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Sodium Arsenite Downregulates Transcriptional Activity of AP-1 and CRE Binding Proteins in IL-1β-Treated Caco-2 Cells by Increasing the Expression of the Transcriptional Repressor CREMα

Dan D. Hershko,[1] Bruce W. Robb,[1,2] Guang-jo Luo,[1] Eric S. Hungness,[1,2] and Per-Olof Hasselgren[3]*

[1]Shriners Hospitals for Children, Cincinnati, Ohio
[2]Department of Surgery, University of Cincinnati, Cincinnati, Ohio
[3]Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts

Abstract In recent studies, sodium arsenite (SA) inhibited IL-6 production in cultured intestinal epithelial cells, at least in part by downregulating the activity of nuclear factor-kappaB (NF-κB). The influence of SA on the activity of other transcription factors regulating the interleukin-6 (*IL-6*) gene in enterocytes is not known. We tested the effect of SA on the activity of CCAAT/enhancer binding protein (C/EBP), activating protein-1 (AP-1), and CRE binding proteins in IL-1β-treated Caco-2 cells. DNA binding activity was determined by electrophoretic mobility shift assay (EMSA) and transcriptional activity by transfecting cells with luciferase reporter plasmids containing promoter constructs with binding sites for the individual transcription factors. DNA binding activity for all three transcription factors was increased after treatment with SA or IL-1β. In contrast, SA inhibited transcriptional activity of AP-1 and CRE binding proteins but not C/EBP. Additional experiments provided evidence that the inhibition of AP-1 and CRE mediated transcriptional activity was associated with, and probably caused by, increased expression of the transcriptional repressor cyclic AMP response element modulator (CREM)α. The present results are consistent with the concept that SA inhibits IL-6 production in stimulated enterocytes by downregulating the transcriptional activity of several, but not all, IL-6-related transcription factors. Because of the multiple important biological functions of IL-6 in the enterocyte and gut mucosa, methods to regulate enterocyte IL-6 production have significant clinical implications. J. Cell. Biochem. 90: 627–640, 2003.

Key words: sodium arsenite; IL-6; transcription factors; cytokines; enterocytes; mucosa; intestine

Recent research in our and other laboratories suggest that the intestinal mucosa and enterocyte are active participants in the inflammatory responses to sepsis and severe injury [Swank and Deitch, 1996; Hasselgren, 1998]. For example, mucosal and enterocyte production of various cytokines and acute phase proteins is increased and transcription factors commonly involved in the regulation of inflammatory genes are activated in gut mucosa during sepsis [Molmenti et al., 1993; Meyer et al., 1995; Wang et al., 1998; Pritts et al., 2002]. Studies in our laboratory have provided evidence that mucosal interleukin-6 (IL-6) production and nuclear factor-kappaB (NF-κB) activity are increased during inflammation caused by sepsis or endotoxemia [Meyer et al., 1995; Pritts et al., 1998, 2000, 2002; Wang et al., 1998]. In other experiments, we found that treatment of cultured Caco-2 cells, a human intestinal epithelial cell line [Rousset, 1986], with IL-1β resulted in activation of the transcription factors activating protein-1 (AP-1), CCAAT/enhancer binding protein (C/EBP), and

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*Correspondence to: Per-Olof Hasselgren, MD, Department of Surgery, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, ST9 919, Boston, MA 02215.
E-mail: phasselg@bidmc.harvard.edu

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NF-κB concomitant with upregulated IL-6 production [Parikh et al., 1997a,b; Hungness et al., 2000, 2002a,b]. Mucosal and enterocyte IL-6 production is of particular interest because of the multiple important biological functions of IL-6, including regulation of intestinal acute phase protein synthesis and IgA production [Beagley et al., 1989; Molmenti et al., 1993]. Previous reports of both pro- and anti-inflammatory properties of IL-6 support the concept that IL-6 is a potent pleiotropic cytokine that plays an important role in the response to inflammation [Papanicolaou et al., 1998].

Because of the multiple biological roles of IL-6, both locally in the mucosa and systemically, mechanisms influencing the regulation of enterocyte IL-6 production have important clinical implications. The *IL-6* gene is regulated by multiple transcription factors, including NF-κB, C/EBP, AP-1, and cyclic AMP response element (CRE) binding proteins [Vanden Berghe et al., 2000]. Sodium arsenite (SA) is a multipotent drug that has been shown in previous studies to influence signaling pathways, transcription factors, and genes commonly involved in inflammation [Bernstam and Nriagu, 2000]. We recently reported that SA decreased IL-6 production and inhibited NF-κB activity in cultured Caco-2 cells treated with the pro-inflammatory cytokine IL-1β [Hershko et al., 2002a,b]. In contrast, the influence of SA on the activity of other transcription factors regulating the *IL-6* gene in stimulated enterocytes is not known. This is significant because studies in other cell types suggest that IL-6-related transcription factors may be differentially regulated by various treatments and that the role of the individual transcription factors in the regulation of the *IL-6* gene varies with cell type and stimulus [Vanden Berghe et al., 1999].

Arsenite can induce multiple biological effects, both at the cellular and tissue level, including stimulation of the mitogen-activated protein (MAP) kinase signaling pathway, induction of the heat shock response, and inhibition of NF-κB [reviewed by Bernstam and Nriagu, 2000]. Examining the metabolic effects of SA is important not only from a biological standpoint but from a clinical standpoint as well. In previous studies, treatment with SA improved survival in septic rats [Hauser et al., 2001]. In other experiments, SA protected the small intestine against ischemia-reperfusion injury by inhibiting the synthesis of inflammatory cytokines [Tsuruma et al., 1999]. In addition, there is increasing evidence that arsenic derivatives may be useful in the treatment of certain cancers in patients [Waxman and Anderson, 2001]. Thus, a better understanding of the metabolic effects of SA and their mechanisms is important from both a biological and clinical standpoint.

The purpose of the present study was to test the hypothesis that SA regulates the activation of C/EBP, AP-1, and CRE binding proteins in IL-1β-stimulated Caco-2 cells. Results suggest that SA inhibits the transcriptional activity of AP-1 and CRE binding proteins secondary to upregulated expression of the transcriptional repressor cyclic AMP response element modulator (CREM)α. In contrast, SA did not influence the IL-1β-induced activation of C/EBP. The results suggest that inhibited IL-6 production in SA-treated enterocytes reflects downregulated activity of multiple, but not all, IL-6-related transcription factors.

MATERIALS AND METHODS

Materials

Caco-2 cells were purchased from American Type Culture Collection (Rockville, MD). Dulbecco's modified Eagle's medium (DMEM), nonessential amino acids, low-endotoxin fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin, and TRIZOL were purchased from Gibco-BRC (Grand Island, NY). Human recombinant IL-1β was from Endogen (Woburn, MA) and SB 203580 from Calbiochem (LaJolla, CA). All other chemicals, unless otherwise stated, were from Sigma (St. Louis, MO).

