Journal of Cellular Biochemistry 84:750–758 (2002)
DOI 10.1002/jcb.10096

Arsenite Stimulates Cyclooxygenase-2 Expression Through Activating IκB Kinase and Nuclear Factor κB in Primary and ECV304 Endothelial Cells

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Abstract Epidemiological studies have shown that chronic exposure to arsenic can result in liver injury, peripheral neuropathy, arteriosclerosis, and an increased incidence of cancer of the lung, skin, bladder, and liver. The overexpression of inducible cyclooxygenase-2 (Cox-2) has been associated with vascular inflammation and cellular proliferation. However, the effect of arsenite on Cox-2 gene expression in endothelial cells was left to be investigated. Western Blot analysis of HUVECs revealed a two-fold induction of Cox-2 protein by arsenite. This induction was associated with a two-fold increase of prostaglandin E_2 in the media. Furthermore, the level of Cox-2 mRNA was correspondingly elevated as demonstrated by both Northern blot and reverse transcriptase-polymerase chain reaction (RT-PCR) analyses. Transfection of an immortalized human endothelium cell line (ECV304) with Cox-2 reporter gene constructs demonstrated that the transcription of Cox-2 gene was enhanced by arsenite. This induction was attenuated by pyrrolidine dithiocarbamate (PDTC), an inhibitor of NFκB. In addition, electrophoretic mobility shift assays indicated that NFκB activity was induced by arsenite. The kinase activity assay also indicated that IκB kinase (IKK) activity was induced by arsenite. These findings indicated that the induction of Cox-2 gene transcription by arsenite was through the stimulation of NFκB activity. Arsenite could induce IKK activity, which leads to the phosphorylation and degradation of IκB in ECV304 cells. Therefore, it appears that IKK signaling pathway is involved in arsenite-mediated Cox-2 expression. J. Cell. Biochem. 84: 750–758, 2002.

Key words: arsenite; cyclooxygenase-2; IκB kinase; NFκB; endothelial cells.

Cyclooxygenase (Cox), also known as prostaglandin (PG) H synthase (EC 1.14.99.1), catalyzes the rate-limiting steps in the formation of PG endoperoxides [Smith et al., 1996]. Two isoforms of Cox have been described. Cyclooxygenase-1 (Cox-1) is expressed constitutively in most tissues, whereas cyclooxygenase-2 (Cox-2), the other isoform, is not expressed under physiological conditions in most organs but is induced by cytokines and mitogens during inflammatory process [Vane et al., 1998]. These two Cox isoforms share about 60% sequence similarity at the amino acid level, and the residues that are critical for enzyme function in both isoforms are highly conserved [Hla and Neilson, 1992]. Since both Cox genes encode active PG synthase with Cox and hydroperoxidase activities, Cox-2 expression is regulated by different mechanisms as compared to Cox-1 [Hla and Neilson, 1992].

The Cox-2 gene has been cloned, and there are two transcription factor nuclear factor-κB (NFκB) consensus sites in the promoter region [Appleby et al., 1994]. NFκB exists in a latent form in the cytoplasm of unstimulated cells comprising a transcriptional active dimer bound to an inhibitor, inhibitor-κB (IκB). Following stimulation with the cytokine, the NFκB is activated through the phosphorylation and degradation of IκB proteins [Baeuerle and Baltimore, 1996]. Recently, a high molecular mass multi-subunit IκB kinase (IKK) has been

Grant sponsor: National Science Council; Grant number: NSC 89-2320-B-038-076.

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Received 14 May 2001; Accepted 10 November 2001

found to phosphorylate IκBα and IκBβ. Two catalytic subunits (termed IKK1 and IKK2) of IKK have been identified, cloned, and shown to be widely expressed in human tissues. It has been demonstrated that IKK is the kinase involved in the signal-inducible degradation of IκB [Didonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Zandi et al., 1997].

