行政院國家科學委員會補助專題研究計畫成果報告

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※三價砷對人類磷脂質過氧化物麩氨基硫氧化酵 ※
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計畫類別：■個別型計畫　　□整合型計畫

計畫編號：NSC89-2320-B-041-016

執行期間：89 年 8 月 1 日至 90 年 7 月 31 日

計畫主持人：黃暉升

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中 華 民 國　90 年 10 月 23 日

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Abstract Phospholipid hydroperoxide glutathione peroxidase (PHGPx) is unique in the substrate specificity among the glutathione peroxidase family, because it can interact with lipophilic substrates including the peroxidized phospholipids and cholesterol and reduce these hydroperoxide to hydroxide compounds. However, what kinds of ligand can regulate the PHGPx expression is still unknown. In our preliminary results, we found that sodium arsenite induced PHGPx down-regulation, $p21^{WAF1/CIP1}$ up-regulation, and finally apoptosis in A431 cells and EA.hy926 cells. Over-expression of PHGPx could prevent the arsenite-induced PHGPx down-regulation, $p21^{WAF1/CIP1}$ up-regulation, and apoptosis in A431 cells. *N*-Acetyl-L-cysteine could also significantly prevent the arsenite-induced PHGPx down-regulation and $p21^{WAF1/CIP1}$ up-regulation. Therefore, we concluded that ROS were involved in the arsenite-induced PHGPx down-regulation, $p21^{WAF1/CIP1}$ up-regulation and apoptosis in A431 cells.

Keywords arsenite, ROS, PHGPx, $p21^{WAF1/CIP1}$

中文摘要：

在過氧化物麩氨基硫過氧化酵素的家族中，人類磷脂質過氧化物麩氨基硫過氧化酵素對受質特別有專一性，因為它可以還原一些親脂性的受質，譬如過氧化的磷脂質或膽固醇。過度表現人類磷脂質過氧化物麩氨基硫過氧化酵素在細胞內也可以保護細胞免受過氧化的傷害，然而至今卻尚未找到可以調控其表現的物質。在我的初步結果發現三價砷可以抑制人類磷脂質過氧化物麩氨基硫過氧化酵素的表現、促進抑癌基因 p21 **的表現、最後造成** A431 **及** EA.hy926 **的細胞凋亡；而過度表現** PHGPx **可以預防這些現象的發生。除此利用** *N*-Acetyl-L-cysteine **這種抗氧化劑也可以預防這些現象的發生。因此我們推論活性過氧化物參與在砷化物對人類磷脂質過氧化物麩氨基硫過氧化酵素表現的抑制、抑癌基因** p21 **表現的促進，以及** A431 **及** EA.hy926 **的細胞凋亡。**

關鍵詞：砷；活性過氧化物；人類磷脂質過氧化物麩氨基硫過氧化酵素；抑癌基因 p21

INTRODUCTION

Sodium arsenite exists ubiquitously in our environment, and various forms of arsenic circulate in soil, water, air, and living organisms. It has been reported that high arsenic levels in drinking water (0.35-1.14 mg/liter) will induce neurotoxicity, liver injury, peripheral vascular disease (known as blackfoot disease), and increase risks of cancer of skin, bladder, kidney, lung and colon (Bagla, *et al.*, 1996). However, in the treatment of acute promyelocytic leukemia (APL), two arsenic compounds, including arsenic trioxide (As_2O_3) and

arsenic disulfide, used in some traditional Chinese remedies are very effective (Huang, *et al.*, 1995). The mechanism of arsenite on APL might be through induction of apoptosis in the leukemia cell (Chen, *et al.*, 1996). Recent studies indicate that arsenite may generate ROS to induce apoptosis in Chinese hamster ovary cells (Wang, *et al.*, 1996) and NIH3T3 cells (Chen, *et al.*, 1998). However, the mechanism is still unclear.