Cell Culture

Caco-2 cells were grown in 5% CO_2 at 37°C in DMEM supplemented with 10% FBS, nonessential amino acids, 6 mM glutamine, 10 mM HEPES, 10 μg/ml apo-transferrin, 1 mM pyruvate, 24 mM $NaHCO_3$, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells, between passages 5 and 25, were seeded at a density of 100,000 cells/cm^2 onto 6-well culture plates for ELISA and luciferase assays or onto 10 cm tissue culture plates (both from Falcon-Becton Dickinson, Franklin Lakes, NJ) for Western blotting and electrophoretic mobility shift assay (EMSA). The cells were grown for 72 h to 90% confluence before use.

Before experiments, cells were washed three times with serum-free DMEM and then pretreated for 1 h with serum-free medium containing 500 μM SA. This concentration of SA resulted in maximal inhibition of IL-6 production in IL-1β-treated Caco-2 cells in a recent study from this laboratory [Hershko et al., 2002a]. After exposure to SA, the cells were washed with phosphate buffered saline (pH 7.4) whereafter IL-1β (0.5 ng/ml) was added to the culture medium for 4 h. Treatment of cultured Caco-2 cells with 0.5 ng/ml of IL-1β resulted in maximal IL-6 production in previous experiments [Parikh et al., 1997a].

Determination of Cell Viability

Cell viability was determined by measuring mitochondrial respiration, assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) to formazan as described previously [Szabo et al., 1996]. Cell viability was not influenced by any of the experimental conditions in the present study (data not shown). It may be argued that if SA influences mitochondial respiration, the MTT test may not be optimal to test cell viability under the present experimental conditions. It should be noted, however, that a previous study suggests that SA can induce at least some biological effects, including induction of the heat shock response and thermotolerance, independent of any potential effect on mitochondrial respiration [Landry et al., 1985]. In addition, none of the treatments in the present study, including treatment with SA, reduced cell viability determined by the MTT test. The concern that changes in the MTT test by SA would reflect a specific effect on mitochondrial respiration, rather than an overall effect on cell viability, would, of course, be more significant if the SA treatment had resulted in reduced MTT activity.

Preparation of Cytoplasmic and Nuclear Extracts

Cells were harvested by scraping into ice-cold phosphate buffered saline, pH 7.4, and pelleted by centrifugation at 3,800*g* for 5 min. Cells were then suspended in one packed-cell volume of lysis buffer containing 10 mM HEPES, pH 7.9, 10 mM KC1, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.2% (v/v) Nonidet P-40, 1 mM DTT, 0.5 mM PMSF, 100 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1.5 μM pepstatin A, 1.4 μM transepoxysuccinyl-L-leucylamidol, 4 μM bestatin, 2.2 μM leupeptin, 0.08 μM aprotinin, 0.0045 μM microcystin LR, 0.46 μM cantharidin, and 0.2 μM (−)-*p*-bromotetramisole. After incubation on ice for 5 min with intermittent vortexing, the nuclear pellet was isolated by centrifugation at 3,800*g* for 5 min. The supernatant was removed and saved as the cytoplasmic fraction. The pellet was resuspended in 1 cell volume of extraction buffer containing 20 mM HEPES, pH 7.9, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$ 25% glycerol (v/v), 1 mM DTT, 0.5 mM PMSF, 100 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1.5 μM pepstatin A, 1.4 μM transepoxysuccinyl-L-leucylamidol, 4 μM bestatin, 2.2 μM leupeptin, 0.08 μM aprotinin, 0.0045 μM microcystin LR, 0.46 μM cantharidin, and 0.2 μM (−)-*p*-bromotetramisole and incubated on ice for 30 min with intermittent vortexing. The nuclear debris was pelleted by centrifugation at 16,000*g* for 20 min and the supernatant was saved as the nuclear fraction. Protein concentrations of nuclear and cytoplasmic extracts were determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as standard.

Western Blot Analysis

Aliquots of nuclear or cytoplasmic fractions containing 25 μg of protein were boiled in equal amounts of loading buffer (125 mmol/L Tris-hydrochloride, pH 6.8, 4% sodium dodecyl sulphate, 20% glycerol, and 10% 2-mercaptoethanol) for 3 min, then separated by electrophoresis on 8–16% Tris-glycine gradient gel (Invitrogen, San Diego, CA). A protein ladder (See-Blue; Invitrogen) was included as a molecular weight marker. The proteins were transferred to nitrocellulose membranes (Xcell II Blot Module; Invitrogen), which were blocked in 5% nonfat dried milk in Tris-buffered saline (pH 7.6), containing 0.05% Tween-20 for 1 h. The membranes were then incubated with antibody against CREMα (Santa Cruz Laboratories, Santa Cruz, CA), phosphorylated CREB (pCREB) (PhosphoPlus CREB [Ser 133]; New England BioLabs, Beverly, MA), or phosphorylated activating transcription factor-1 (pATF-1) (Santa Cruz Laboratories) for 1 h and then washed three times with TTBS before incubation with peroxidase-conjugated secondary antibody for 45 min. Following successive washes, the membranes were incubated in

enhanced chemiluminescence reagents and exposed on radiographic film (Eastman-Kodak, Rochester, NY).

Electrophoretic Mobility Shift Assay

EMSA was performed as described in detail previously [Hungness et al., 2000, 2002a,b; Hershko et al., 2002a,b]. Aliquots of the nuclear fractions (7.5 μg protein) were incubated in buffer containing 12% glycerol (v/v), 12 mM HEPES, pH 7.9, 4 mM Tris-HCl, pH 7.9, 1 mM EDTA, 1 mM dithiothreitol, 25 mM KCl, 5 mM $MgCl_2$, 0.04 μg/μl poly [d(I-C)] (Boehringer Mannheim, Indianapolis, IN), and Tris-EDTA buffer, pH 7.4. C/EBP gel shift oligonucleotide 5′-TGC AGA TTG CGC AAT CTG CA-3′, AP-1 gel shift oligonucleotide 5′-CGC TTG ATG ACT CAG CCG GAA-3′, and CRE gel shift oligonucleotide 5′-AGA GAT TGC CTG ACG TCA GAC AGC TAG-3′ were purchased from Santa Cruz Laboratories. Probes were end-labeled with (^{32}P) γATP using polynucleotide kinase T4 (Gibco BRL, Grand Island, NY). End-labeled probe was purified from unincorporated (^{32}P) γATP using a purification column (Bio-Rad Laboratories) and recovered in Tris-EDTA buffer, pH 7.4. Labeled probe was added to nuclear extracts and the samples were incubated for 30 min on ice. Samples were then subjected to electrophoretic separation on a nondenaturing 5% poly-acrylamide gel at 30 mA using Tris-borate EDTA buffer (0.45 M Tris-borate, 0.001 M EDTA, pH 8.3). Blots were dried at 80°C for 3 h and analyzed by exposure to PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA). Supershift analysis of C/EBP EMSA was performed by adding antibodies to C/EBPβ or δ; supershift analysis of AP-1 EMSA was performed by adding an antibody to one of the AP-1 subunits Jun-B, Jun-D, c-Jun, c-Fos, or Fra-1; supershift analysis of CRE EMSA was performed by adding an antibody to CREB, ATF-1, or CREMα to the reaction.

Plasmids and Transfections

RSV-CREMα and RSV-KCREB expression vectors were kindly provided by Dr. R. Goodman, Oregon Health Sciences University, Portland, OR. Plasmids containing a 3× tandem repeat of AP-1 binding sequences or a 3× tandem repeat of C/EBP binding sequences upstream from a firefly luciferase gene were kindly provided by Dr. H. Wong, Children's Hospital Medical Center (Cincinnati, OH). A luciferase reporter plasmid containing a 5× tandem repeat of CRE binding sites was obtained from Invitrogen.

