*J. Microbiol. Biotechnol.* (2008), **18**(2), 392–395

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY



# Regulation of NO from Endothelial Cells by the Decrease of Cellular cAMP Under Arsenite Exposure

**Lee, Soo Youn and Jiho Min***

*Division of Environmental and Chemical Engineering, Chonbuk National University, Jeonju 561-756, Korea*

Received: November 6, 2007 / Accepted: December 31, 2007

**In an attempt to delineate the direct effect of arsenite-induced endothelial dysfunction on nitric oxide (NO) production, confluent bovine aortic endothelial cells (BAEC) were incubated with arsenite, and endothelial NO synthase expression and NO production were measured. Exposure of arsenite decreased NO production for up to 24 h. This decrease was accompanied by decreases in cAMP, protein kinase A (PKA) activity, and furthermore, significant reduction of pCREB. In conclusion, this study is the first to demonstrate that exposure of arsenite decreases NO production by a reduction of pCREB and PKA activity that may be mediated by cAMP, leading to endothelial dysfunction.**

**Keywords:** Endothelial nitric oxide synthase, arsenite, nitric oxide, cAMP, PKA

Heavy metals presented in environments can become injurious to human health [2, 16]. In areas where there is a high level of human activities on soils (such as agriculture and grazing), studies are therefore required to monitor the toxicity of such metals in the soils in order to identify the point in time when toxicity problems become real [8, 9, 16].

In particular, vascular endothelial cells (ECs) have long been considered as the primary target in the process of cardiovascular disease (CVD) induced by arsenic exposure, and epidemiological studies have indicated a strong association of high incidences of CVD with inorganic arsenic exposure [1, 12, 18]. It has also been reported that there were decreased serum concentrations of nitric oxide (NO) metabolites (nitric oxide synthase [NOS] activity indicators) among the population in an endemic area of chronic arsenic poisoning in Inner Mongolia [12]. Vascular ECs generate NO from L-arginine *via* a calcium-dependent endothelial NOS (eNOS) [5, 10, 11]. NO plays an important role in vasodilation, and in ECs, NO is catalyzed from the amino acid L-arginine and molecular oxygen by eNOS [13, 17]. Furthermore, recent *in vivo* studies revealed that arsenite exposure significantly suppressed the reduction of blood pressure by acetylcholine infusion, possibly *via* the inhibitory effect of arsenite on eNOS activity in ECs [18]. Other studies also indicated that arsenic exposure caused the impairment of NO formation in rabbits and suppressed the activity of human eNOS *in vitro*. Results from these studies strongly suggested that long-term arsenic exposure could cause reduced eNOS activity and result in decreased NO production.

We therefore hypothesized that impairment of vascular eNOS activity by arsenite could be one of the important mechanisms on vascular dysfunction. However, the biophysical mechanisms of arsenite implications in vascular diseases are not yet explained. Therefore, in our study, we will show how arsenite induces endothelial dysfunction by downregulation of eNOS in vascular endothelial cells. Here, we show for the first time that arsenite decreases the cellular level of cAMP, and this is associated with the decrease of NO production by cAMP-dependent protein kinase A (PKA)-mediated eNOS regulation.

Minimal essential medium (MEM), Hank's balanced salt solution (HBSS), newborn calf serum (NCS), penicillin-streptomycin, L-glutamine, trypsin-EDTA solution, and other plastic wares for cell culture were obtained from Gibco-BRL (Grand Island, NY, U.S.A.). Sodium arsenite ($NaAsO_2$) for acute exposure was purchased from Sigma-Aldrich, Co. Antibodies against eNOS and β-actin were purchased from Transduction Laboratories (Lexington, KY, U.S.A.) and Santa-Cruz Inc., respectively. Antibodies against pCREB (phosphorylated at Ser133) and CREB were purchased from Cell Signaling Technology, Inc. (Beverly, MA, U.S.A.).

