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Arsenite Stabilizes IκBα and Prevents NF-κB Activation in IL-1 β-Stimulated Caco-2 Cells Independent of the Heat Shock Response

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Abstract Recent studies suggest that sodium arsenite downregulates NF-κB activity by inhibiting phosphorylation and subsequent degradation of IκBα. Many effects of sodium arsenite are secondary to induction of heat shock proteins. The role of the heat shock response in arsenite-induced inhibition of NF-κB, however, is not known. We examined the involvement of the heat shock response in arsenite-induced inhibition of NF-κB activity in IL-1β-stimulated Caco-2 cells, a human colorectal adenocarcinoma cell line with enterocytic properties. Treatment of the cells with IL-1β resulted in increased IκB kinase activity, reduced levels of IκBα and increased NF-κB DNA binding activity. Sodium arsenite blocked all of these responses to IL-1β without inducing changes in heat shock factor activity or heat shock protein levels. Results from additional experiments showed that the protective effect of sodium arsenite on IκBα was not influenced by the oxygen radical scavenger catalase or by inhibitors of the MAP-kinase signaling pathway. The present results suggest that sodium arsenite stabilizes IκBα and prevents NF-κB activation in IL-1β-stimulated Caco-2 cells independent of the heat shock response. In addition, stabilization of IκBα by sodium arsenite does not require oxygen radical formation or activation of the MAP kinase signaling pathway. J. Cell. Biochem. 84: 687–698, 2002.

Key words: intestine; inflammation; cytokines; stress response

The intestinal mucosa and enterocyte are important participants in the inflammatory response to endotoxemia, sepsis, and severe injury [Deitch, 1992]. There is evidence that metabolic and inflammatory changes in gut mucosa may influence the outcome in patients with critical illness by increasing mucosal permeability and bacterial translocation resulting in increased risk for multiple organ failure [Wang et al., 1998].

One of the molecular hallmarks of inflammation is activation of the transcription factor nuclear factor kappa B (NF-κB) [Baldwin, 1996; Barnes and Karin, 1997; Ghosh et al., 1998; Karin, 1998]. The most common form of NF-κB is the p50/p65 heterodimer that is typically sequestered in the cytoplasm as an inactive complex with a member of the inhibitory IκB family, most commonly inhibitory κBα (IκBα). NF-κB is rapidly activated by a number of stimuli, including endotoxin and proinflammatory cytokines such as IL-1β and TNF-α. NF-κB, in turn, regulates many genes involved in the inflammatory response, including genes for acute phase proteins and various cytokines. Inflammatory stimuli activate NF-κB by first activating IκB kinase (IKK), which consists of homo- and heterodimers of IKK α and IKK β [Woronicz et al., 1997; Karin, 1999]. Among these, there is evidence that IKK β plays the most important role in NF-κB activation during

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inflammation [Rothwarf et al., 1998; Li et al., 1999]. Activation of IKK results in phosphorylation of IκBα, subsequently leading to ubiquitination and degradation by the proteasome of the inhibitory protein. This process liberates NF-κB allowing it to translocate to the nucleus where it binds to its cognate site and transactivates the downstream genes.

In recent studies in our laboratory, NF-κB was activated in intestinal mucosa of endotoxemic mice [Pritts et al., 1998]. Local inflammation as well has been reported to be associated with mucosal NF-κB activation [Rogler et al., 1998]. In other studies from our and other laboratories, stimulation in vitro of cultured human enterocytes with IL-1β or TNF-α upregulated NF-κB DNA binding activity and increased the production of IL-6 and IL-8 at the transcriptional level [Parikh et al., 1997; Jobin et al., 1999a].

Because of the central role of NF-κB in the inflammatory response, much research has focused on attempts to downregulate NF-κB activity. Several different strategies have been described by which IκBα degradation and NF-κB activation were inhibited, for example treatment with IL-10 [Wang et al., 1995; Schottelius et al., 1999], glucocorticoids [Scheinman et al., 1995], curcumin [Jobin et al., 1999b], aspirin [Yin et al., 1998], or induction of the heat shock (stress) response [Shanley et al., 2000]. In recent studies, treatment of cytokine-stimulated cells with arsenite prevented the degradation of IκBα and inhibited the activation of NF-κB [Kapahi et al., 2000; Roussel and Barchowsky, 2000; Yoo et al., 2000]. In experiments in our laboratory, treatment of mice in vivo with sodium arsenite blocked the activation of NF-κB in intestinal mucosa during endotoxemia [Pritts et al., 2000]. Studies in cultured human respiratory epithelial cells [Yoo et al., 2000], embryonic kidney cells [Roussel and Barchowsky, 2000], and HeLa cells [Roussel and Barchowsky, 2000] suggest that inhibition of IKK activity is the principle mechanism by which arsenite protects IκBα and prevents NF-κB activation.