Caco-2 cells (10^5 cell/cm^2) were seeded onto 6-well culture dishes and grown to 50% confluence before transfection. The Lipofectin (Gibco-BRL) transfection method was used. Briefly, LipofectAmine Plus was incubated with serum-free OPTIMEM and 1 μg of plasmid at room temperature for 15 min. The Caco-2 cells were washed three times with serum-free medium, and the 4:1 lipid–DNA complexes were added to the cells. After incubation at 37°C for 4 h, the culture medium was changed to DMEM supplemented with 10% FBS and incubated for an additional 24 h at 37°C. After the cells had been washed with serum free DMEM, they were treated with IL-1β (0.5 ng/ml) or SA 500 μM in serum free medium for 8 h. For luciferase assays, cells were washed twice with phosphate buffered-saline, pH 7.4, and 250 μl of Luciferase Cell Culture Lysis Reagent (Promega, Madison, WI) was added to each well for 15 min, after which the cells were harvested and stored at −70°C. For measurement of luciferase activity, the samples were thawed and centrifuged at 14,000*g* for 2 min. Supernatant (30 μl) was combined with 100 μl of Luciferase Assay Substrate (Promega) in Sarstedt 12 × 75 mm tubes in duplicate and read for 10 s on a Berthold AutoLumat LB953 luminometer.

Determination of IL-6

IL-6 was determined by ELISA using a commercially available kit (Endogen, Cambridge, MA). The limit of detection was 1 pg/ml.

Statistical Analysis

Results are expressed as mean ± standard error of the mean. Student's *t*-test or ANOVA followed by Tukey's test was used for statistical analysis. $P < 0.05$ was considered statistically significant. Experiments were performed at least three times for evidence of reproducibility.

RESULTS

Treatment of cultured Caco-2 cells with IL-1β increased C/EBP and AP-1 DNA binding activity determined by EMSA (Fig. 1). These results confirm previous reports from this laboratory [Hungness et al., 2000, 2002b] and are consistent with the concept that multiple

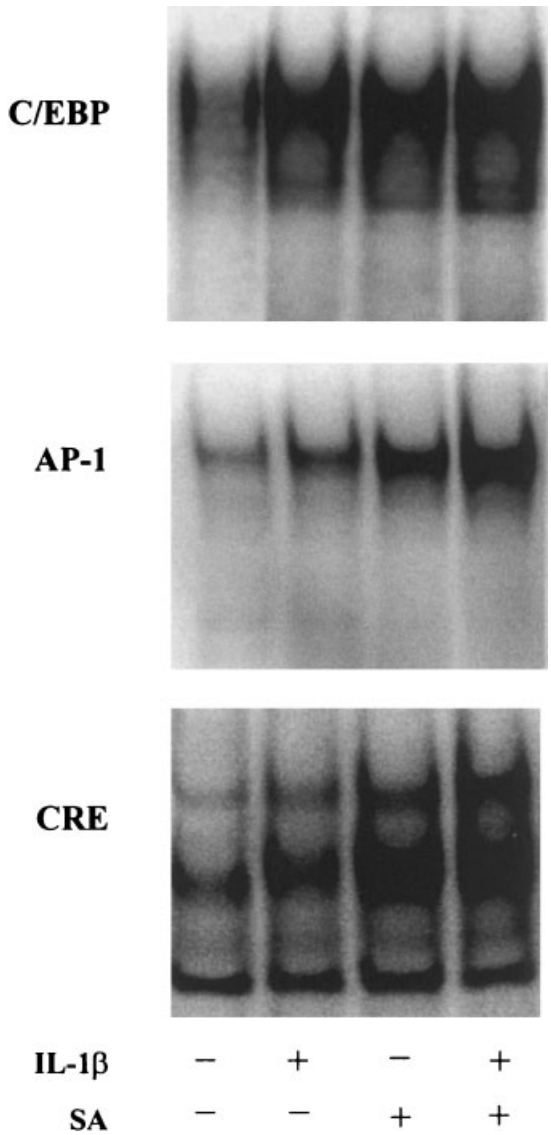

Fig. 1. The effects of sodium arsenite (SA) and IL-1β on DNA binding activity of CCAAT/enhancer binding protein (C/EBP), activating protein-1 (AP-1), and cyclic AMP response element (CRE) binding proteins. Cultured Caco-2 cells were treated for 1 h with SA (500 μM) or 4 h with IL-1β (0.5 ng/ml) as described in Materials and Methods whereafter DNA binding activity was determined by electrophoretic mobility shift assay (EMSA). Similar results were observed in three consecutive experiments.

transcription factors that regulate the *IL-6* gene are activated in the enterocyte during inflammation. This was further supported by increased binding of nuclear proteins to a CRE oligonucleotide in IL-1β-treated Caco-2 cells (Fig. 1, lower panel). It should be noted that although increased DNA binding activity of AP-1 and CRE binding proteins was seen in IL-1β-treated cells in three consecutive experiments, the effect of IL-1β on AP-1 and CRE binding proteins was consistently less pronounced than the effect on C/EBP DNA binding.

When cells were treated with SA alone, DNA binding activity of the three transcription factors studied here was increased (Fig. 1). SA did not seem to further increase the IL-1β-induced DNA binding of C/EBP and CRE binding proteins whereas the effects of IL-1β and SA on AP-1 were additive.

In previous experiments, we found evidence that upregulated C/EBP DNA binding activity in IL-1β-treated Caco-2 cells was at least in part regulated by the mitogen activated protein (MAP) kinase p38 [Hungness et al., 2002b]. Arsenite, in turn, is a well-known stimulator of the p38 MAP kinase pathway, mainly reflecting inhibited activity of phosphatases that normally decrease basal p38 MAP kinase activity [Cavigelli et al., 1996; Samet et al., 1998]. In order to test whether the p38 MAP kinase pathway was involved in the regulation of C/EBP, AP-1, and CRE binding proteins under the present experimental conditions, cells were treated with the p38 inhibitor SB 203580. This treatment decreased the IL-1β- and SA-induced increase in DNA binding activity of C/EBP, AP-1, and CRE binding proteins, suggesting that p38 kinase activation at least in part mediated the effects of both IL-1β and SA on transcription factor DNA binding activity (Fig. 2). It should be noted, however, that the inhibitory effect of SB 203580 on IL-1β-induced DNA binding of C/EBP and AP-1 was less pronounced than the effect on SA-induced DNA binding activity of the same transcription factors, suggesting that p38 kinase activity was less important for IL-1β-induced activation of C/EBP and AP-1 than for arsenite-induced activation. Interestingly, SB 203580 inhibited IL-1β- and arsenite-induced DNA binding of CRE binding proteins to the same extent (Fig. 2, bottom panel).