Bovine aortic ECs (BAEC) were isolated exactly as previously described [8] and maintained in MEM supplemented with 20% NCS at 37°C under 5% $CO_2$-20% $O_2$ air. The concentration of 10 μM arsenite was selected, with the highest concentration resulting in 20% decrease of total protein level in the exposed BAECs ($LC_{20}$) through the acute

*Corresponding author
Phone: 82-63-270-2436; Fax: 82-63-270-2309;
E-mail: jihomin@chonbuk.ac.kr

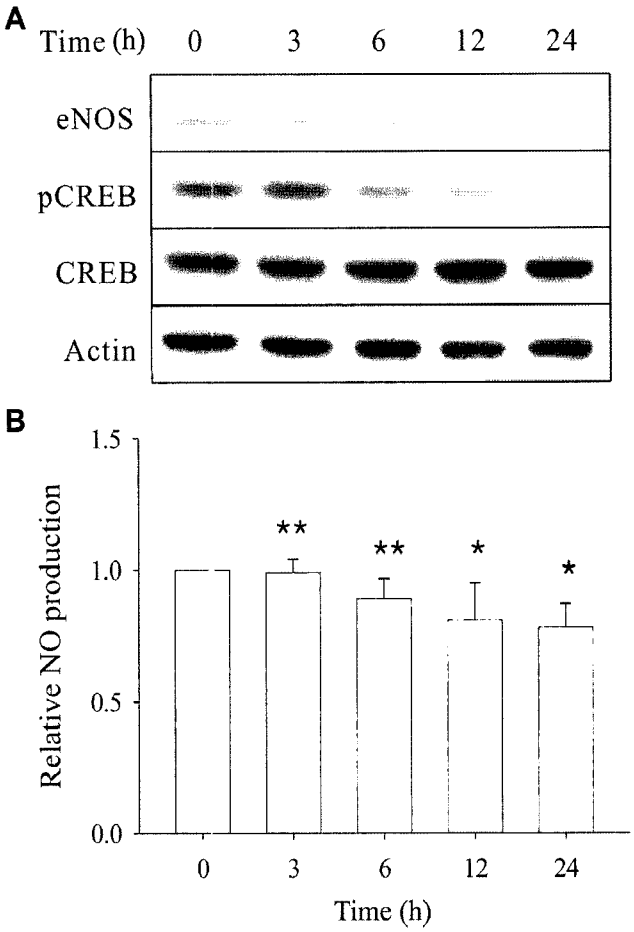


**Fig. 1.** Arsenite inhibits NO production (**A**) and the level of eNOS protein in BAEC (**B**).

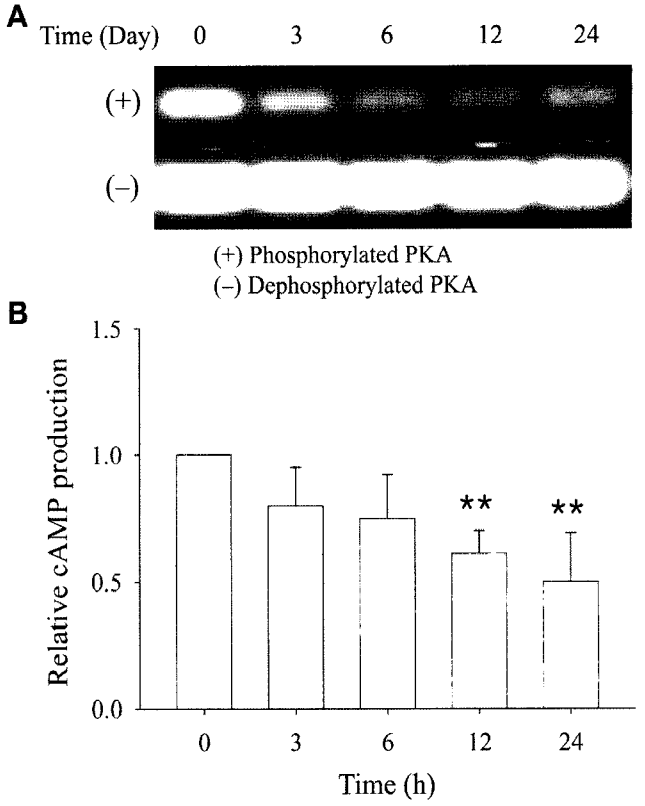


**Fig. 2.** Arsenite inhibits the activity of PKA (**A**) and the decreases the level of intracellular cAMP (**B**).

toxicity assay (data not shown). NO production by BAEC was measured as described in previous studies [3, 14], with minor modifications.