Arsenite is capable of activating a wide range of cellular responses, including production of oxygen radicals and stimulation of the mitogen-activated protein (MAP) kinase signaling pathway [Bernstam and Nriagu, 2000]. One of the best known consequences of arsenite treatment is induction of the heat shock response [Ribeiro et al., 1994; Wong and Wispe, 1997], and in many studies, the effect of arsenite was mimicked by hyperthermia-induced stress response leading to the assumption that the effects of arsenite were secondary to the induction of heat shock proteins. The role of the heat shock response in arsenite-induced inhibition of NF-κB, however, is not fully understood. In previous reports, inhibition of NF-κB activity after treatment of mice in vivo [Pritts et al., 2000] or of cells in vitro [Yoo et al., 2000] with sodium arsenite was associated with increased expression of heat shock proteins but a cause–effect relationship between the increased levels of heat shock proteins and inhibited NF-κB activity was not established. In a recent report in which arsenite blocked NF-κB activation in TNF-α-stimulated HeLa cells by binding to cystein-179 in the IKK β activating loop, heat shock protein levels were not determined and the potential role of the heat shock response was not discussed [Kapahi et al., 2000].

In the present study, we tested the potential role of the heat shock response in arsenite-induced inhibition of NF-κB activity in IL-1β-stimulated Caco-2 cells, a human intestinal epithelial cell line [Rousset, 1986; Pinto et al., 1994]. In addition, the involvement of oxygen radicals and the MAP kinase signaling pathway in sodium arsenite-induced protection of IκBα was examined. Our results suggest that sodium arsenite can prevent NF-κB activation independent of the heat shock response and that this effect of sodium arsenite does not require production of oxygen radicals or activation of the MAP kinase signaling pathway.

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-BRL (Grand Island, NY). Human recombinant IL-1β was purchased from Endogen (Woburn, MA). Rabbit polyclonal anti-IκBα, anti-heatshock protein (HSP) 90 and anti-HSP 70 antibodies, goat anti-rabbit secondary antibodies, and the enhanced chemiluminescence kit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal

anti-phospho-IκBα antibody was from New England Biolabs (Beverly, MA). The proteasome inhibitor MG-132 [Lee and Goldberg, 1998] and all other chemicals, unless otherwise stated, were from Sigma (St. Louis, MO).

Cell Culture

Caco-2 cells, a human colon adenocarcinoma cell line that displays enterocyte-like features in culture [Rousset, 1986; Pinto et al., 1994] were grown at 37°C in 5% CO_2 in DMEM supplemented with 10% FBS, nonessential amino acids, 6 mM glutamine, 10 mM Hepes, 10 mg/ml apotransferrin, 1 mM pyruvate, 24 mM $NaHCO_3$, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells, between passages 5 and 25, were seeded at a density of 100,000 cells/cm^2 onto 10 cm^2 tissue culture plates and were grown for 72 h to 90% confluence before use.

Experimental Conditions

Prior to experiments, cells were washed three times with serum-free DMEM and then pretreated at 37°C with serum-free medium containing sodium arsenite (500 μM) for various periods of time up to 60 min. After exposure to sodium arsenite, the cells were allowed to recover for 1 h in sodium arsenite-free medium, whereafter, IL-1β (0.5 ng/ml) was added to the culture medium for 30 min. Treatment of cultured Caco-2 cells with this concentration of IL-1β resulted in rapid IκBα degradation and NF-κB activation in recent studies from our laboratory [Parikh et al., 1997, 2000; Moon et al., 1999]. In other experiments, cells were preincubated with cycloheximide (100 μg/ml), quercetin (100 μM), or the HSP 90 inhibitor geldanamycin (300 nM) for 1 h before arsenite was added. To test the involvement of oxygen radicals in sodium arsenite-induced protection of IκBα, cells were incubated in the presence of the oxygen radical scavenger catalase (100 μg/ml). To test the potential role of the MAP kinase signaling pathway, cells were treated with 30 μM PD98059 (for ERK) or 30 μM SB203580 (for p38) as described previously [Elbirt et al., 1998]. This experiment was based on a recent report in which treatment of cultured LMH cells (a chicken hepatoma cell line) with sodium arsenite resulted in a rapid (within 10–20 min) activation of ERK and p38 [Elbirt et al., 1998]. All experiments were performed at least three times to ensure reproducibility.

Preparation of Cytoplasmic and Nuclear Extracts

Nuclear and cytosplasmic fractions were prepared as previously described [Parikh et al., 2000]. All steps were carried out on ice. 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 KCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1.5 mM $MgCl_2$, 0.2% (v/v) Nonidet P-40, 1 mM 1,4-dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (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 3,800g for 5 min. The supernatant was removed and saved as the cytoplasmic fraction. The pellet was resuspended in 1 cell volume of extract 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. Protein concentrations of nuclear and cytoplasmic extracts were determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a standard.

Western Blot Analysis

Aliquots of the cytoplasmic fractions containing 25 μg of protein were boiled in equal amounts of loading buffer (125 mM Tris-HCl, 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, San Diego, CA) was included as a molecular weight marker. The proteins were transferred

to nitrocellulose membranes (Xcell II Blot Module; Novex), which were blocked with 5% non-fat dried milk in Tris-buffered saline, pH 7.6 (TBS) containing 0.05% Tween-20 (TTBS) for 1 h. The membranes were incubated with rabbit polyclonal anti-IκBα, anti-HSP 70, or anti-HSP 90 for 1 h and then washed three times with TTBS before incubation with peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 45 min. The blots were washed three times in TTBS for 5 min, incubated in enhanced chemiluminescence reagents, and exposed on radiographic film (Eastman-Kodak, Rochester, NY).