We next examined which individual members of the transcription factor families were affected by SA. Within the C/EBP family of transcription factors, at least six different isoforms have been identified [Lekstrom-Himes and Xanthopoulos, 1998]. We recently found that it is mainly C/EBPβ and δ that are involved in the inflammatory response in enterocytes [Hungness et al., 2002b]. Here, we therefore performed supershift analysis by adding an antibody to C/EBPβ or δ. In addition, nuclear levels of C/EBPβ and δ proteins were determined by Western blot

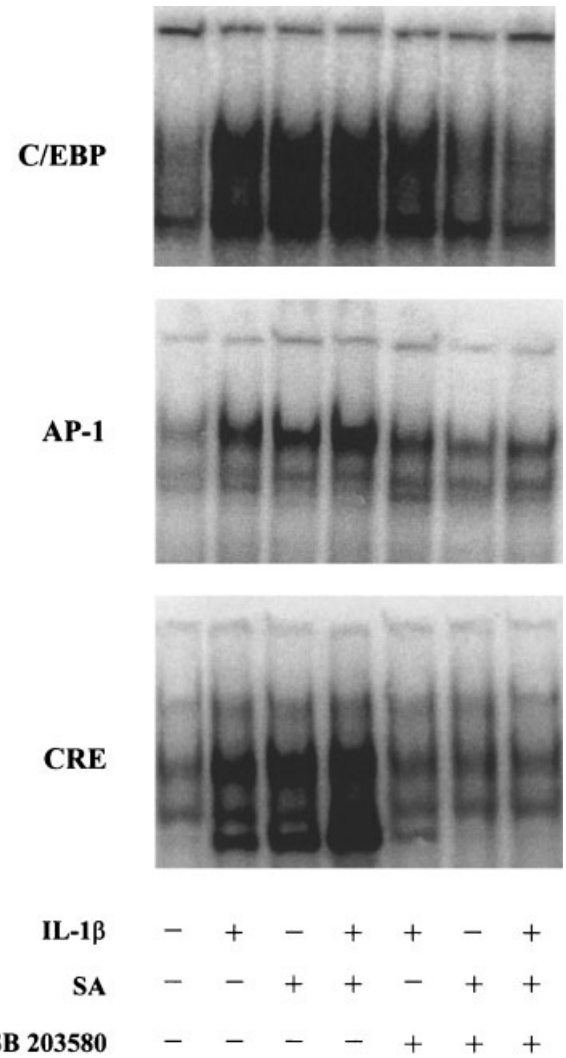

Fig. 2. The effect of SB 203580 (10 μM) on DNA binding activity of C/EBP, AP-1, and CRE binding proteins in Caco-2 cells treated with SA (500 μM) or IL-1β (0.5 ng/ml). Cells were treated as described in Figure 1 except that some cells were exposed to SB 203580 during the treatment with IL-1β and SA. Similar results were observed in three repeated experiments.

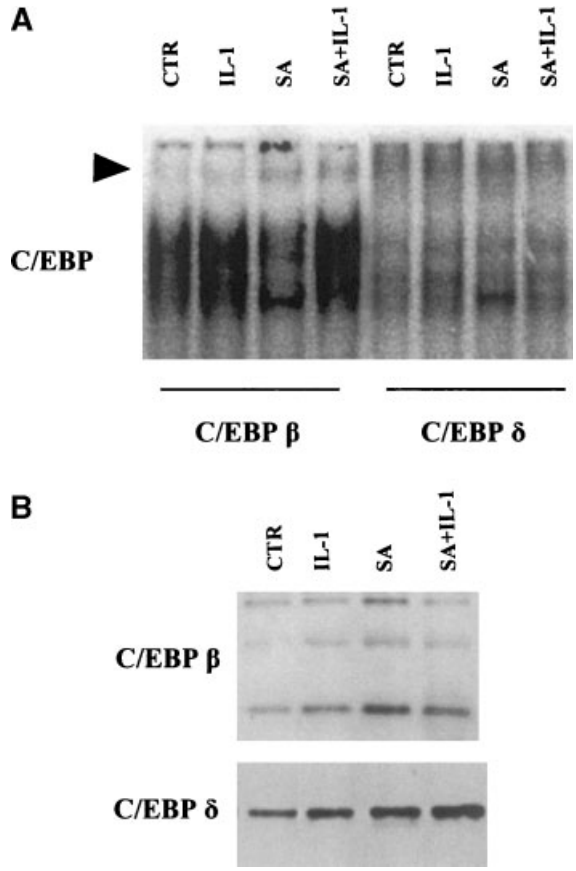

Fig. 3. **A**: Supershift analysis of EMSA for C/EBP in Caco-2 cells treated with IL-1β, SA, or SA + IL-1β as described in Materials and Methods and in the legend to Figure 1. Supershift was induced by adding antibody to C/EBPβ or δ to the EMSA reaction as indicated under the blots. Arrowhead indicate position of supershifted band. **B**: Nuclear concentrations of C/EBPβ and C/EBPδ determined by Western blotting in Caco-2 cells treated with IL-1β or SA as described in Materials and Methods in the legend to Figure 1. The three bands noted in the C/EBPβ blot, correspond to C/EBPβ isoforms reported previously with molecular weights of approximately 38, 35, and 20 kDa [Ossipow et al., 1993; Hungness et al., 2002b].

analysis. Results from these experiments suggest that SA increased DNA binding activity and protein levels for C/EBPβ whereas no obvious effect on C/EBPδ expression or activity was observed (Fig. 3). Note that the three bands seen on the Western blots for C/EBPβ are similar to previous reports from our and other laboratories of multiple isoforms of C/EBPβ with molecular weights of approximately 38, 35, and 20 kDa [Ossipow et al., 1993; Hungness et al., 2002b].

When supershift analysis was performed using antibodies to various AP-1 subunits, results showed that SA alone or in combination with IL-1β increased the DNA binding activity of AP-1 complexes containing c-Jun, c-Fos, and Fra-1 (Fig. 4). Note that the supershifted band for c-Fos was located higher than the other supershifted bands in the upper but not the lower panel of Figure 4. Although we have no definitive explanation for this apparent discrepancy, it is possible that the different positions of the bands in the two experiments reflects variations in the length of time during which electrophoresis was carried out. It should also be noted that it will be important in future

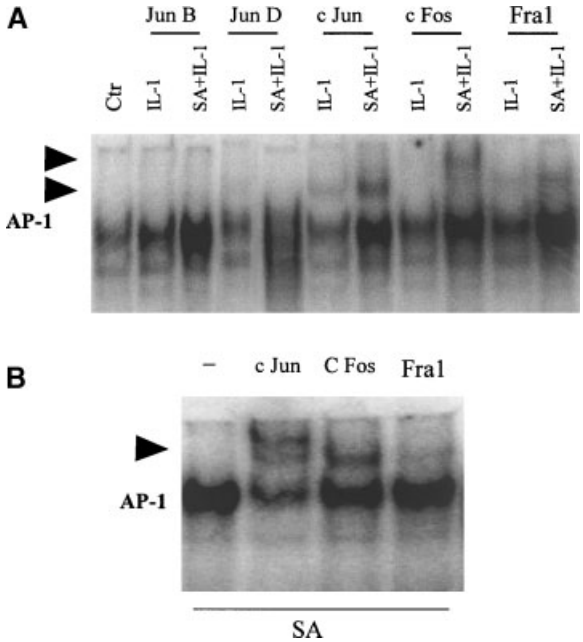

Fig. 4. **A**: Supershift analysis of EMSA for AP-1 in Caco-2 cells treated with IL-1β or SA + IL-1β (SA + IL-1) as described in Materials and Methods and in the legend to Figure 1. Supershift was induced by adding antbodies to various AP-1 subunits as indicated above the gels. **B**: Supershift analysis of EMSA for AP-1 in Caco-2 cells treated with 500 μM SA for 1 h as described in Materials and Methods.

experiments to supershift for FosB and Fra-2 for completeness.