Arsenic, a known human carcinogen and teratogen, is ubiquitously present in the environment, where it occurs mainly as compounds of arsenite (As^{3+}) and arsenate (As^{5+}). Epidemiological studies have shown that chronic exposure to arsenic can result in liver injury, peripheral neuropathy, arteriosclerosis, and an increased incidence of cancer of the lung, skin, bladder, and liver [Penn, 1990; Engel and Smith, 1994; Engel et al., 1994]. At a molecular level, arsenite acts as a sulfhydryl reagent that binds to free thiol (-SH) groups [Snow, 1992]. All protein tyrosine phosphatase contain such -SH groups [Walton and Dixon, 1993], and it has been shown that arsenite inhibits c-Jun N-terminal kinase (JNK) phosphatase, presumably via this mechanism [Cavigelli et al., 1996]. Arsenite also activates extracellular signal-regulated kinase (ERK) and p38 in addition to the stress-activated protein kinase pathway [Rouse et al., 1994; Ludwig et al., 1998].

Considerable evidence has accumulated to suggest that arsenite may act as a carcinogen [Snow, 1992; Hartwig, 1995], mutagen [Hei et al., 1998] and effective activator of mitogen-activated protein kinase (MAPK) [Rouse et al., 1994]. However, molecular mechanism for the effects of arsenic on chronic disease is incompletely understood. In this study, we investigated the ability of arsenite not arsenate to increase Cox-2 protein expression and rates of PGE_2 production in human umbilical vein endothelial cells (HUVEC). In vitro cultured ECV304 cells were employed to further investigate the signal transduction pathway that arsenite-induced Cox-2 gene expression and shows that it is at least partially mediated through the activation of IKK and NFκB/IκB system.

METERIALS AND METHODS

Reagents

Sodium arsenite, sodium arsenate, and pyrrolidine dithiocarbamate (PDTC) were obtained from Sigma Chemical (St. Louis, MO). SB203580 was obtained from TOCRIS (Ballwin, MO). Isotopes were obtained from Amersham (Arlington Heights, IL).

Cell Culture and Arsenic Treatment

HUVEC cells were isolated as described by Barchowsky et al. [1996] and grown in medium 199 with 20% endotoxin-free heat-inactivated fetal calf serum (FCS) and 2 mM L-glutamine [Barchowsky et al., 1996]. The immortalized human endothelial cell line ECV304 cells were obtained from the Culture Collection and Research Center of Food Industry Research and Development Institute (Hsinchu, Taiwan). ECV304 cells were cultured in medium 199 (HEPES modification) containing 10% endotoxin-free heat-inactivated FCS (GIBCO, Grand Island, NY), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine, and cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. When the cells reached a confluent monolayer, various concentrations of the arsenic dissolved in water were added.

Determination of PGE_2

The control and treated culture medium was collected, centrifuged, and stored at −70°C, until tested. The level of PGE_2 released into culture media was quantified using a specific enzyme immunoassay (EIA) according to the manufacturer's instructions (Amersham), and the level of PGE_2 was normalized by cell numbers.

Western Blots

Protein extraction and Western blot analysis were done as described [Tsai et al., 1999a]. Total proteins (for IKK1, IκBα, phosphorylated-IκBα, IκBβ, Cox-1, and Cox-2) containing 30–50 μg of protein were analyzed on blots and incubated overnight at 0°C with 10% bovine serum albumin (BSA) in phosphate-buffered saline and then incubated with anti-IκBα, IκBβ, IKK1, and Cox-1 polyclonal antibodies (Santa Cruz Biochemicals, Santa Cruz, CA), anti-phospho(Ser32)-specific IκBα polyclonal antibody (New England Biolabs, Beverly, MA) or anti-Cox-2 monoclonal antibody (Transduction Laboratories, Lexington, KY). IKK1, IκBα, IκBβ, Cox-1, and Cox-2 protein were detected by chemiluminescence (ECL, Amersham).

RT-PCR and Northern Blot

RNA extraction and Northern blot analysis were done as described [Tsai et al., 1999b]. The RT-PCR was performed by one-step protocol of

Pharmacia Biotech Ready-To-Go PAR Beads (Atlantic Avenue, CA). Total RNA (2 μg), primers (5 pmol), and DEPC-treated water were added to RT-PCR Beads in a final volume of 50 μl. The amplification cycles of Cox-2 were 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min. The amplification cycles of β-actin were 94°C for 30 s, 62°C for 45 s, and 72°C for 2 min. The PCR products were separated by electrophoresis on a 1.2% agarose gel after 35 cycles and visualized by ethidium bromide staining. Amplification of β-actin served as a control for sample loading and integrity. PCR was performed on the cDNA using the following sense and antisense primers, respectively: Cox-2: AATCCTTGCTGTTCCCACCCATGTCAA and GAATCATCTCTGCCTGAGTATC TT; β-actin: GATATCGCCGCGCTCGTCGTCGAC and CAGGAAGGAAGGCTGG AAGAGTGC. Total RNA (25 μg) per lane was analyzed on blots and hybridized with Cox-2 fragment, which was labeled with ^{32}P by using a Random Primer Labeling kit (Amersham). After hybridization, the membrane was washed, dried, and autoradiographed with Kodak X-ray film (Rochester, NY).