Electrophoretic Mobility Shift Assays (EMSA)

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. NF-κB gel shift oligonucleotide 5′ AGT TGA GGG GAC TTT CCC AGG C 3′ was purchased from Santa Cruz Laboratories (Santa Cruz, CA). Oligonucleotides corresponding to a known heat shock response element 5′ GCC TCG AAT GTT CGC GAA GTT TCG 3′ were synthesized by the University of Cincinnati DNA Core Facility. 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% polyacrylamide 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).

IKK Activity

IKK activity was determined by measuring the phosphorylation of the fusion protein glutathione S-transferase-IκBα (gst-IκBα) by immunoprecipitated IKK as described by Kapahi et al. [2000]. Whole cell extracts were prepared by lysis in 150 μl lysis buffer consisting of 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1% Triton X-100, 0.1% Nonidet P-40, 10% glycerol with protease and phosphatase inhibitors (1 mM PMSF, 0.1 mM Na_3V0_4, 2 mM *p*-nitrophenyl phosphate (PNPP), 30 μl/ml aprotinin) for 30 min at 4°C. Samples were centrifuged for 10 min at 10,000*g* at 4°C. Supernatants were collected and protein concentration was determined as described above. Protein (300 μg) from each sample was incubated for 1 h at 4°C with anti-IKKγ antibody (Santa Cruz Biotechnology). Twenty-five microliters of protein agarose beads (Santa Cruz Biotechnology) were added and the incubation continued for an additional 2 h. Beads were then pelleted for 2 min at 1,100*g* at 4°C and washed three times with wash buffer (20 mM Tris, pH 8.0, 3 mM EGTA, 3 mM EDTA, 250 mM NaCl, 0.05% Nonidet P-40 with protease and phosphatase inhibitors (1 μl/ml Sigma Protease Inhibitor Mix, 0.5 mM 4-(2-aminoethyl)-benzene sulfonyl fluoride, 0.1 mM Na_3VO_4, 2 mM PNPP, 20 mM β-glycerophosphate)). Beads were finally washed with kinase buffer (20 mM HEPES, pH 7.7, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT, 0.1 mM Na_3VO_4, 2 mM PNPP, 20 mM β-glycerophosphate). Following washings, IKK activity was assayed for 30 min at 30°C in kinase buffer with 6 μg gst-IκBα (kindly provided by Dr. Hector Wong, Children's Hospital Medical Center, Critical Care Division, Cincinnati, Ohio) and (γ-^{32}P) ATP. Reactions were stopped on ice and the samples were prepared for electrophoresis with the addition of 2× Laemmli buffer and boiling for 4 min. After electrophoresis on 8–16% Tris-glycine gradient gels (Invitrogen), gels were dried and exposed to PhosphorImager screens and radiographic film.

Cell Transfection and Luciferase Assay

An HSP-70 promoter-luciferase reporter plasmid was kindly provided by Dr. Hector Wong. The reporter plasmid contained the HSP 70 promoter upstream from a firefly luciferase gene in a pGL2 vector (Promega, Madison, WI). Caco-2 cells (100,000 cell/cm^2) were seeded onto six-well culture dishes, and grown to 50% confluence prior to transfection. The Lipofectin (GIBCO, Grand Island, NY) transfection method was used. Briefly, the DNA was diluted to a concentration of 1 μg/well. LipofectAmine Plus was incubated with serum-free OptiMEM and 1 ng/well of plasmid at room temperature for 15 min. The Caco-2 cells were

washed three times with serum free medium, and the 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. Cells were exposed to arsenite (500 μM) for 1 h followed by 1 or 7 h recovery in serum free medium without sodium arsenite. After recovery, cells were washed twice with PBS. Next, 250 μl of lysis buffer (Stratagene, La Jolla, CA) was added to each well for 15 min, after which the cells were gently scraped into 1.5 ml Eppendorf tubes and stored at −70°C. For measurement of luciferase activity, the samples were thawed and centrifuged at 14,000*g* for 2 min. Thirty microliters of supernatant were then combined with 100 μl of Luciferase substrate in Sarstedt 12 × 75 mm tubes in duplicate and read for 10 s on a Berthold AutoLumat LB953 luminometer (PerkinElmer, Gaithersburg, Maryland).

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 [Parikh et al., 2000]. Cell viability was not influenced by any of the experimental conditions in the present study (data not shown).

RESULTS

When Caco-2 cells were treated with 0.5 ng/ml of IL-1β for 30 min, cytoplasmic levels of IκBα were reduced and NF-κB DNA binding activity was upregulated (Fig. 1A), in line with previous reports from our laboratory [Parikh et al., 1997, 2000]. These effects of IL-1β were abolished in cells treated with sodium arsenite.