We next examined the effect of SA on the activity of different CRE binding proteins. Results from that experiment showed that SA mainly increased the DNA binding activity of CREMα (Fig. 5). This conclusion was made from comparison between cells treated with SA + IL-1β and cells treated with IL-1β alone (rather than untreated control cells). Although not ideal, this comparison was reasonable considering the fact that IL-1β increased (rather than decreased) overall DNA binding activity of CRE binding proteins (see Figs. 1 and 2). The influence of SA on the DNA binding activity of ATF-2, an additional CRE binding protein, remains to be determined.

Because changes in transcription factor DNA binding activity, determined by EMSA, do not necessarily reflect changes in transcriptional activity, we next examined the influence of IL-1β and SA on C/EBP, AP-1, and CRE binding protein transcriptional activity. To achieve this, cells were transfected with luciferase reporter plasmids containing promoter constructs of multiple tandem repeats of binding sites for the individual transcription factors. Surprisingly, changes in the DNA binding activity (by EMSA) of AP-1 and CRE binding proteins induced by SA were not reflected by similar changes in transcriptional activity. Thus, SA reduced basal transcriptional activity and completely abolished the increase in transcriptional activity of AP-1 and CRE binding proteins induced by IL-1β (Fig. 6). In contrast, changes in C/EBP transcriptional activity induced by

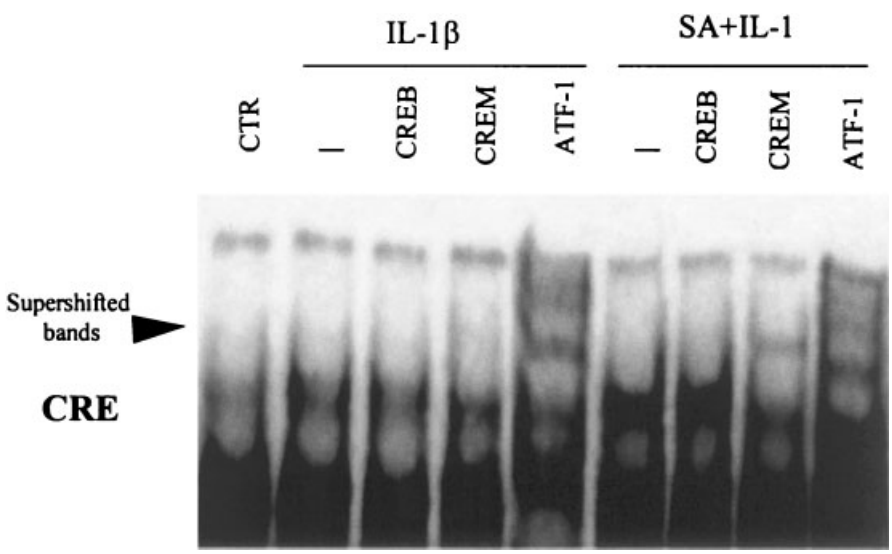

Fig. 5. Supershift analysis of EMSA for CRE binding proteins in Caco-2 cells treated with IL-1β or SA + IL-1β (SA + IL-1) as described in Materials and Methods and in the legend to Figure 1. The induction of supershift was tested by adding antibodies against CREB, cyclic AMP response element modulator (CREM)α, or ATF-1 to the EMSA reaction as indicated above the gels. Similar results were observed in three repeated experiments.

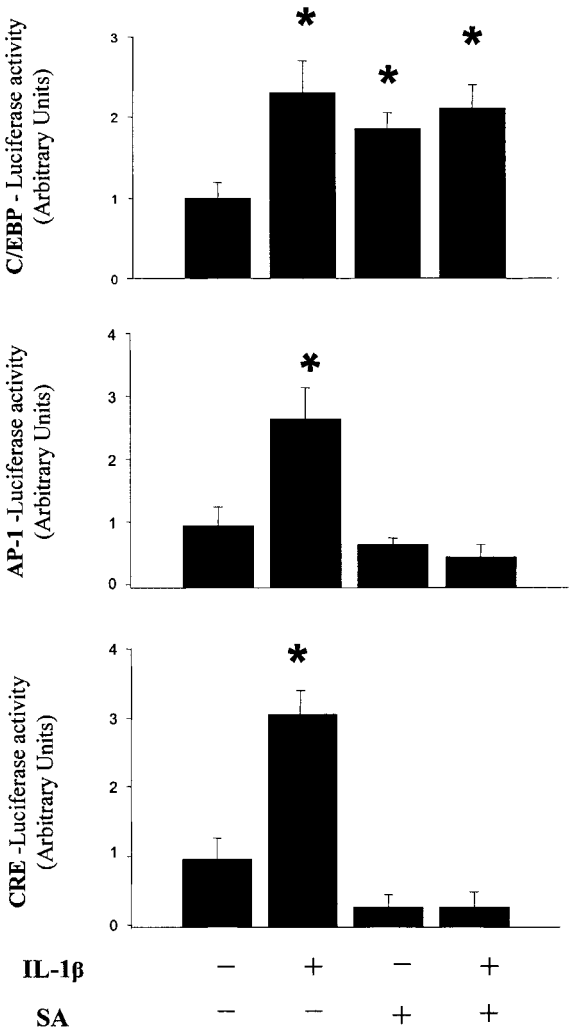

Fig. 6. The effect of IL-1β and SA on transcriptional activity of C/EBP (**upper panel**), AP-1 (**middle panel**), and CRE binding proteins (**lower panel**). Caco-2 cells were transfected with luciferase reporter plasmids containing promoter constructs of tandem repeats of C/EBP, AP-1, and CRE responsive elements as described in Materials and Methods and luciferase activity was measured after treatment for 8 h with 0.5 ng/ml of IL-1β, 500 μM of SA or SA + IL-1β. Results are mean ± SEM with n = 4–6 in each group. *$P < 0.05$ versus control (−IL-1β, −SA).

IL-1β and SA were similar to the changes in DNA binding activity of the same transcription factor determined by EMSA.

Inhibited AP-1 and CRE binding protein transcriptional activity in SA-treated enterocytes is a novel finding, and it was, therefore, important to explore potential mechanisms. Because the CRE binding protein CREB is activated by phosphorylation at the Ser 133 site [Gonzalez and Montminy, 1989], we first tested whether SA decreased the phosphorylation of CREB. The cellular levels of pCREB were determined by Western blot analysis and results from that experiment showed that SA increased, rather than decreased, the expression of pCREB (Fig. 7). Treatment of the cells with IL-1β also increased cellular levels of pCREB and when the Caco-2 cells were exposed to both IL-1β and SA, an additive effect was noticed. Nuclear levels of phosphorylated ATF-1 (pATF-1), another member of the family of CRE binding proteins, were also increased by SA (Fig. 7). Because these results suggest that inhibited transcriptional activity of CRE binding proteins after treatment with SA does not reflect reduced levels of pCREB or pATF-1, other potential mechanisms were tested.