Plasmids

The human Cox-2-promoter plasmid contain a 967 bp fragment, −917 to +49 relative to transcription start, was amplified from human genomic DNA using the primer 5′-3′GGACATTTAGCGTCCCTGCA (sense), 5′-3′GAGTTCCTGGACGTGCTCCT (anti-sense). Each 5′ amplimer contained a XmaI site and each 3′ amplimer contained a HindIII site, such that the resulting PCR product, upon digestion with XmaI/HindIII could be ligated in frame into the unique XmaI/HindIII site present within the pGL2Enhance plasmid (Promega Corp., Madison, WI). PGL2Enhance was linerized to the above XmaI/HindIII-digested PCR products using standard techniques. Sequences were confirmed identity by ABI PRISM 377 DNA Analysis System (Perkin-Elmer Corp., Taiwan Branch).

Transient Transfection and Luciferase Assay

The luciferase assay was performed as described by Tsai et al. [1999a] with some modification. ECV304 cells were seeded in a 24-well tissue culture plate, and were transfected with the Cox-2 promoter gene using lipofectamine reagent (GIBCO, NRL, Life Technologies, Inc.). As an internal control for transfection efficiency, pcDNA3-LacZ plasmid was co-transfected and the β-galactosidase was measured in the experiment. After 12 h, we replaced the medium with fresh, complete medium for 12 h. Cells were then incubated with the arsenite for 6 h before the addition of 200 μl of lysis buffer (0.5 M HEPES pH 7.8, 5% Triton-N 101, 1 mM $CaCl_2$, 1 mM $MgCl_2$). Luminescence was measured on a Top Counter Microplate Scintillation and Luminescence Counter (Packard 9912V) in singlephoton counting mode for 0.1 min/well. Data was expressed as mean ± SE of the fold of minimal luciferase activity observed with control as determined by three independent experiments.

Electrophoretic Mobility Shift Assays (EMSA) for NFκB

Nuclear and cytoplasmic extracts were prepared according to a modified method of Tsai et al. [1999b]. Each 2 μg of nuclear extract was mixed with the labeled double-stranded NFκB oligonucleotide, 5′-AGTTGAGGGGACTTTCCCAGGC-3′, and incubated at room temperature for 20 min. The incubation mixture was included 1 μg of poly (dI-dC). The DNA–protein complex was electrophoresed on 6% nondenaturing polyacrylamide gels in 0.5 × Tris/borate/EDTA buffer. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. Radioactive bands were detected by autoradiography.

IKK Assay

Protein extraction and IKK analysis were done as described [Tsai et al., 1999a]. The IKK1 and IKK2 were immunoprecipitated with IKK-specific antibody (Santa Cruz Biotechnology) for 18 h at 4°C. The IKK–antibody complex was then precipitated with protein-A agarose, and then it was reacted with its substrate-IκBα-GST (Santa Cruz Biotechnology). The reaction was terminated by the addition of 5 × sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) sample-buffer and boiling for 10 min. Proteins were separated on 12% SDS–PAGE and autoradiography of the dried gel was performed at −70°C.

RESULTS

Induction of Cox-2 Protein and PGE_2 Levels in HUVEC by Arsenite

After treatment with various concentrations of arsenic for 20 h, the cells were lysed and their

