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TITLE: INHIBITION OF MITOTIC-SPECIFIC HISTONE PHOSPHORYLATION BY SODIUM ARSENITE

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Inhibition of Mitotic-Specific Histone Phosphorylation by Sodium Arsenite

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ABSTRACT

Synchronized cultures of Chinese hamster cells (line CHO) were used to measure the effects of 10μM sodium arsenite on histone phosphorylation. This treatment caused cell proliferation to be temporarily arrested, after which the cells spontaneously resumed cell proliferation in a radiomimetric manner. Immediately following treatment, it was found that sodium arsenite affected only mitotic-specific H1 and H3 phosphorylations. Neither interphase, nor mitotic, H2A and H4 phosphorylations were affected, nor was interphase H1 Phosphorylation affected. The phosphorylation of H1 was inhibited only in mitosis, reducing H1 phosphorylation to 38.1% of control levels, which was the level of interphase H1 phosphorylation. The phosphorylation of both H3 variants was inhibited in mitosis, the less hydrophobic H3 to 19% and the more hydrophobic H3 to 24% of control levels. These results suggest that sodium arsenite may inhibite cell proliferation by interfering with the cyclin B/p34^{cdc2} histone kinase activity which is thought to play a key role in regulating the cell cycle. It has been proposed by our laboratory that H1 and H3 phosphorylations play a role in restructuring interphase chromatin into metaphase chromosomes. Interference of this process by sodium arsenite may lead to structurally damaged chromosomes resulting in the increased cancer risks known to be produced by arsenic exposure from the environment.

INTRODUCTION

Arsenic is mobilized to the environment during energy production based on coal, oil shale, and geothermal sources. It also enters the environment from industrial sources such as smelting operations and agricultural applications of organoarsenicals in herbicides, insecticides, fungicides, and algicides. This environmental arsenic represents a potential health hazard of unknown magnitude since arsenicals are associated with increased risks of cancer of the skin, respiratory tract, hematopoietic system and lymphatic system in humans, and they are teratogenic and embryotoxic in a variety of animal species (see references in Gurley, et al., 1980, 1983a, 1986; Spall, et al., 1986).

To answer questions concerning the environmental impact of arsenic, we have previously used characterized cell cultures to measure the effects of arsenic on biological parameters, such as cell proliferation, cell metabolism, and chromosome constituents, that might be relevant to the cancer problem (Gurley, et al. 1978b, 1980, 1981a, 1981b, 1983a, 1986; Spall et al., 1986). In those studies we found that low concentrations of sodium arsenite (10μM $NaAsO_2$) induced culture growth kinetics that were radiomimetic, i. e. they caused a cell division delay period followed by spontaneous recovery of cell division in the continued presence of the sodium arsenite (Gurley, et al., 1979, 1980). During this division delay, histone Hl phosphorylation was reduced to 40% of control levels. This phosphorylation recovered to the control levels just before recovery of cell division (Gurley, et al., 1979, 1980). We paid particular attention to the effects of arsenic on this growth related histone phosphorylation, since previous experiments in our laboratory had correlated histone phosphorylation with cell division in cultured CHO Chinese hamster cells (Gurley and Walters, 1971; Gurley et. al., 1974; 1978a; 1978b). These results had led to the suggestion that histone phosphorylation is vitally involved in the cell proliferation process. The observation that sodium arsenite both inhibited H1 phosphorylation and induced a cell division delay supported this hypothesis.

In the work reported here we have used cell cultures synchronized in the cell cycle to determine whether the inhibition of histone H1 phosphorylation by sodium arsenite is restricted to a particular phase of the cell cycle or whether it is inhibited throughout the cell cycle. If the inhibition is restricted to one phase of the cell cycle it would imply that sodium arsenite specifically inhibits one histone kinase from among the plethora of histone kinases known to be operating during cell proliferation. Such specificity would make sodium arsenite a valuable tool for studying the mechanisms that control cell proliferation.

MATERIALS AND METHODS

Cell Cultures

Chinese hamster cells (line CHO) were grown exponentially in suspension culture as previously described (Gurley et al., 1980). Culture growth was monitored by measuring the culture's cell concentration with a Coulter counter.