Because IκBα degradation is preceded by phosphorylation, we next examined the effects of IL-1β and sodium arsenite on cellular levels of phosphorylated IκBα. In order to enhance the possibility to detect phosphorylated IκBα, some cells were treated with 50 μM of the proteasome inhibitor MG-132 [Lee and Goldberg, 1998]. When cells were treated with both IL-1β and MG-132, a slower migrating band appeared on the Western blot when the anti-IκBα antibody was used (Fig. 1B, lane 4, upper panel). This extra band most likely represented phosphorylated IκBα, an interpretation that was confirmed when the specific anti-phospho-IκBα antibody was used (Fig. 1B, lane 4, lower panel). Increased levels of phosphorylated IκBα in MG-132 treated cells are consistent with accumulation of phosphorylated IκBα "proximal" to the

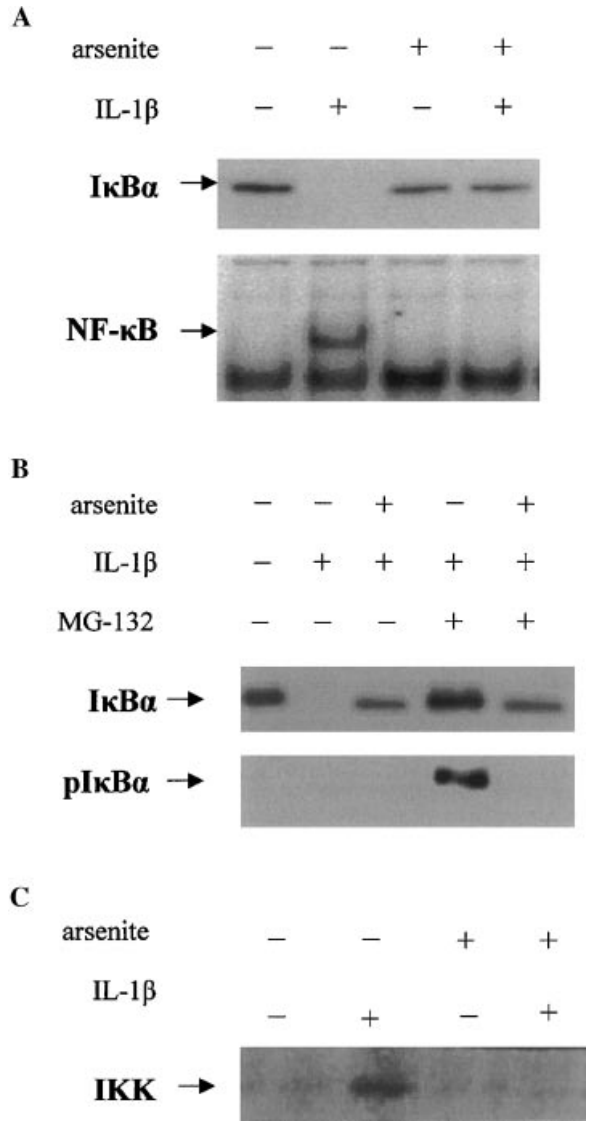

Fig. 1. Sodium arsenite blocks the effects of IL-1β on IKK activity, phosphorylated IκBα and IκBα levels and NF-κB activity. **A**: Cultured Caco-2 cells were treated with sodium arsenite (500 μM) for 1 h, were allowed to recover for 1 h in arsenite-free medium, and were then treated for 30 min with 0.5 ng/ml of human recombinant IL-1β. IκBα was determined by Western blotting and NF-κB DNA binding activity by EMSA. **B**: Cells were treated with sodium arsenite and IL-1β as described above. In addition, some cells were treated with the proteasome inhibitor MG-132 (50 μM) for 1 h before addition of sodium arsenite or IL-1β. IκBα and phosphorylated IκBα (pIκBα) were determined by Western blotting. **C**: IKK activity in Caco-2 cells treated with sodium arsenite and IL-1β as described above.

inhibited proteasome. The accumulation of phosphorylated IκBα in cells treated with MG-132 and IL-1β was prevented by sodium arsenite, confirming recent reports of arsenite-induced stabilization of IκBα at the level of phosphorylation [Kapahi et al., 2000]. Further support for this concept was found when IKK activity was determined. Thus, treatment of the Caco-2 cells with IL-1β upregulated IKK activity. This effect of IL-1β was abolished by sodium arsenite (Fig. 1C).

Because in other cell types, arsenite-induced downregulation of NF-κB was associated with increased levels of heat shock proteins, in particular HSP 70 [Yoo et al., 2000], we next examined whether treatment of Caco-2 cells with sodium arsenite resulted in upregulated HSP 70 levels. Surprisingly, the same treatment that resulted in protection of IκBα and downregulation of NF-κB activity (i.e., treatment with sodium arsenite for 1 h followed by recovery for 1 h) did not result in increased HSP 70 levels (Fig. 2A). This result suggests that sodium arsenite can prevent IκBα degradation and NF-κB activation in the absence of increased HSP 70 levels. To test whether the Caco-2 cells were able to increase HSP 70 levels after treatment with sodium arsenite, protein levels were measured 4 and 24 h after the 1 h period of sodium arsenite treatment. At both these time points, HSP 70 levels were increased (Fig. 2A) suggesting that the lack of increase 1 h after treatment did not reflect an inherent inability of the cells to produce heat shock proteins. Interestingly, treatment with IL-1β of cells that had recovered for 4 or 24 h after sodium arsenite resulted in reduced IκBα levels despite the increased HSP 70 levels (Fig. 2A). This result further supports the concept that there is no absolute correlation between sodium arsenite-induced protection of IκBα and HSP 70 levels.