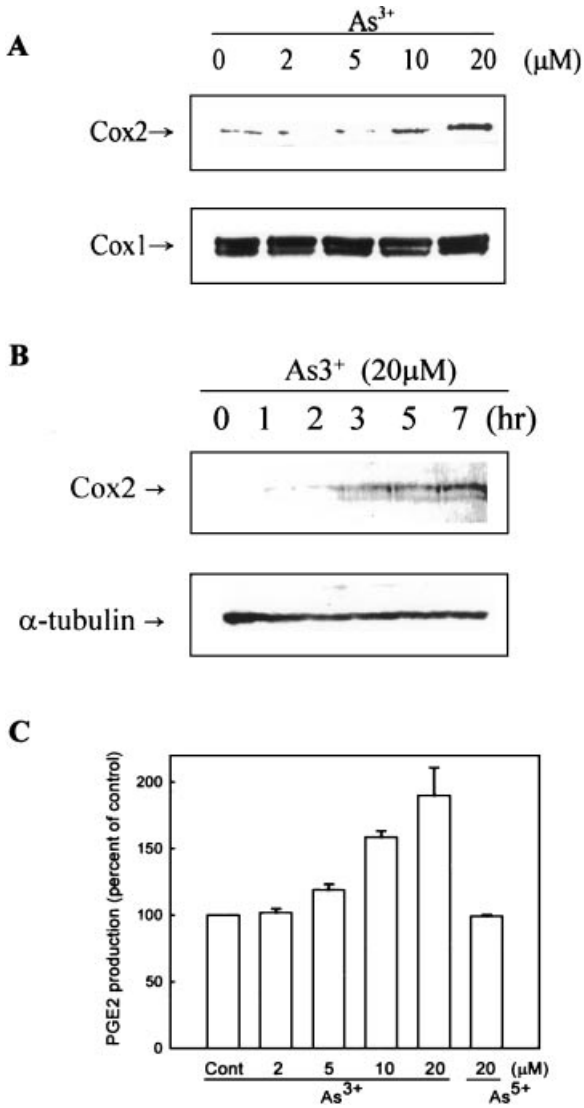

Fig. 1. **A**: Western blot analysis of Cox-2 protein in HUVEC. The cells were treated with various concentrations of sodium arsenite for 20 h. Total protein (25 μg/lane) was separated on 8% SDS–PAGE and blotted with specific Cox-2 and Cox-1 antibodies as described in Materials and Methods. Similar results were obtained from three independent experiments. **B**: Total protein was extracted from HUVEC treated with sodium arsenite (20 μM) for indicated time and assayed for Cox2 and α-tubulin protein expression by Western blots as described in Materials and Methods. Signals for α-tubulin for each lane are as control. Similar results were obtained from three independent expreriments. **C**: Sodium arsenite stimulates the formation of PGE_2 in HUVEC. The cells were treated with different concentrations of sodium arsenite for 15 h. Production of PGE_2 was determined by ELA as described in Materials and Methods. The values were expressed as mean ± SE from three independent experiments.

Cox-1 and Cox-2 levels were estimated by Western blot analysis as described in Materials and Methods. Figure 1A showed that treatment of HUVEC with sodium arsenite for 20 h induced the Cox-2 protein expression in a dose-dependent manner. Similar results were observed 5 h after induction of arsenite (data not shown). In contrast, the expression of Cox-1 was not changed by induction with arsenite in HUVEC. The effect of sodium arsenate on HUVEC was also tested, and the expression of Cox-1 and Cox-2 was not affected by sodium arsenate (data not shown). In addition, a time course of Cox-2 protein induction by arsenite was illustrated in Fig. 1B. This result showed that significant increase of Cox-2 protein expression was shown within 3 h of stimulation. To determine if increased Cox-2 expression correlates with PG production, we treated HUVEC with arsenite for 15 h and examined the levels of PGE_2 in medium. Arsenite increased the PGE_2 level greater than two-fold in condition media as compared with media of control, whereas arsenate was inactive under the similar condition (Fig. 1C).

Induction of Cox-2 Gene Expression by Arsenite

In order to investigate whether the increase of Cox-2 activity was due to induction of Cox-2 mRNA, the RT-PCR analysis and Northern blot for total RNA samples extracted from ECV304 cells were carried out. The Cox-2 mRNA of ECV304 cells response to arsenite is illustrated in Figure 2. Both RT-PCR and Northern blotting revealed that arsenite remarkably increased Cox-2 mRNA expression. Figure 2B demonstrated that Cox-2 mRNA was induced within 3 h in these cells. Significant increase of Cox-2 mRNA expression was seen within 1 h of stimulation peaking around 3 h and gone by 5 h.