PHGPx, a selenium-dependent glutathione peroxidase, belongs to glutathione peroxidase family, which consists of five isozymes. Among these isozymes, PHGPx is unique in the substrate specificity because it can interact with lipophilic substrates including the peroxidized phospholipids and cholesterol and reduce these hydroperoxide to hydroxide compounds (Ursini, *et al.*, 1985; Thomas, *et al.*, 1990).

In this paper, we found that sodium arsenite down-regulated PHGPx expression. In the same conditions, sodium arsenite also induced CDK inhibitor $p21^{WAF1/CIP1}$ (p21) up-regulation. It was also found arsenite induced apoptosis in A431 cell and EA.hy926 after 24 hour treatment. We found PHGPx-overexpressed transfectant (G4) and *N*-Acetyl-L-cysteine (a thiol-containing antioxidant) could prevent the arsenite-induced PHGPx down-regulation, p21 up-regulation, and apoptosis in A431 cells. Therefore, we concluded that ROS were involved in the arsenite-induced PHGPx down-regulation, p21 up-regulation and apoptosis in A431 cells.

RESULTS

Western blot and activity analysis of human PHGPx

A cDNA encoding the entire amino acid-coding region of human PHGPx was obtained, and the expression vector was constructed as described under Materials and Methods. In figure 1A, the enzymatic activity of G4 cells (PHGPx-overexpression) was almost twice of that in V8 cells (neo) and normal A431 cells. The same result was also observed in PHGPx protein expression (Fig. 1B).

Overexpression of PHGPx resistant to sodium arsenite-induced PHGPx down-regulation

The effect of sodium arsenite on the PHGPx promoter activity, mRNA expression, protein production and enzyme activity were studied. With the aid of PHGPx luciferase reporter, we found sodium arsenite could down-regulate PHGPx promoter activity in dose- and time-dependent manners in V8 cells (Fig. 2A). The conditions of the cells were fine even though the luciferase activity of the V8 cells treated with 30 μM sodium arsenite for 8 h was about a quarter to the cells treated with vehicle. However, the luciferase activity of the G4 cells treated with 30 μM sodium arsenite for 8 h was only one half to the cells treated with vehicle (Fig. 2A). That is to say, the less extent of down-regulation was observed in PHGPx over-expressed cells when the cells were treated with sodium arsenite. The same results were also observed in mRNA expression (Fig. 2B), protein production

(Fig. 2C), and enzyme activity (Fig. 2D). We suggested that PHGPx could remove ROS, which was involved in the arsenite-induced PHGPx down-regulation.

Sodium arsenite induced p21 up-regulation

To further prove the function of cells was fine in the assay system, the effect of sodium arsenite on the p21 expression was studied. Figure 3A showed that the cells treated with 15 μM sodium arsenite for 7 h could up-regulate p21 promoter activity about 2.5 fold to the cells treated with vehicle. The arsenite-induced p21 protein production also showed a time- and dose-dependent manner, which paralleled with its effect on the promoter expression (Fig. 3B).

PHGPx-overexpressed mutant resistant to sodium arsenite-induced p21 up-regulation

To prove the ROS was also involved in the arsenite-induced p21 up-regulation. The promoter activity of p21 in V8 and G4 cells treated with various dose of arsenite at 8 h was performed. The luciferase activity of p21 in V8 cells treated with 30 μM sodium arsenite for 8 h was about a triple to the cells treated with vehicle. However, the luciferase activity of p21 in G4 cells treated with 30 μM sodium arsenite for 8 h was below one half to the V8 cells in the same condition (Fig. 4). The same effect was also found even though in the vehicle. We hypothesized that PHGPx could prevent arsenite-induced p21 up-regulation through ROS scavenging.

