IL-1. Thus I examined the effect of OA, an inhibitor of protein phosphatases 1 and 2A [31], on the induction of acute-phase proteins by IL-6 plus IL-1 α or IL-6 alone in the two hepatoma cell lines, Hep 3B and NPLC/PRF/5. In the Hep 3B cell line, IL-6 alone induces only fibrinogen and α_1 -PI, whereas induction of CRP and SAA requires a co-operative interaction between IL-6 and IL-1 [6]. In contrast, in the NPLC/PRF/5 cells IL-6 alone is capable of inducing all four acute-phase proteins [6].

In Hep 3B cells, OA inhibited induction of CRP, SAA and fibrinogen by IL-6 plus IL-1 α in a dose-dependent manner, with a linear response between 5 and 20 nM (Figs. 1 and 2). The induction of CRP by IL-6 plus IL-1 α was most sensitive to the

Fig. 1. Dose-response relationship of the effect of OA on induction of acute-phase proteins by IL-6 plus IL-1 α in Hep 3B cells

Hep 3B cells were incubated with IL-6 (29 ng/ml) plus IL-1 α (0.67 ng/ml) in the absence or presence of various concentrations of OA for 18–24 h and labelled with L-[^{35}S]methionine. Radiolabelled CRP, SAA, fibrinogen (FIBR) and α_1 -PI secreted into the culture medium were analysed by immunoprecipitation followed by fractionation of the immunoprecipitate on SDS/polyacrylamide gels, fluorography and autoradiography of the dried gels as described in the Experimental section. The relative intensities of the autoradiographic signals were determined by densitometric scanning. The value for IL-6 plus IL-1 α alone was normalized to 100 %. The ordinate represents the percentage induction of acute-phase proteins by IL-6 plus IL-1 in the presence of OA compared with that in the absence of OA (control). Values correspond to the means $\pm$ S.D. of three independent experiments carried out in duplicate.

inhibitory effect of OA. At a concentration of 10 nM-OA almost complete inhibition of the induction of CRP by IL-6 plus IL-1 α was observed, whereas induction of SAA and fibrinogen was inhibited by only 40–50 %. The induction of fibrinogen by IL-6 alone was also inhibited by OA in a dose-dependent manner in Hep 3B cells (Fig. 3). In this cell line, concentrations of OA above 20 nM were toxic and synthesis of total proteins secreted into the culture medium was inhibited by 50–60 % at 30 nM-OA and by about 90 % at 100 nM. OA up to 20 nM had no significant effect on the induction of α_1 -PI by IL-6 plus IL-1 α (Figs. 1 and 2) or by IL-6 alone (Fig. 3).

In the NPLC/PRF/5 cells, OA inhibited induction of CRP, fibrinogen and α_1 -PI by IL-6 in a dose-dependent manner (20–80 nM), albeit at higher concentrations than in Hep 3B cells (Fig. 4). Induction of SAA was not examined in this cell line. Almost complete inhibition of these three acute-phase proteins was observed at a concentration of 80 nM-OA. In the NPLC/PRF/5 cell line also the induction of CRP by IL-6 was most sensitive to the inhibitory effect of OA. A concentration of 40 nM-OA inhibited induction of CRP by IL-6 by 60–70 %, whereas induction of fibrinogen and α_1 -PI were minimally inhibited (10–25 %). In this cell line concentrations of OA up to 160 nM were not toxic and did not affect the synthesis of total proteins secreted into the culture medium.

The results obtained in this study strongly suggest that induction of at least some acute-phase proteins by IL-6 alone or in combination with IL-1 is mediated by activation of protein phosphatase 1 and/or 2A, thus supporting the above hypothesis that dephosphorylation of a key protein(s) may represent an important mechanism regulating the synthesis of acute-phase proteins. Both OA and PMA, acting via distinct mechanisms (inhibition of protein phosphatases 1 and 2A and activation of PKC respectively) would lead to increased phosphorylation of

Fig. 2. Effect of PKC activators on induction of acute-phase proteins by IL-6 plus IL-1 in Hep 3B cells

Autoradiograph of a representative experiment in Hep 3B cells incubated with IL-6 (29 ng/ml) plus IL-1 α (0.67 ng/ml) in the absence or presence of various concentrations of OA. L-[³⁵S]Methionine-labelled CRP, SAA, fibrinogen (FIBR) and α_1 -PI synthesized and secreted into the culture medium were determined by immunoprecipitation, SDS/PAGE and autoradiography as described in the legend to Fig. 1. C represents control cells incubated in medium alone.

several cellular proteins, some of which could lead to repression of gene transcription or inhibition or a post-transcriptional event involved in the regulation of acute-phase protein synthesis. In another independent study [29] it has been demonstrated that one of the IL-6-stimulated signal transduction pathways responsible for inhibiting proliferation of haematopoietic cells involves dephosphorylation of the retinoblastoma gene product. This observation also supports my finding that IL-6-stimulated signal transduction pathways may involve activation of protein phosphatases, since dephosphorylation of the retinoblastoma gene product could be accomplished by activation of protein phosphatases or inhibition of protein kinases by IL-6.