Induction of Transfected Cox-2 Promoter Luciferase Reporter Construct by Arsenite and PDTC Blocks This Induction

Transient transfection of Cox-2 reporter gene construct was performed in ECV304 cells to further demonstrate arsenite-mediated induction of Cox-2 transcription. Figure 3A showed that arsenite yielded progressive luciferase expression with a dose-dependent manner. The similar result was also obtained with transient transfection in HUVEC (data not shown). These results demonstrated that arsenite-mediated Cox-2 protein expression was through inducing the transcription of Cox-2. Since it is known that the expression of Cox-2 involves sequence-specific binding of cis-acting transcription factors, particularly NFκB. We

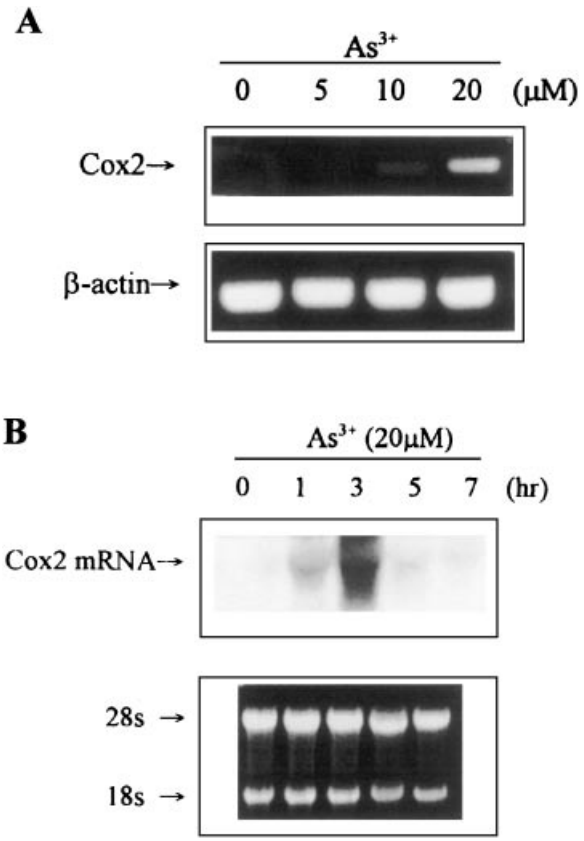

Fig. 2. RT-PCR analysis of the expression of Cox-2 mRNA. **A**: The ECV304 cells were treated with various concentrations of sodium arsenite for 4 h. Total RNA was extracted from treated cells and the Cox-2 mRNA expression was determined as described in Materials and Methods. **B**: Total RNA was extracted from ECV304 cells treated with sodium arsenite (20 μM) for indicated time and assayed for Cox-2 mRNA expression by Northern blot analysis. Blots were hybridized to ^{32}P-labeled Cox-2 probes as described in Materials and Methods. Signals for 18S rRNA and 28S rRNA for each lane are shown as control.