Effect of NAC on the regulation of PHGPx and p21 promoter activity by sodium arsenite

To reconfirm our hypothesis that ROS was involved in the arsenite-induced effects. A431 cells transfected with pGP4-2 (0.5 μg) and -galactosidase plasmid (0.2 μg) were incubated with *N*-acetyl-L-cysteine (NAC), a thiol-containing antioxidant, for 1 h before adding arsenite. We found NAC was able to prevent PHGPx from arsenite-induced gene down-regulation (Fig. 5A), but also prevent p21 from arsenite-induced gene up-regulation (Fig. 5B). Therefore, we concluded that arsenite-induced PHGPx down-regulation and p21 up-regulation were through ROS production.

Induction of apoptosis by arsenite

After exposure to various concentration of arsenite, internucleosomal DNA fragments, a characteristic feature of apoptosis, was evaluated in A431 cells and EA.hy926. Figure 6 showed that arsenite treatment produced a dose-dependent increase of DNA fragment in A431 cells (Fig. 6A) and EA.hy926 (Fig. 6B). These results indicated that arsenite could induce apoptosis in a dose-dependent manner not only in A431 cells, but also in EA.hy926 endothelial cells.

PHGPx-overexpressed mutant resistant to sodium arsenite-induced apoptosis

To determine whether PHGPx was able to prevent arsenite-induced apoptosis, V8 and G4 cells were exposed to 30 μM of arsenite for various hours, and the ratio of sub-G1 was measured by flow cytometry analysis (Fig. 7). PHGPx-overexpressed G4 cells were more resistant to arsenite-induced cell death, and the sub-G1 ratio in G4 cells (7.2 %) was less than that in parental V8 cells (11.27 %) under 30 μM arsenite treatment. Based on these findings, we proposed that PHGPx removed ROS, thereby preventing the arsenite-induced cell death signals.

DISCUSSION

Two pieces of evidence were provided in this study to indicate that ROS played an important role in the arsenite-induced PHGPx down-regulation, p21 up-regulation and apoptosis in A431 cells. Firstly, overexpression of PHGPx in A431 cells could prevent arsenite-induced PHGPx down-regulation, p21 up-regulation, and apoptosis in A431 cells. Secondly, *N*-Acetyl-L-cysteine, a thiol-containing antioxidant inducing glutathione production as well as removing ROS, also inhibited arsenite-induced effects. Taken together, we concluded that ROS was involved in the arsenite-induced PHGPx down-regulation, p21 up-regulation and apoptosis in A431 cells.

Another novel finding was arsenite could down-regulate PHGPx expression through ROS production. In this study, we found arsenite down-regulated PHGPx promoter and mRNA expression in a time- and dose-dependent manner. The responsive elements in the PHGPx promoter regulated by ROS and the signal transduction pathway following arsenite treatment in the A431 cells should be further clarified.

Furthermore, to study the mechanism of arsenite-induced pathophysiological phenomena, the relationship between ROS and arsenite-induced PHGPx down-regulation, p21 up-regulation, and apoptosis in A431 cells should be elucidated in the future.

REFERENCE:

1. Bagla, P.; Kaiser, J. *Science* **274:** 174-175; 1996.
2. Huang, S. L.; Guo, A. X.; Xiang, Y.; Wang, X. B.; Ling, H. J.; Fu, L. *Chin. J. Hematol.* **16:**26; 1995.
3. Chen, G. Q.; Zhu, J.; Shi, X. G.; Ni, J. H.; Zhong, H. J.; Si, G. Y. Jin, X. L. *Blood* **88:**1052-1061; 1996.
4. Wang, T. S.; Kuo, C. F.; Jan, K. Y.; Huang, H. *J. Cell. Physiol.* **169:**256-268; 1996.
5. Chen, W.; Martindale, J. L.; Holbrook, N. J.; Liu, Y. *Mol. Cell. Biol.* **18:**5178-5188; 1998.
6. Ursini, F.; Maiorino, M.; Gregolin, C. *Biochim. Biophys. Acta* **839:**62-70; 1985.
7. Thomas, J. P.; Maiorino, M.; Ursini, F.; Girotti, A. W. *J. Biol. Chem.* **265:**454-461; 1990.