To measure the PKA activity, whole-cell lysates of BAEC were prepared as described previously, with minor modifications [6]. The samples extracted from BAECs exposed with arsenite were stored at −80°C until cAMP assay using the ELISA kit and manual (R&D Systems Europe Ltd., Abingdom, U.K.).

In an attempt to delineate the direct effect of arsenite on NO production, BAECs were incubated with 10 μM arsenite for 24 h, and eNOS expression and NO production were measured. Arsenite decreased NO production in a time-dependent manner (Fig. 1A). As shown in Fig. 1A, 10 μM arsenite decreased the phosphorylation of CREB with no alteration of CREB expression until 24 h, and CREB phosphorylation was significantly decreased with the same patterns of eNOS protein. Taken together, our data suggest that the stimulatory effect of arsenite on eNOS expression and NO production in BAECs is mediated at least in part by CREB phosphorylation.

Next, to determine whether CREB phosphorylation by arsenite is dependent on PKC activity in BAECs, PKA activity was analyzed in BAECs after treatment with 10 μM arsenite. As shown in Fig. 2A, the PKA activity showed the same pattern as with the eNOS protein and NO production shown in Fig. 1. It means that in response to decreasing PKA activity, there was a significant reduction in the NO production and eNOS protein by arsenites.

Binding of cAMP to the regulatory subunit of PKA induces dissociation of the holoenzyme and subsequent phosphorylation of key substrates by the catalytic subunit [4, 15, 19, 20]. Therefore, cAMP is the most important substrate to increase the PKA activity. In this study, to further evaluate the effect of decrease of PKA activity, eNOS protein level, and NO production by arsenite treatment, cellular cAMP formation was determined in BAECs. As shown in Fig. 2B, BAECs treated by arsenite for 24 h significantly showed a relative decreased level of cAMP. Therefore, it suggests that eNOS protein expression and NO production by arsenite may respond to alter cAMP as key molecules.

We already noted that treatment of BAECs with arsenite decreased NO, eNOS protein, PKA activity, and cAMP levels. Therefore, to restore the all-reduction phenomena by arsenite treatment, 5-bromo-cAMP was treated for 1 h in arsenite-treated BAECs. As shown in Fig. 3, stimulation with 5-bromo-cAMP markedly increased intracellular cAMP levels in the arsenite-treated BAECs. Indeed, cAMP