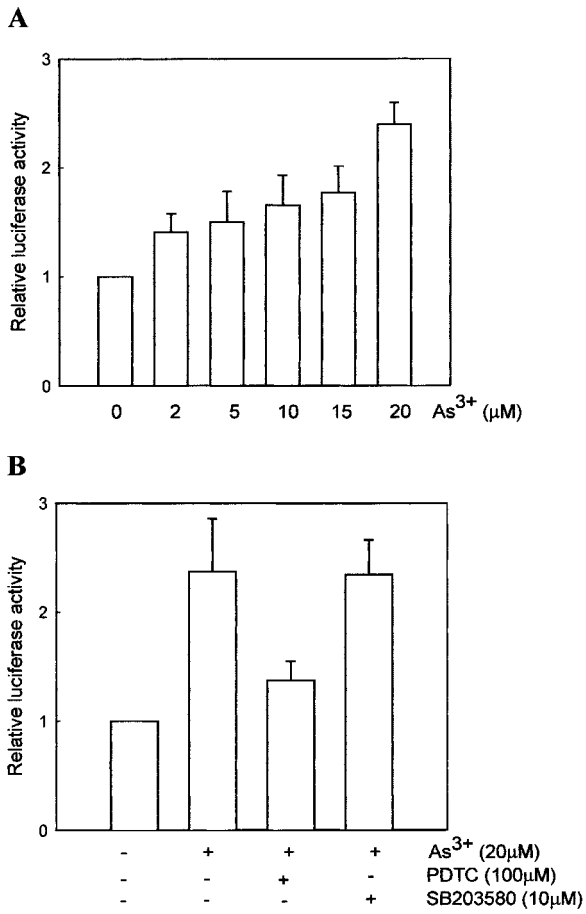

Fig. 3. **A**: Effects of sodium arsenite on the Cox-2 promoter activity in ECV304 cells. The cells were co-transfected with human Cox-2 promoter and pcDNA3-LacZ constructs. After 24 h transfection, cells were treated with different concentrations of sodium arsenite for 6 h. Cells were harvested and the levels of luciferase activities were determined as described in Materials and Methods. The luciferase activity was normalized by β-galactosidase activities. **B**: Effects of PDTC and SB203580 on the Cox-2 promoter activity and NFκB binding activity in response to sodium arsenite. ECV304 cells were co-transfected with human Cox-2 promoter and pcDNA3-LacZ constructs. After 24 h transfection, cells were pretreated with PDTC (100 μM) or SB203580 (10 μM) before addition of 20 μM sodium arsenite. An untreated control and a control treated with sodium arsenite only were included in each experiment, and the cells were harvested 6 h after addition of sodium arsenite. The levels of luciferase activities were determined as described in Materials and Methods. The luciferase activity was normalized by β-galactosidase activity.

used PDTC, a well-known inhibitor of the activation of the NFκB transcription factor complex, to investigate whether NFκB activation was essential for arsenite-induced Cox-2 gene expression. Figure 3B showed that the pre-exposure of ECV304 cells to PDTC markedly reduced the Cox-2 promoter activity induced by sodium arsenite treatment. In contrast, SB203580, an inhibitor of $p38^{mapk}$, had little effect on arsenite-induced Cox-2 promoter activity. These results indicated that NFκB activity was essential for the arsenite induced Cox-2 gene expression.

Arsenite Activates NFκB Activity and IKK Activity in ECV304 Cells

To further investigate if arsenite selectively induced activation of NFκB, analysis of NFκB binding activity by EMSA was performed. The different time course of nuclear extracts were prepared from 20 μM arsenite-treated ECV304 cells and were tested for gel retardation in EMSA. In addition, the specificity of protein–DNA complex for the NFκB sequence was determined by competition binding experiments (data not shown). Figure 4A showed that