In order to further test the potential role of HSP 70 in the effects caused by sodium arsenite, cells were treated with 100 μM quercetin. This substance is a flavonoid compound that was shown in previous studies to suppress the heat shock response secondary to downregulation of the transcription factor heat shock factor (HSF) [Nagai et al., 1995]. In this experiment, cells were allowed to recover 4 h after the arsenite treatment to allow for the induction of HSP 70. When cells were treated with quercetin, the induction of HSP 70 was blocked (Fig. 2B, compare lanes 3 and 6), but the protective effect of sodium arsenite on IκBα levels and the inhibition of NF-κB activation persisted (Fig. 2B, lanes 5 and 6). Quercetin alone did not influence IκBα levels or NF-κB DNA binding (Fig. 2B, lanes 1 and 4), but slightly reduced IL-1β-induced NF-κB DNA binding activity (lanes 2 and 5). Taken together, the results shown in Fig. 2B lend additional support to the notion that sodium arsenite prevents NF-κB activation independent of HSP 70.

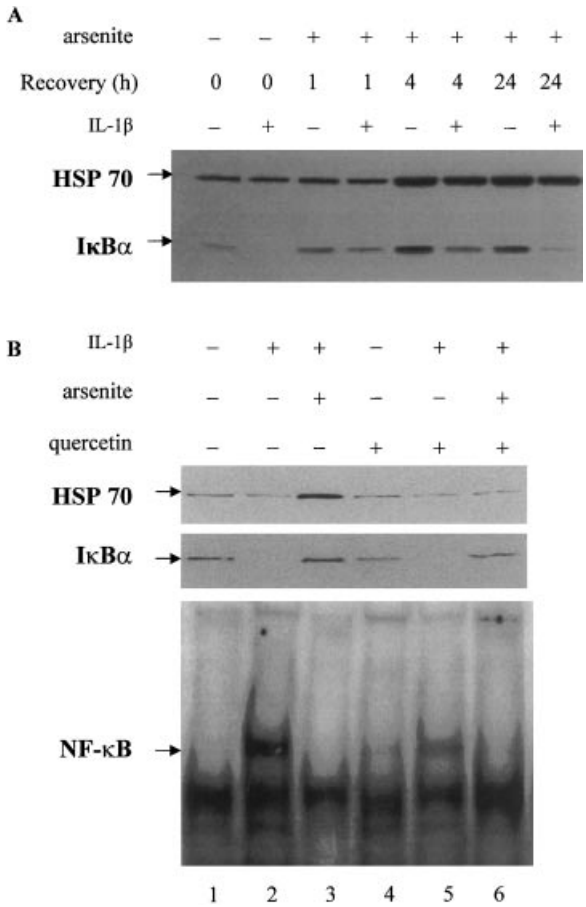

Fig. 2. Sodium arsenite blocks the effects of IL-1β on IκBα levels independent of HSP 70 levels. **A**: Cultured Caco-2 cells were treated with 500 μM sodium arsenite for 1 h, were allowed to recover for 1, 4, or 24 h in arsenite-free medium, and were then treated for 30 min with 0.5 ng/ml of human recombinant IL-1β. HSP 70 and IκBα levels were determined by Western blotting. **B**: Cultured Caco-2 cells were treated with 100 μM quercetin for 1 h before 500 μM sodium arsenite was added to the cells for 1 h. After recovery for 4 h in sodium arsenite-free medium, cells were treated with 0.5 ng/ml recombinant IL-1β for 30 min, whereafter, IκBα levels and NF-κB DNA binding activity were measured.

Although HSP 70 is the most extensively studied inducible heat shock protein, the stress response results in increased production of a

number of heat shock proteins, including HSP 90 [Roychowdhury et al., 1992]. Treatment of the Caco-2 cells with sodium arsenite for 1 h followed by recovery in arsenite-free medium for 1 h did not result in increased cytoplasmic HSP 90 levels (Fig. 3A), suggesting that the inhibited IκBα degradation and NF-κB activation did not reflect high HSP 90 levels. This was further supported by experiments in which cells were treated with the HSP 90 antagonist geldanamycin [Vasilevskaya and O'Dwyer, 1999]. Thus, sodium arsenite prevented the degradation of IκBα in IL-1β-treated cells in the presence of 300 nM geldanamycin (Fig. 3B).