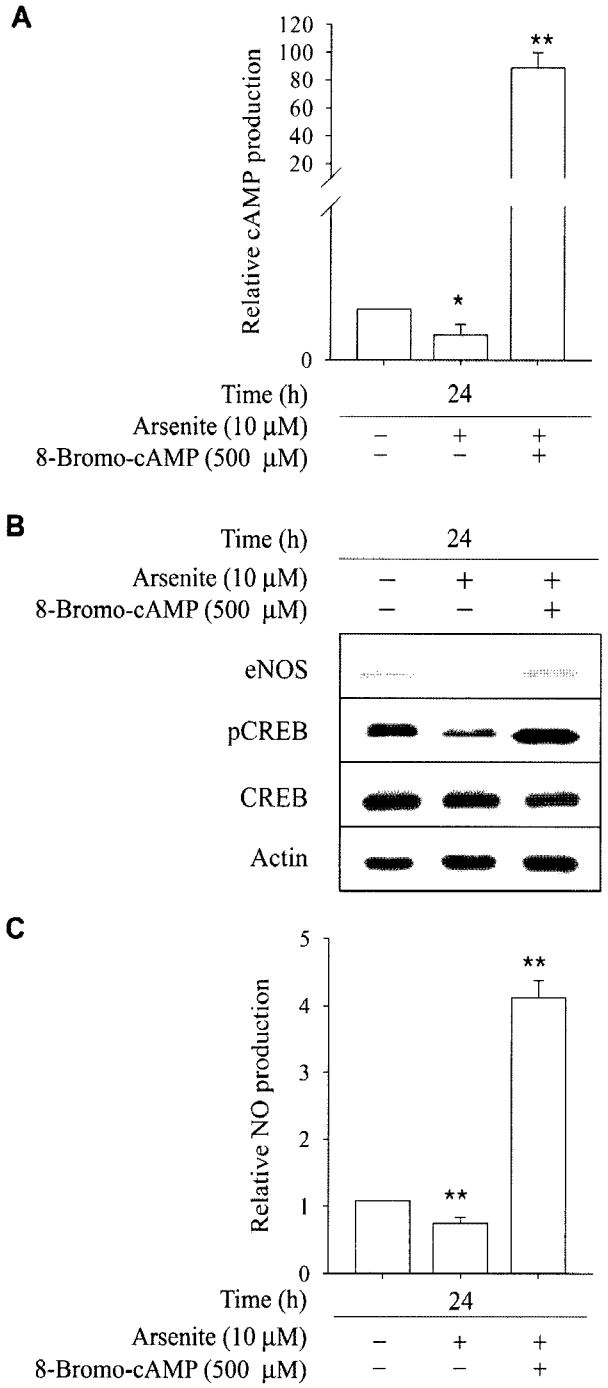


**Fig. 3.** The effect of 5-bromo-cAMP on intracellular cAMP levels (**A**) in arsenite-exposured BAECs was to restore the eNOS protein expression (**B**) and NO production (**C**).

accumulation by treating with 5-bromo-cAMP resulted in the increase of PKA activity (Fig. 3A). This also elicited a marked increase in the phosphorylation of CREB, and then we could observe the increase of eNOS protein level by activating CREB (Fig. 3B). Finally, it found that the activation of cAMP level restored NO production from BAECs treated with CSE for 4 days up to the level of NO produced from BAECs treated with CSE for 1 day (Fig. 3C).

Our current finding, that arsenite decreases pCREB-mediated eNOS gene transcription for up to 24 h under acute exposure period, deserves particular attention, because cAMP decreased NO production *via* either phosphorylation of eNOS [15, 19] or expression of eNOS [19, 20], suggesting a direct effect of cAMP on eNOS modulation. In this previous study, PKA was found to phosphorylate CREB, and PKA inhibitors completely blocked the effect of hypoxia on eNOS gene transcription and NO production [14]. In addition, it was also found that the phosphorylation of CREB was restored in arsenite-exposed BAECs, after 5-bromo-cAMP exposure (Fig. 3B), and thus our study suggests that arsenite decreases NO production by inactivating pCREB-mediated eNOS gene transcription by reducing the cAMP-dependent PKA pathway.

In summary, the present study has shown that arsenite reduced both the expression of eNOS and NO production by reducing cAMP in ECs, which is associated with endothelial dysfunction. These findings suggest that the inhibition of eNOS by arsenite in ECs is an early event that may antecede the full development of COPD.

## Acknowledgment

This work was supported by a research grant of the Chunbuk National University in 2006 (Grant #105522), awarded to Prof. Jiho Min. The Author is grateful for their support.

## References

1. Barua, R. S., J. A. Ambrose, S. Srivastava, M. C. DeVoe, and L.-J. Eales-Reynolds. 2003. Reactive oxygen species are involved in smoking-induced dysfunction of nitric oxide biosynthesis and upregulation of endothelial nitric oxide synthase. *Circulation* **107:** 2342–2347.
2. Chang, J.-S., I.-H. Yoon, and K. W. Kim. 2007. Isolation and *ars* detoxification of arsenite-oxidizing bacteria from abandoned arsenic-contaminated mines. *J. Microbiol. Biotechnol.* **17:** 812–821.
3. Cho, D.-H., Y. J. Choi, S. A. Jo, and I. Jo. 2004. Nitric oxide production and regulation of endothelial nitric-oxide synthase phosphorylation by prolonged treatment with troglitazone. *J. Biol. Chem.* **279:** 2499–2506.
4. Cho, H. J., J. Y. Cho, M. H. Rhee, and H.-S. Kim. 2007. Inhibitory effects of cordyceptin (3'-deoxyadenosine), a component of *Cordyeps militaris*, on human platelet aggregation induced by thapsiargin. *J. Microbiol. Biotechnol.* **17:** 1134–1138.
5. Harrison, D. G. 1997. Cellular and molecular mechanisms of endothelial cell dysfunction. *J. Clin. Invest.* **100:** 2153–2157.
6. Kang, B.-H., I. Jo, S. Y. Eun, and S. A. Jo. 2003. Cyclic AMP-dependent protein kinase A and CREB are involved in neuregulin-induced synapse-specific expression of acetylcholine receptor gene. *Biochem. Biophys. Res. Commun.* **304:** 758–765.