The differential dose-response relationships for OA in inhibiting cytokine-induced synthesis of acute-phase proteins in the Hep 3B and NPLC/PRF/5 cell lines, and the differential effects of OA on induction of α_1 -PI by cytokines in the two cell lines, suggest that: (i) the two cell lines may contain different amounts of protein phosphatases 1 and/or 2A, and (ii) induction of different acute-phase proteins may require activation of different phosphatases. This could also explain why PMA, which almost completely abolished induction of CRP, SAA and fibrino-

Fig. 3. Effect of OA on induction of fibrinogen and α_1 -PI by IL-6 in Hep 3B cells

Autoradiograph of a representative experiment in Hep 3B cells incubated with IL-6 (29 ng/ml) in the absence or presence of various concentrations of OA. L-[³⁵S]Methionine-labelled fibrinogen (FIBR) and α_1 -PI synthesized and secreted into the culture medium were determined by immunoprecipitation, SDS/PAGE and autoradiography as described in the legend to Fig. 1. C represents control cells incubated in medium alone.

Fig. 4. Effect of various concentrations of OA on induction of acute-phase proteins by IL-6 in NPLC/PRF/5 cells

Autoradiograph of a representative experiment in NPLC/PRF/5 cells incubated with IL-6 (29 ng/ml) in the absence or presence of various concentrations of OA. L-[³⁵S]Methionine-labelled CRP, SAA, fibrinogen (FIBR) and α_1 -PI synthesized and secreted into the culture medium were determined by immunoprecipitation, SDS/PAGE and autoradiography as described in the legend to Fig. 1. C represents control cells incubated in medium alone.

gen by IL-6 plus IL-1 or IL-6 alone in Hep 3B cells, minimally inhibited induction of CRP, fibrinogen and α_1 -PI in the NPLC/PRF/5 cell line [31].

Induction of CRP and SAA in the Hep 3B cell line requires both IL-6 and IL-1, whereas induction of these acute-phase proteins in the NPLC/PRF/5 cell line requires only IL-6. This indicates that the signal transduction mechanisms in these two

cell lines differ. It is possible that Hep 3B cells are defective in one of the IL-6-stimulated signal transduction pathways that is essential for the induction of CRP and SAA, but not fibrinogen or α_1 -PI, since fibrinogen and α_1 -PI are induced by IL-6 alone in both cell lines. Alternatively, it is possible that the signal transduction pathways activated by IL-1 in Hep 3B cells (which in combination with IL-6 leads to induction of CRP and SAA) is constitutively activated in NPLC/PRF/5 cells. In either case it would imply that induction of CRP and SAA requires activation of more than one parallel signal transduction pathway, which could act independently or converge at some step downstream. One of these pathways, which is mediated by protein phosphatases 1 and/or 2A, is stimulated by IL-6, and not by IL-1, since: (i) induction of fibrinogen in both cell lines and of α_1 -PI in NPLC/PRF/5 cells by IL-6 alone is inhibited by OA, and (ii) IL-6 has been shown to dephosphorylate the retinoblastoma gene product [29], probably by activation of protein phosphatase(s). The other signal transduction pathway(s) required for induction of CRP and SAA is not known, but may be mediated by activation of tyrosine kinases, since IL-6 has been shown to phosphorylate a 160 kDa cellular protein on a tyrosine residue, and our previous studies demonstrated that staurosporine, an inhibitor of PKC and tyrosine kinases [32,33], inhibits induction of CRP and SAA, but not of fibrinogen, in Hep 3B cells.

In summary, this study demonstrates that the induction of CRP, SAA, fibrinogen and α_1 -PI by IL-6, alone or in combination with IL-1, is mediated by a novel mechanism which involves activation of protein phosphatases 1 and/or 2A by IL-6. Furthermore, our results indicate that induction of CRP and SAA requires activation of additional, as yet unidentified, signal transduction pathways.

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