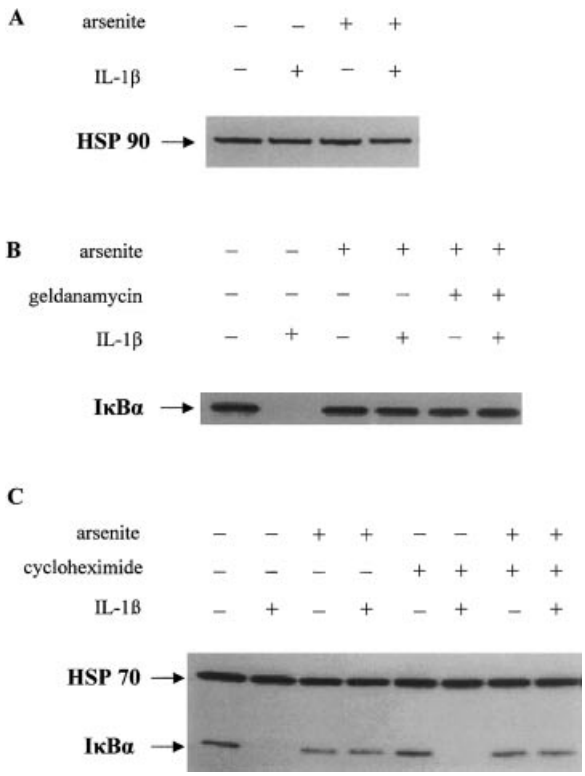

Fig. 3. The stabilization of IκBα by sodium arsenite does not require increased HSP 90 levels or ongoing protein synthesis. **A**: Cultured Caco-2 cells were treated with 500 μM sodium arsenite for 1 h, were allowed to recover for 1 h in arsenite-free medium, and were then treated for 30 min with 0.5 ng/ml of human recombinant IL-1β. HSP 90 levels were determined by Western blotting. **B**: Caco-2 cells were treated as described above except that some cells were exposed to 300 nM of the HSP 90 inhibitor geldanamycin. IκBα levels were determined by Western blotting. **C**: Cultured Caco-2 cells were treated with 100 μg/ml cycloheximide for 1 h before treatment with 500 μM sodium arsenite for 1 h. Cells were then allowed to recover for 1 h in arsenite-cycloheximide-free medium and were then treated for 30 min with 0.5 ng/ml of human recombinant IL-1β. HSP 70 and IκBα levels were determined by Western blotting.

An additional way to examine whether the effect of sodium arsenite on IκBα and NF-κB requires the induction of heat shock protein(s) is to block protein synthesis. When cells were treated with the protein synthesis inhibitor cycloheximide, the protective effect of sodium arsenite on IκBα in IL-1β-treated cells was sustained, indicating that de novo synthesis of heat shock proteins (or any other proteins) is not needed for sodium arsenite to exert its protective effect on IκBα (Fig. 3C).

The production of heat shock proteins is regulated by the transcription factor HSF [Wong and Wispe, 1997]. To further examine the association between sodium arsenite-induced protection of IκBα and the heat shock response, we next compared the effects of sodium arsenite on induction of HSF and NF-κB DNA binding activities and IκBα levels. A protective effect on IκBα levels and inhibition of NF-κB activity were noticed already after treatment of the cells with sodium arsenite for 5 min, whereas, activation of HSF occurred at a later time point (Fig. 4A). Note that in this experiment, cells were treated with 500 μM sodium arsenite for 5 min or 60 min, whereafter, cells were washed and immediately treated with 0.5 ng/ml IL-1β in sodium arsenite-free medium for 30 min.

Because DNA binding activity measured by EMSA does not always reflect gene activation, we next transfected Caco-2 cells with an HSP 70 promoter-luciferase reporter plasmid. When these cells were treated with sodium arsenite for 1 h followed by recovery in sodium arsenite-free medium for 1 h (the same experimental conditions that resulted in stabilization of IκBα in IL-1β-treated Caco-2 cells; see Fig. 1), no increase in luciferase activity was seen, whereas, in cells that recovered for 7 h after sodium arsenite, a 25-fold increase in luciferase activity was noted (Fig. 4B). Taken together, these results indicate that the protective effect of sodium arsenite on IκB-α occurs before HSF is activated, further dissociating the effects of sodium arsenite on IκBα levels and NF-κB activation from the induction of the heat shock response.

In addition to inducing the heat shock response, sodium arsenite activates other cellular responses as well, including production of oxygen radicals and activation of the MAP kinase signaling pathway [Bernstam and Nriagu, 2000], and there is evidence that

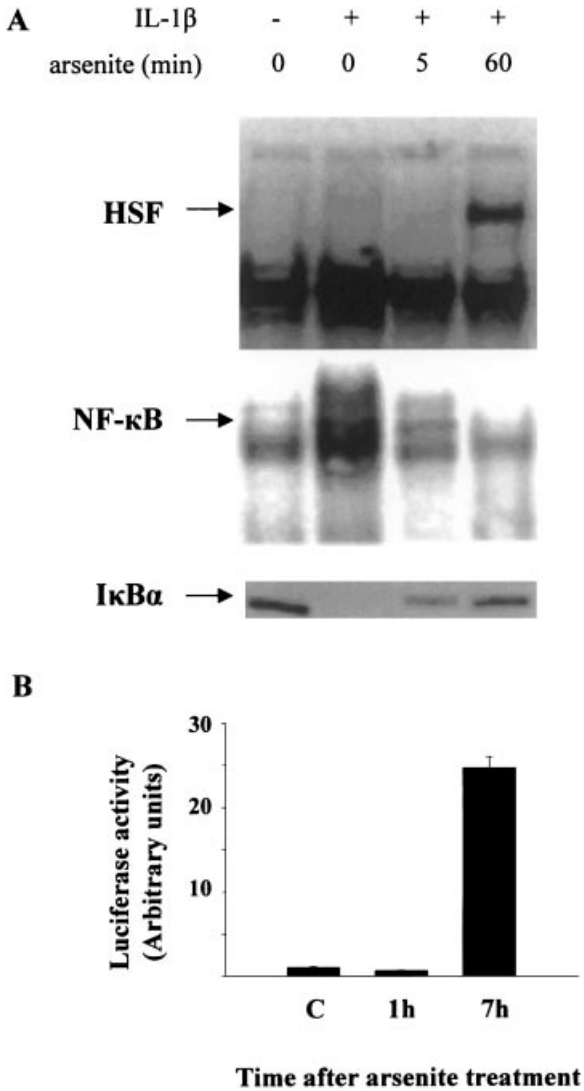

Fig. 4. Sodium arsenite blocks the effects of IL-1β on IκBα levels and NF-κB activity independent of HSF activity. **A**: Cultured Caco-2 cells were treated for 5 or 60 min with 500 μM sodium arsenite and were then treated for 30 min with 0.5 ng/ml human recombinant IL-1β in arsenite-free medium. HSF and NF-κB DNA binding activity was determined by EMSA and IκBα levels by Western blotting. **B**: Caco-2 cells were transfected with an HSP 70 promoter-luciferase reporter plasmid and were then treated for 1 h with sodium arsenite. Cells were then allowed to recover in arsenite-free medium for 1 or 7 h, whereafter, luciferase activity was determined as a measure of HSP gene activation. Control cells (C) were not exposed to sodium arsenite. Results are means ± SEM with n = 3 for each observation.