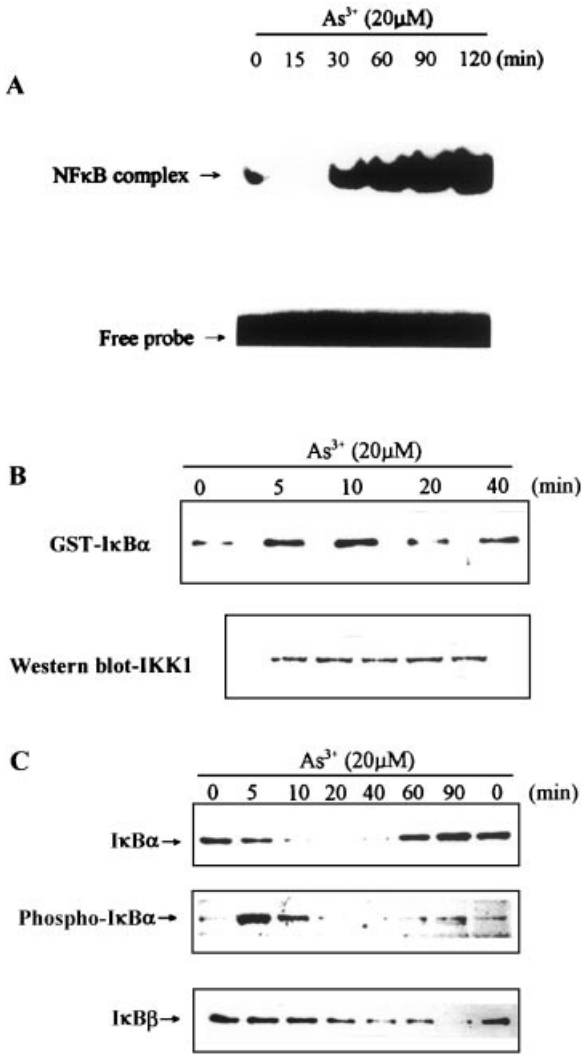

Fig. 4. **A**: Arsenite increases NFκB activity. Gel mobility shift assay for NFκB in nuclear extracts from ECV304 cells. The nuclear extracts from ECV304 cells treated with sodium arsenite (20 μM) for indicated time and assayed for NFκB binding activity as described in Material and Methods. Data are shown one of the three typical experiments. **B**: Arsenite stimulates IKK activity in ECV304 cells. Whole cell extracts were prepared from ECV304 cells treated with sodium arsenite (20 μM) for indicated time. IKK1 activity was carried out as described in Materials and Methods. Similar results were obtained in three independent experiments. **C**: Arsenite stimulates phosphorylation and degradation of IκBs in ECV304 cells. Western blot analysis of total protein extracts from ECV304 cells treated with sodium arsenite (20 μM) for indicated time. Extracts were separated on 10% SDS–PAGE and analyzed as described in Materials and Methods. Similar results were obtained in three independent experiments.

NFκB binding activity was downregulated in the first 15 min and then upregulated up to 120 min in these cells. Detectable increase of NFκB binding activity was seen after 30 min of stimulation. This result suggested that in the latter phase arsenite induced Cox-2 gene expression through upregulation of NFκB activity. Because IKK is involved in the activation of NFκB, we further determine whether the induction of NFκB activity by arsenite was due to its effect on the IKK activity. In order to directly measure IKK1 activity in ECV304 cells, IKK1 protein was immunoprecipitated from cell extracts, and the kinase activity in the immunocomplex was assayed by using recombinant GST-IκBα (1-317) as a substrate. As shown in Figure 4B the ECV304 cells treated with 20 μM sodium arsenite exhibited an immediate increase in IKK activity that peaked between 5 and 10 min and steadily declined to basal levels by 45 min. It has been reported that IKK could phosphorylate IκBα and IκBβ, then trigger the degradation of IκBs, and finally the degradation of IκBs resulted in the NFκB activation. We then studied whether arsenite affects phosphorylation and degradation of IκBs. After various time lengths of activation of ECV304 cells by arsenite, IκBs and phosphorylated-IκBα were detected by specific IκBα, IκBβ, and Ser32-phosphorylated-IκBα antibodies. The results were illustrated in Figure 4C Western blotting of cell extracts showed that stimulation with arsenite caused an immediate increase in the levels of phosphorylated form of IκBα that peaked at 5 min. This result was consistent with the IKK activity assay. The data in Figure 4C demonstrated that arsenite induced degradation of IκBα peaked between 10 and 45 min and steadily recovered to basal levels by 60 min. In addition, the degradation of IκBβ induced by arsenite was peaked between 20 and 90 min. Thus, these results suggested that IKK signaling cascade was involved in arsenite-induced NFκB activation. Taken together, these data strongly implicate that IKK and NFκB/IκB signaling pathway are essential for the arsenite-induced Cox-2 gene expression.

PDTC Blocks the Induction of Cox-2 Promoter Activity by Sodium Arsenite

Thus, these results further confirmed that NFκB activity was important for the arsenite-induced Cox-2 gene expression. Taken together, these data strongly implicate that IKK and NFκB/IκB signaling pathway is one of the important factors that contributes to arsenite-induced Cox-2 gene expression.

DISCUSSION

Epidemiological studies have suggested that low concentrations of arsenic, which do not cause cell death, promote vascular disease, and tumor formation [Bencko, 1987; Penn, 1990; Snow, 1992; Engel and Smith, 1994; Engel et al., 1994]. Arsenic in deep-well drinking water has been associated with endemic peripheral vascular disease, hepatic veno-occlusive disease, and perisinusoidal fibrosis [Labadie et al., 1990]. Mechanisms for the effects of arsenic on proliferative diseases are unclear. However, the toxin-induced tumorigenesis and atherosclerotic plaque formation may share common mechanisms involving altered gene expression in endothelial cells. In this study, arsenite potently induced both Cox-2 protein and mRNA expression and enhanced the activity of transient transfected Cox-2 reporter construct. These data strongly indicate that arsenite could enhance Cox-2 gene expression in human endothelial cells. This finding may provide a new molecular basis for understanding the effects of arsenite on vascular and proliferative disease.