Various isoforms of CREM constitute a group of proteins in the family of CRE DNA binding proteins that are transcriptional repressors [Foulkes et al., 1991]. In a recent study, overexpression of CREMα in keratinocytes inhibited CRE transcriptional activity [Rutberg et al., 1999]. The results from the supershift experiments described above suggested that CREMα DNA binding activity was increased by SA (see Fig. 5). We next tested whether increased CREMα expression may be a mechanism by which SA decreased transcriptional activity of CRE binding proteins under the present experimental conditions. Treatment of the Caco-2 cells with SA resulted in upregulated nuclear CREMα levels determined by Western blotting (Fig. 8A). In contrast, IL-1β did not influence the nuclear content of CREMα. No CREMα was detected in the cytoplasmic fraction of control cells or cells treated with IL-1β or SA.

In order to further test the role of CREMα in the arsenite-induced inhibition of CRE-mediated transcriptional activity, cells were co-transfected with a CREMα expression plasmid and a luciferase reporter plasmid containing multiple tandem repeats of CRE binding sites. The increase in CRE transcriptional activity induced by IL-1β was completely abolished in cells overexpressing CREMα, similar to the effect of treatment with SA (Fig. 8B). CREMα overexpression even reduced CRE-luciferase activity below control level, suggesting that CRE binding proteins regulate gene transcription also under basal conditions.

Taken together, the results shown in Figure 8A,B suggest that inhibited CRE-mediated transcriptional activity in Caco-2 cells treated with SA reflects increased levels of

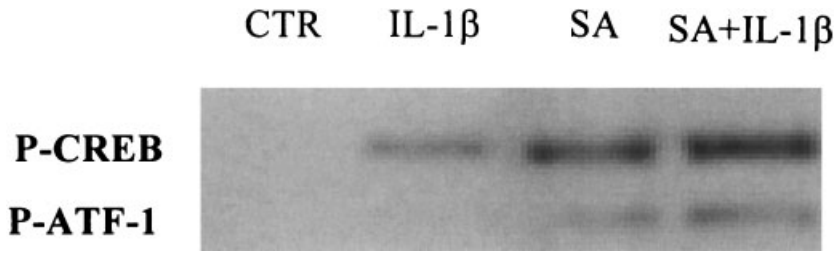

Fig. 7. Nuclear concentrations of phosphorylated CREB (pCREB) and pATF-1 determined by Western blotting in Caco-2 cells treated with IL-1β, SA, or SA + IL-1β as described in Figure 6. Almost identical results were observed in three repeated experiments.

CREMα. An additional way to test that concept would be to block CREMα in SA-treated cells. Recent studies in other cell types suggest that the naturally occurring CREB mutant KCREB can dimerize with CREMα and prevent the transcriptional repression caused by CREMα [Rutberg et al., 1999]. To test if a similar mechanism may be operational in Caco-2 cells, we co-transfected cells with a KCREB expression plasmid and a luciferase reporter/CRE promoter construct. When cells overexpressing KCREB were treated with IL-1β, the inhibitory effect of SA on transcriptional activity was blocked (Fig. 8C). This result is consistent with a model in which the transcriptional repressor CREMα is inactivated by KCREB. Interestingly, basal CRE-mediated transcriptional activity was increased in cells overexpressing KCREB (not shown) indicating that basal levels of CREMα may participate in the regulation of CRE-mediated transcriptional activity in Caco-2 cells. This was further supported by the present observation that KCREB did not only prevent the inhibitory effect of SA but increased CRE-luciferase activity above control level (Fig. 8C).

The DNA binding sites for AP-1 and CRE binding proteins have a high degree of homolgy and there is evidence in other cell types that members of the two families of transcription factors can overlap with regards to DNA binding [Rutberg et al., 1999]. This raises the possibility that the reduced AP-1 transcriptional activity noticed in arsenite-treated cells (see Fig. 6) may reflect increased CREMα levels. To test this hypothesis, we first examined whether AP-1 and CRE binding proteins overlap in their DNA binding in cultured Caco-2 cells. When EMSA was performed using a labeled AP-1 consensus oligonucleotide probe, the shifted band was competed out with an excess amount of unlabeled AP-1 oligonucletide (as expected) (Fig. 9).

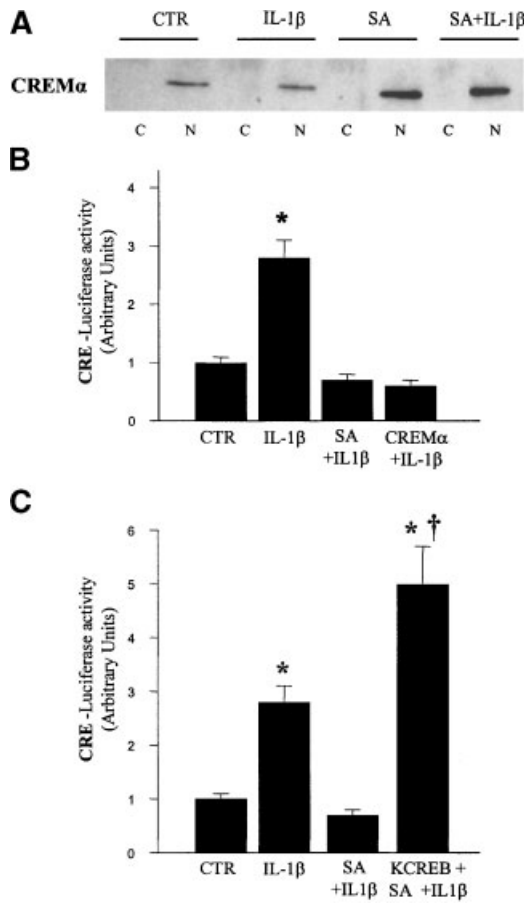

Fig. 8. **A**: Nuclear (N) and cytoplasmic (C) concentrations of CREMα determined by Western blotting in Caco-2 cells treated with IL-1β, SA, or SA + IL-1β as described in Figure 6. **B**: Luciferase activity in IL-1β-treated Caco-2 cells that had been transfected with empty vector (three left bars) or a CREMα expression plasmid (bar number four) and treated with IL-1β or SA + IL-1β (SA + IL-1β) as described in Figure 6. Results are mean ± SEM with n = 4–6 in each group. *$P < 0.05$ versus all other groups. **C**: Luciferase activity in Caco-2 cells treated with IL-1β, SA + IL-1β, or SA + IL-1β after transfection of the cells with a KCREB expression plasmid (bar number four). Cells in the other groups (three left bars) had been transfected with an empty vector. Results are mean ± SEM with n = 4–6 in each group. *$P < 0.05$ versus ctr; +, $P < 0.05$ versus IL-1β and versus SA + IL-1β.