certain metabolic effects of sodium arsenite are caused by these mechanisms [Huot et al., 1995; Elbirt et al., 1998]. To test whether the sodium arsenite-induced stabilization of IκBα reflected oxygen radical production or activation of the MAP kinase signaling pathway, cells were treated with the oxygen radical scavenger catalase, the MAP kinase inhibitor PD98059 (for ERK) or SB203580 (for p38). Sodium arsenite protected IκBα levels in IL-1β-treated Caco-2 cells also in the presence of these inhibitors (Fig. 5) indicating that the effect of sodium arsenite did not reflect oxygen radical production or activation of the MAP kinase signaling pathway. Note that in this experiment, IκBα levels were somewhat reduced in cells treated with catalase. Although the mechanism for this finding is not known at present, the results clearly show that IκBα levels were not further reduced by IL-1β in these cells.

DISCUSSION

The results in the present study suggest that arsenite can block IKK activity, stabilize IκBα, and downregulate NF-κB activity in IL-1β-treated Caco-2 cells independent of the heat shock response. This conclusion is based on several lines of evidence: (1) IκBα levels were maintained and NF-κB DNA binding activity was inhibited in arsenite-treated cells without increased levels of HSP 70 or HSP 90; (2) cycloheximide did not prevent the effects of sodium arsenite on IκBα levels indicating that de novo synthesis of proteins (including heat shock proteins) was not needed for the effects of sodium arsenite; (3) quercetin did not prevent the effects of sodium arsenite on IκBα levels and NF-κB activity despite inhibited HSP 70 production; (4) the effects of sodium arsenite on IκBα levels and NF-κB DNA binding activity

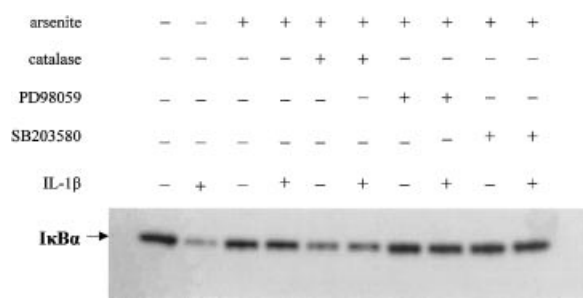

Fig. 5. Sodium arsenite blocks the effect of IL-1β on IκBα levels independent of oxygen radical production and MAP kinase signaling. Cultured Caco-2 cells were treated with 500 μM sodium arsenite for 1 h, were allowed to recover for 1 h in arsenite-free medium, and were then treated for 30 min with 0.5 ng/ml human recombinant IL-1β in the absence or presence of 100 μg/ml of the oxygen radical scavenger catalase, 30 μM PD98059 or 30 μM SB203580 to inhibit MAP kinase. IκBα levels were determined by Western blotting.

occurred before HSF was activated. To our knowledge, the present study is the first report providing evidence that arsenite prevents cytokine-induced NF-κB activation in Caco-2 cells (or any other cell line) independent of the heat shock response.

The Western blot analyses and EMSAs performed here were based on a fixed amount of protein added to the assays. Thus, at least from a theoretical standpoint, it is possible that the results were influenced by changes in cellular protein concentrations or cellular growth rate. Although the same number of cells were always seeded in the wells and experiments were performed when cells were 90% confluent, further studies are needed to make certain that changes in cell growth or cellular protein concentration did not influence the present results.

It should be noted that the present results do not rule out that sodium arsenite can induce the heat shock response in Caco-2 cells and that other effects of sodium arsenite than inhibition of IKK activity and stabilization of IκBα may be mediated by heat shock proteins. Indeed, in the present study, increased HSP 70 levels were observed in cells that were allowed to recover 4 or 24 h after sodium arsenite treatment, consistent with a relatively slow induction of the heat shock response by sodium arsenite under these experimental conditions. Importantly, the stabilizing effect of sodium arsenite on IκBα started to decrease 4 h after arsenite treatment and was almost abolished after 24 h despite increased HSP 70 levels at the same time points (Fig. 2). These observations further support the concept that the anti-inflammatory effect of arsenite is not mediated by heat shock proteins.

Recent studies suggest that arsenite stabilizes IκBα by blocking IKK activity [Kapahi et al., 2000; Roussel and Barchowsky, 2000; Yoo et al., 2000], but the potential role of the heat shock response in this effect of arsenite was not clarified in those studies. In one report, treatment of respiratory epithelial cells with sodium arsenite (1 mM) or hyperthermia (43°C) induced increased cellular levels of HSP 70 and prevented TNF-α-induced activation of IKK [Yoo et al., 2000]. From those results, the authors speculated that HSP 70 may bind to IKK and inhibit its activity. However, the authors also reported that no binding between HSP 70 and IKK was observed by immunoprecipitation, indicating (but not proving) that HSP 70 did not mediate the inhibitory effect on IKK.