7. Kim, H. J., E. Chatani, Y. Goto, and S. R. Paik. 2007. Seed-dependent accelerated fibrillation of α-synuclein induced by periodic ultrasonication treatment. *J. Microbiol. Biotechnol.* **17:** 2027–2032.
8. Kim, H. P., J. Y. Lee, J. K. Jeong, S. W. Bas, H. K. Lee, and I. Jo. 1999. Nongenomic stimulation of nitric oxide release by estrogen is mediated by estrogen receptor α localized in caveolae. *Biochem. Biophys. Res. Commun.* **263:** 257–262.
9. Kim, Y. S., J. Min, H. N. Hong, J. H. Park, K. S. Park, and M. B. Gu. 2007. Analysis of the stress effects of endocrine disrupting chemicals (EDCs) on *Escherichia coli*. *J. Microbiol. Biotechnol.* **17:** 1390–1393.
10. Kubes, P., M. Suzuki, and D. N. Granger. 1991. Nitric oxide: An endogenous modulator of leukocyte adhesion. *Proc. Natl. Acad. Sci. USA* **95:** 4651–4655.
11. Kugiyama, K., H. Yasue, K. Okumura, H. Ogawa, K. Fujimoto, K. Nakao, M. Yoshimura, T. Motoyama, Y. Inobe, and H. Kawano. 1996. Nitric oxide activity is deficient in spasm arteries of patients with coronary spastic angina. *Circulation* **94:** 266–272.
12. Lee, M. Y., B. I. Jung, S. M. Chung, O. N. Bae, J. Y. Lee, J. D. Park, J. S. Yang, H. Lee, and J. H. Chung. 2003. Arsenic-induced dysfunction in relaxation of blood vessels. *Environ. Health Perspect.* **111:** 513–517.
13. Loscalzo, J. and G. Welch. 1995. Nitric oxide and its role in the cardiovascular system. *Prog. Cardiovasc. Dis.* **38:** 87–104.
14. Min, J., Y.-M. Jin, J.-S. Moon, M.-S. Sung, S. A. Jo, and I. Jo. 2006. Hypoxia-stimulated transcriptional activation of the eNOS gene is mediated through the Tax-responsive element (TRE) in endothelial cells. *Hypertension* **47:** 1189–1196.
15. Niwano, K., M. Arai, N. Koitabashi, S. Hara, A. Watanabe, K. Sekiguchi, T. Tanaka, I. Iso, and M. Kurabayashi. 2006. Competitive binding of CREB and ATF2 to cAMP/ATF responsive element regulates eNOS gene expression in endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **26:** 1036–1042.
16. Nordberg, G. F., T. Jin, F. Hong, A. Zhang, J. P. Buchet, and A. Bernard. 2006. Biomarkers of cadmium and arsenic interactions. *Toxicol. Appl. Pharmacol.* **2:** 191–197.
17. Radomski, M. W., R. M. Palmer, and S. Moncada. 1987. Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet* **2:** 1057–1058.
18. Tsou, T. C., F. Y. Tsai, Y. W. Hsieh, L. A. Li, S. C. Yeh, and L. W. Chang. 2005. Arsenite induces endothelial cytotoxicity by down-regulation of vascular endothelial nitric oxide synthase. *Toxicol. Appl. Pharmacol.* **208:** 277–284.
19. Zhang, X. and T. H. Hintze. 2000. cAMP signal transduction cascade, a novel pathway for the regulation of endothelial nitric oxide production in coronary blood vessels. *Arterioscler. Thromb. Vasc. Biol.* **21:** 797–803.
20. Zhang, X. L., H. Tada, Z. Wang, and T. H. Hintze. 2002 cAMP signal transduction, a potential compensatory pathway for coronary endothelial NO production after heart failure. *Arterioscler. Thromb. Vasc. Biol.* **22:** 1273–1278.