Currently, the role of Cox and prostanoids in the pathogenesis of athrogenesis are poorly understood. The induction of Cox-2 had been considered as an example of a vasoprotective and potentially anti-atherogenic endothelial gene [Pomerantz and Hajjar, 1989]. However, it has been rationalized on grounds of a hypothesis that vasoprotective mechanism are part of an early atherogenic response, which is initially designed to serve its defensive function and then regress [Ross, 1993; Zembowicz et al., 1995]. In contrast to endothelium, the induction of Cox-2 in macrophages and vascular smooth muscle cells may lead to the production of mainly proinflammatory prostanoids that may contribute to the vascular damage [Radomski et al., 1987]. In this study, we found that arsenite could induce the Cox-2 gene expression in endothelium cells. It can be conceived that the induction of Cox-2 by arsenite is a back-up mechanism necessary to support the elevated synthesis of vasoprotective prostacyclins by stressed endothelial cells, then triggered an early atherogenic response. We also found that arsenite did induce Cox-2 protein expression in both macrophages and vascular smooth muscle cells (data not shown). This finding provided further evidence to support that arsenite could mediate vascular disease through the induction of Cox-2 gene expression. In addition, previous reports have shown that NFκB/IκB may play a key role in regulating vascular pathophysiology [Linder and Collins, 1996]. The inflammatory, procoagulant, and proliferative aspects of endothelial dysfunction in atherosclerosis may be linked by the fact that many of the genes for leukocyte adhesion molecules associated with initiation of the atherosclerotic lesion have functional NFκB binding sites [Collins, 1993]. In this study, we also showed that arsenite could up-regulate NFκB/IκB pathway. This may be an additionally important factor in arsenite-mediated vascular disease. Nevertheless, we still need to further investigate whether or not arsenite induce other leukocyte adhesion molecules.

There are two NFκB consensus sites in the promoter region of Cox-2, and NFκB has been considered to play an important role in the control of Cox-2 transcription [Appleby et al., 1994; Newton et al., 1997]. In this study, we found that arsenite could enhance the binding activity of NFκB, although the NFκB binding activity was slightly repressed by sodium arsenite at 15 min (Fig. 4A). It seems that arsenite may exert biphasic induction of NFκB binding activity. Arsenite, therefore, could slightly reduce the endogenous NFκB binding activity in the early stage. However, our results clearly showed that arsenite enhanced the NFκB activity profoundly in the later stage. This finding is consistent with previous work, which showed that arsenite could induce NFκB activation in cultured aortic endothelial cells [Barchowsky et al., 1996]. Recently, it has been suggested that transcriptional regulation might be conducted through the cooperation of more than one trans-acting factor with regulated assembly of multi-protein complex on enhancers and promoters. The complex nature of these processes is considered to result in an elaborate fail-safe mechanism for controlling gene expression [Tjian and Maniatis, 1994]. In this study, we clearly showed that NFκB in the human Cox-2 gene was involved in arsenite-induced promoter activity (Fig. 3A). This result was supported by our findings that arsenite could cause an increased NFκB binding activity, and that treatment of cells with an NFκB inhibitor, PDTC, could result in an reduction of the arsenite-induced Cox-2 promoter activity. Therefore, we postulated that the induction of Cox-2 gene expression was through upregulation of

NFκB activity. However, we do not rule out the possibility that other sites may contribute to the arsenite-induced promoter activity.

Previous works indicate that arsenite can activate ERK, JNK, and p38 MAP kinase in many cell types [Cavigelli et al., 1996; Liu et al., 1996]. Nevertheless, no study on IKK has been reported previously. Here we clearly showed that arsenite could potently induce IKK activity (Fig. 4B). IKK has recently been shown to play an important effector of NFκB/IκB pathway and a key enzyme involved in signal transduction [Didonate et al., 1997]. We assume that IKK signaling cascade is involved in the arsenite-mediated Cox-2 gene expression. The finding supports this assumption that arsenite could induce the phosphorylation and degradation of IκB (Fig. 4C), and arsenite could stimulate NFκB activity by using EMSA (Fig. 4A). In addition, the NFκB inhibitor-PDTC, not the $p38^{mapk}$ inhibitor-SB203580, could attenuate the arsenite-induced Cox-2 promoter activity (Fig. 3B). This result further confirmed that IKK activation might be involved in arsenite-induced Cox-2 gene expression. However, we still cannot completely rule out the possibility that other different signaling pathways are also involved in arsenite-induced Cox-2 gene expression.

In summary, we have identified a novel IKK activating pathway, which is mediated by the arsenite-induced stress response in human endothelial cells. This pathway may be important for the arsenite-mediated Cox-2 gene expression, which leads to the induction of cancer, neuropathy, and arteriosclerosis.

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