Additional evidence for an inhibitory effect of sodium arsenite on IKK activity was reported in a study in which treatment of human bronchial epithelial or embryonic kidney cells with arsenite (500 μM) blocked TNF-α-induced IKK activity [Roussel and Barchowsky, 2000]. In another report, Kapahi et al. [2000] found that treatment of HeLa cells with sodium arsenite (12.5 μM) blocked TNF-α-induced IKK activity by modification of the catalytic subunit of IKK β at a site that includes cysteine-179. The potential involvement of the heat shock response in arsenite-induced inhibition of IKK was not examined or discussed in either of those reports. The result in the present study that inhibition of NF-κB activity by sodium arsenite did not require increased levels of heat shock proteins and was not mediated by oxygen radicals or MAP kinase signaling supports the report of Kapahi et al. [2000] in which it was found that sodium arsenite binds directly to cysteine-179 in the activation loop of the IKK catalytic subunits.

Although most previous reports examined the effect of arsenite on NF-κB activity in cultured cells in vitro, a recent study from our laboratory suggests that arsenite may stabilize IκBα and prevent NF-κB activation in vivo as well [Pritts et al., 2000]. In that report, treatment of mice with sodium arsenite or hyperthermia blocked endotoxin-induced IκBα degradation and NF-κB activation in intestinal mucosa and because these effects of hyperthermia and sodium arsenite were accompanied by increased mucosal HSP 70 levels, we speculated that the protective effects of both hyperthermia and sodium arsenite were caused by the stress response. Considering the results in the present study, it is possible that our previous findings in vivo reflected an association between increased HSP 70 levels and inhibited NF-κB activation rather than a cause–effect relationship.

In addition to sodium arsenite, several other treatments have been found to downregulate NF-κB activation by blocking IKK activity. For example, treatment of human monocytic cells or the human intestinal epithelial cell line HT-29 with IL-10 blocked TNF-induced activation of IKK [Schottelius et al., 1999]. In the same study, evidence was found that IL-10 blocks NF-κB DNA binding activity by an additional (nuclear) mechanism that is independent of

IκBα phosphorylation and NF-κB nuclear translocation.

Curcumin is the spice that gives the specific flavor and yellow color to curry. In the countries of its origin, curcumin has been used for centuries as a traditional medicine to treat inflammatory diseases [Yegnanarayan et al., 1976]. Recent studies suggest that inhibition of NF-κB may be a mechanism by which curcumin exerts its anti-inflammatory effects [Plummer et al., 1999; Jobin et al., 1999b]. When cultured intestinal epithelial cells were treated with curcumin, the IL-1β-induced activation of IKK was blocked by targeting kinase(s) upstream of IKK [Jobin et al., 1999b].

When HeLa cells were subjected to heat shock (45°C for 15 min), the activation of NF-κB induced by ionizing radiation was blocked secondary to inhibited IKK activity [Curry et al., 1999]. Interestingly, in the same study, pretreatment of the cells with cycloheximide almost completely inhibited the hyperthermia-induced increase in HSP 70 levels, but did not alter the heat shock-induced inhibition of NF-κB activation. From those observations, the authors concluded that de novo protein synthesis is not required for the inhibition of NF-κB in response to hyperthermia and that heat shock proteins are not involved in this process, similar to the conclusion in the present study.

Although inhibition of IKK activity (resulting in inhibited phosphorylation and degradation of IκBα) has been well established as a mechanism by which sodium arsenite treatment results in maintained IκBα levels [Kapahi et al., 2000; Roussel and Barchowsky, 2000; Yoo et al., 2000], it should be noted that maintained IκBα levels may also reflect increased synthesis, in addition to inhibited degradation of the protein. The influence of arsenite on IκBα synthesis is not known. In a recent study, however, we found that treatment of mice with sodium arsenite resulted in increased IκBα mRNA levels in intestinal mucosa, indicating that the synthesis of IκBα was upregulated [Pritts et al., 2000]. Thus, it is possible that maintained IκBα levels after treatment with sodium arsenite reflect both reduced degradation (secondary to inhibited IKK activity and phosphorylation) and stimulated synthesis of the protein. Regardless of which of these mechanisms is most important for stabilization of IκBα levels, the present results strongly suggest that maintained IκBα levels after treatment of cultured Caco-2 cells with sodium arsenite do not reflect induction of the heat shock response.

It should be noted that even if the present study and other reports are consistent with the concept that sodium arsenite blocks IL-1β-induced NF-κB activation by preventing phosphorylation of IκBα, additional mechanisms may be involved. For example, arsenite has been shown to inhibit several steps in the ubiquitin-proteasome pathway [Klemperer and Pickart, 1989; Klemperer et al., 1989], and inhibited proteasome activity may contribute to IκBα stabilization. Results in other reports suggest that sodium arsenite can directly inhibit NF-κB binding to a consensus κB oligonucleotide, indicating that critical sulfhydryls of the NF-κB complex are required for its binding to DNA [Shumilla et al., 1998]. Thus, sodium arsenite may inhibit IL-1β-induced NF-κB activation at multiple levels in addition to the level of IKK.

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