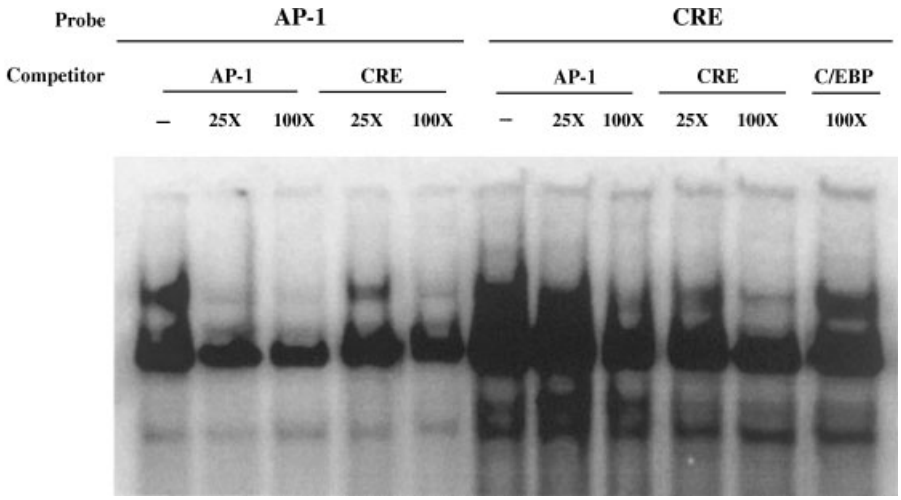

Fig. 9. EMSA for AP-1 and CRE binding proteins in SA-treated Caco-2 cells (500 μM for 1 h). Competition reactions were performed by adding increasing amounts (25 or 100 times excess) of unlabeled AP-1, CRE, or C/EBP probes (competitors) as indicated above the gels. Similar results were observed in repeated experiments.

This effect of unlabeled AP-1 probe was seen already at a 25-fold excess of the probe. A similar, but less pronounced, competition was achieved by adding an excess of unlabeled CRE oligonucleotide, suggesting that AP-1 can bind to CRE as well. A complete competition by the unlabeled CRE probe was seen when a 100-fold excess of the unlabeled CRE probe was added to the reaction. Similarly, when a labeled CRE consensus oligonucleotide probe was used for EMSA, the shifted band was competed out by an excess amount of unlabeled CRE or AP-1 oligonucleotide, supporting the concept that the two transcription factors overlap with regards to their DNA binding (Fig. 9). In contrast, a 100-fold excess of unlabeled C/EBP DNA oligonucleotide did not compete out the CRE EMSA, suggesting that CRE DNA binding proteins do not bind to C/EBP binding sites under the present experimental conditions and confirming the specificity of the competition reactions.

An overlap between AP-1 and CRE binding proteins with regards to their DNA binding sites supports the notion that reduced AP-1 transcriptional activity after treatment with SA may reflect increased levels of CREMα, similar to the reduced CRE-mediated transcriptional activity. To test this possibility, transcriptional activity was determined in Caco-2 cells that were co-transfected with a CREMα expression plasmid and a luciferase reporter plasmid containing a multiple tandem repeat AP-1 promoter construct. The increase in AP-1 transcriptional activity induced by IL-1β was abolished in cells overexpressing CREMα (Fig. 10), supporting the concept that CREMα inhibits AP-1 transcriptional activity in stimulated Caco-2 cells.

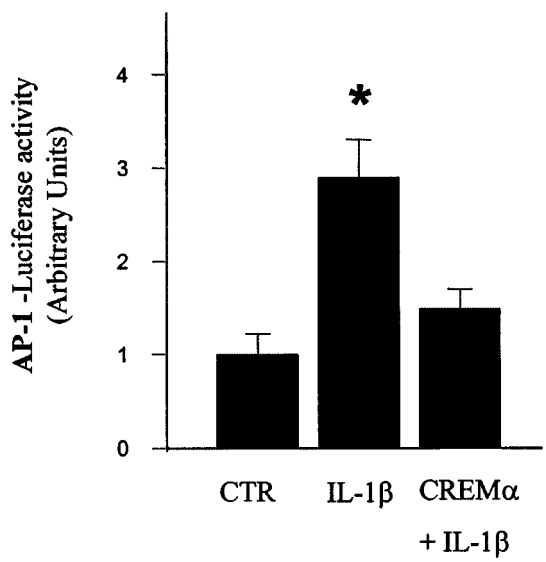

Fig. 10. The effect of CREMα overexpression on AP-1 transcriptional activity in Caco-2 cells treated with IL-1β. Cells were co-transfected with a CREMα expression plasmid and a luciferase reporter/AP-1 promoter plasmid and were then treated with IL-1β (0.5 ng/ml for 8 h) as described in Materials and Methods. Results are mean ± SEM with n = 5 or 6 in each group. *$P < 0.05$ versus other groups.

Taken together, the results reported here suggest that SA reduces the transcriptional activity of CRE binding proteins and AP-1 secondary to increased CREMα expression and that the effects of increased CREMα levels can be prevented by overexpressing KCREB. Because both CRE binding proteins and AP-1 regulate the IL-6 promoter, we hypothesized that overexpressing KCREB would reduce the arsenite-induced inhibition of IL-6 production reported previously [Hershko et al., 2002a]. To test this hypothesis, cells were transfected with a KCREB expression plasmid and then treated with SA and IL-1β followed by determination of IL-6 production. As reported previously [Hershko et al., 2002a], SA blocked the IL-1β-induced IL-6 production in cultured Caco-2 cells (Fig. 11). This inhibitory effect of SASA was blunted in cells transfected with the KCREB expression plasmid. It should be noted that theinhibitory effect of SA on IL-6 production was not completely prevented by KCREB overexpression, indicating that additional mechanisms, other than increased CREMα levels, contribute to the arsenite-induced inhibition of IL-6 production.

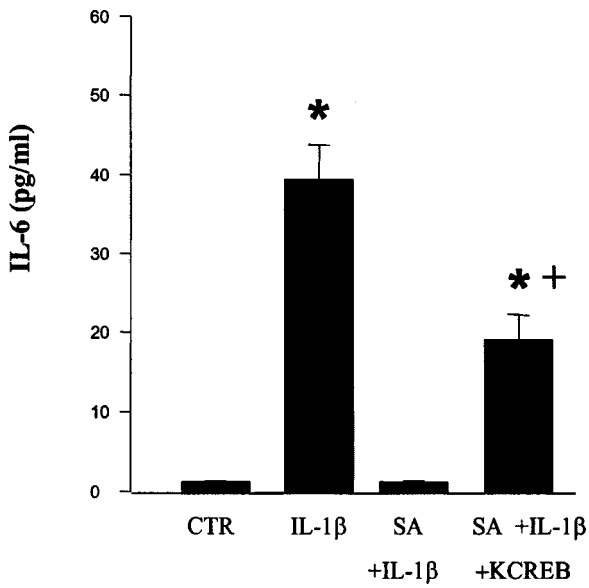

Fig. 11. IL-6 production in cultured Caco-2 cells treated with IL-1β, SA + IL-1β, or SA + IL-1β after transfection of the cells with a KCREB expression plasmid. Cells in the three left bars were transfected with an empty vector. Cells were treated for 2 h with 0.5 ng/ml of IL-1β. Some cells were pretreated for 1 h with 500 μM SA. IL-6 levels were measured by ELISA in the medium at the end of incubation. Results are mean ± SEM with n = 4–6 in each group. *$P < 0.05$ versus ctr; +, $P < 0.05$ versus SA + IL-1β.

DISCUSSION

In a recent study from this laboratory, treatment of cultured Caco-2 cells with SA blocked the IL-1β-induced increase in IL-6 production and this effect of SA was at least in part caused by inhibited NF-κB activity [Hershko et al., 2002a]. The present experiments were undertaken to examine the influence of SA on other transcription factors that regulate the *IL-6* gene, namely, C/EBP, CRE binding proteins, and AP-1. We found that treatment of IL-1β-stimulated Caco-2 cells with SA decreased transcriptional activity of AP-1 and CRE DNA binding proteins and that this effect of SA was associated with, and possibly caused by, increased expression and activity of the transcriptional repressor CREMα. In contrast, SA did not influence C/EBP transcriptional activity in IL-1β-treated cells and actually increased basal C/EBP transcriptional activity. The present results, taken together with recent reports from our laboratory [Hershko et al., 2002a,b], suggest that different IL-6-related transcription factors are differentially regulated by SA and that reduced IL-6 production in Caco-2 cells treated with SA may at least in part be caused by inhibited activity of NF-κB, AP-1, and CRE binding proteins, but not C/EBP.

An interesting observation in the present study was that changes in DNA binding activity determined by EMSA for AP-1 and CRE binding proteins were not reflected by similar changes in transcriptional activity determined by activation of luciferase reporter plasmids containing promoter constructs with transcription factor responsive elements. Similar discrepancies have been reported in other studies as well. For example, when cultured keratinocytes were treated with increasing calcium concentrations, a strong upregulation of DNA binding activity of CRE binding proteins determined by EMSA was noticed after 48 h, whereas, at the same time point, CRE-mediated transcriptional activity was not altered [Rutberg et al., 1999]. These differences most likely reflect the different natures of EMSA and the cell transfection experiments. Whereas EMSA reflects in vitro binding of transcription factor(s) to a segment of transcription factor responsive element, the experiments in which cells are transfected with plasmids containing various promoter and reporter constructs provide information about

the function of the transcription factors under in vivo (intracellular) conditions. From a biological standpoint, changes in transcriptional activity may be more informative than changes in DNA binding by EMSA. This is particularly true if a family of transcription factors (such as the family of CRE binding proteins) is studied that contain both stimulatory and repressive transcription factors.

To the best of our knowledge, the present report is the first to describe increased CREMα expression and inhibited transcriptional activity of AP-1 and CRE DNA binding proteins after treatment of cultured enterocytes with SA. In a recent study, a similar association between CREMα levels and inhibited CRE and AP-1 transcriptional activity as found here was observed in cultured keratinocytes after treatment of the cells with increasing calcium concentrations [Rutberg et al., 1999]. In that study as well, overexpressing KCREB resulted in increased CRE transcriptional activity, consistent with the concept that KCREB can dimerize with and prevent the inhibitory effect of CREMα.

Evidence for cross talk between AP-1 and CRE DNA binding proteins with regards to their DNA binding sites, as reported here, was observed in other studies as well [Rutberg et al., 1999]. The finding in the present report that CRE DNA binding proteins did not seem to bind to C/EBP binding sites differs from a recent study in cultured rat pheochromocytoma P12 cells [Fawcett et al., 1999]. In those experiments, evidence was found for interactions between C/EBP family members and various components of the CREB family of transcription factors in the regulation of Gadd153 (also known as CHOP) in response to sodium aresnite treatment. The reason for these apparently contradictory results is not known at present, but may be differences in cell types being studied.

Although it is well established that the IL-6 gene promoter has a C/EBP responsive element and that C/EBP regulates IL-6 gene transcription [Vanden Berghe et al., 2000], the present results suggest that C/EBP is not involved in the downregulation of IL-6 after treatment with SA. This observation is consistent with previous reports in which evidence was found that the recruitment of different transcription factors regulating the *IL-6* gene may vary depending on stimulus and cell type. For example, in murine fibrosarcoma cells and embryonic kidney cells, TNF-induced IL-6 production was mainly regulated by NF-κB whereas staurosporine-induced IL-6 production was regulated by AP-1, CREB, and C/EBP with no involvement of NF-κB [Vanden Berghe et al., 1999]. The reason for the differential regulation of IL-6-related transcription factors after different stimuli, including SA, is not well understood but it is possible that activation of different signaling pathways by different stimuli plays a role.

The present finding of increased AP-1 DNA binding activity after treatment with SA is similar to a recent report by Cavigelli et al. [1996] in which cultured HeLa S3 cells were treated with 50 μM arsenite. In that study, however, the increased AP-1 DNA binding activity (determined by EMSA) was associated with increased transcriptional activity determined by using a luciferase reporter plasmid containing a promoter construct with an AP-1 responsive element. The reason for these apparently contradictory results (reduced AP-1 transcriptional activity after arsenite treatment in the present study and increased AP-1 transcriptional activity in the study by Cavigelli et al.) is unclear but may be the different cell types being used in the two studies. It should be noted that arsenite did not influence NF-κB DNA binding activity in the HeLa S3 cells [Cavigelli et al., 1996] which is different from the inhibitory effects of arsenite on NF-κB activity in a large number of cell types reported previously [Kapahi et al., 2000; Yoo et al., 2000; Hershko et al., 2002a,b]. Thus, it is possible that the regulation of transcription factors is different in the HeLa S3 cells used by Cavigelli et al. [1996] than in many other cell types.

The present finding that overexpression of KCREB did not completely restore IL-6 production in arsenite-treated cells (see Fig. 11) suggests that some, and perhaps even the majority, of the arsenite-induced inhibition of IL-6 production was caused by a mechanism that is independent of increased CREMα levels. Based on results in a recent report from this laboratory [Hershko et al., 2002a], we propose that this mechanism may be arsenite-induced inhibition of NF-κB activity.

Although the present report provides novel information about the regulation by SA of transcription factors that regulate the *IL-6* gene, several limitations of the study need to be taken into account when the results are

interpreted. First, the experiments were performed in vitro in cultured enterocytes, and it will be important in future studies to determine whether SA regulates the transcription factors examined here in vivo as well. Second, the Caco-2 cells originate from a colon adenocarcinoma cell line and additional experiments need to be performed to examine whether the regulatory mechanisms observed here are specific for the Caco-2 cells. Finally, the cells were studied when they were 90% confluent, rather than fully differentiated. In a recent study, we found that the regulation of IL-6 production was similar in 90% confluent Caco-2 cells and in Caco-2 cells that had been cultured in bicameral transwell chambers for 3 weeks to full differentiation [Moon et al., 2000], suggesting that the use of 90% confluent Caco-2 cells is valid when mechanisms regulating the *IL-6* gene are studied.

Despite several limitations, the present study is important because it provides novel information about arsenite-induced changes in the activity of transcription factors that participate in the regulation of the *IL-6* gene. Considering the multiple important biological effects of IL-6, both locally in gut mucosa and systemically [Beagley et al., 1989; Molmenti et al., 1993; Papanicolaou et al., 1998], and recent reports of protective effects of SA [Tsuruma et al., 1999; Hauser et al., 2001] and the use of SA in the treatment of patients with certain cancers [Waxman and Anderson, 2001], an increased understanding of the transcriptional regulation of IL-6 by SA may have important clinical implications.

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