Synchronization of Cells

1-liter cultures of exponentially growing cells were synchronized in various phases of the cell cycle as previously described (Gurley et al., 1978a). Briefly, the cells were first synchronized in early G1 by maintenance for 36h in isoleucine-deficient medium. These cells were then used for histone phosphorylation measurements in G1 (with or without sodium arsenite), or they were resynchronized near the G1/S boundary by resuspending them in complete medium containing 1mM hydroxyurea.

After 10h in hydroxyrea, the cells were arrested near the G1/S boundary and used for histone phosphorylation measurements or they were released from hydroxyurea blockade by resuspension in fresh medium and allowed to traverse S phase. One hour after this release the cells were used for histone phosphorylation measurements in S phase.

To acquire cultures synchronized in G2, the cells that were released from hydroxyurea blockade were treated with 7.5μg Hoechst 33342/ml of culture. This treatment permits cells to traverse S and then reversibly arrests them in late G2 (Tobey, et al., 1990). After 6 h in Hoechst this culture was used for histone phorphorylation measurements in G2 phase.

To obtain cells synchronized in M, Colcemid was added to cultures 5 h after release from hydroxyurea blockade. These cells traversed S and G2 phases and were arrested and resynchronized in mitosis by metaphase arrest. Three h after adding Colcemid the cells entering M were used for histone phosphorylation measurements during mitosis.

Arsenic Treatment

The synchronized cultures were used to measure the effect of sodium arsenite on histone phosphorylation. To do this, the synchronized l-liter cultures were divided into two 500 ml cultures and sodium arsenite was added to one culture to produce a 10μM $NaAsO_2$ dose. The other 500 ml culture received no arsenic as a control. The $NaAsO_2$ solution (100 times the final concentration in the culture) was prepared fresh in water and sterilized by filtration through a 0.22 μm Millipore Swinnex-13 filter. A 50 ml aliquot of culture was removed from each 500ml culture when arsenic was added and it was incubated in a separate small spinner flask. The aliquot (with or without arsenic) contained no ^{32}P and was used for cell cycle analysis at the end of the 2 h ^{32}P-labeling period (see below) and for cell concentration measurements by Coulter counter.

Labeling Histones with ^{32}P

The 450 ml synchronized cultures (with or without sodium arsenite) were treated with $H_3{}^{32}PO_4$ at a concentration of 20 mCi/liter of culture. After a 2-h labeling period, the cells were removed from their growth medium by centrifugation and used for histone preparation.

Cell Cycle Analysis

The cell cycle distribution of cells in the synchronized cultures was determined by flow cytometry at the end of the ^{32}P-labeling period. To do this, cells in the 50 ml non-labled culture were stained with Mithramycin, a fluorescent DNA stain. The relative DNA content of each cell was then determined with the Los Alamos flow cytometer,

FMF-II, as previously described (Crissman, et al., 1977). The fractions of cells in G1, S, and G2+M were derived from the DNA histograms produced by the flow cytometer using the computer program "Multicycle" (Rabinovitch, 1991). Since the flow cytometer does not discriminate between G2 and M cells, the fraction of cells in M in the Colcemid and Hoechst treated cultures was determined by staining a 1 ml aliquot of culture with acridine orange and counting the percentage of mitotic cells using a fluorescence microscope (Gurley, et al., 1973).

Histone Preparation

Histones were prepared from the 450 ml cultures containing 300,000 cells/ml as described previously (Gurley, et, al. 1983). In these experiments approximately 1.4 x 10^8 cells were homogenized in 0.14M NaCl containing 0.05M sodium bisulfite which prevents histone dephosphorylation. Crude chromatin was prepared from this homogenate and histones were extracted using 0.4N H_2SO_4. The histones were recovered by acetone precipitation and dissolved in aqueous 0.2% trifluoroacetic acid for HPLC.

HPLC of Histones

Histones were purified and fractionated into the five chemically distinct classes (Hl, H2A, H2B, H3, and H4) by a reversed-phase HPLC method developed in our laboratoy (Gurley et al. 1983b). The variations on this method have been previously reveiwed (Gurley, et al., 1990). In this work we used a Waters HPLC system fitted with a 15 cm μBondapak CN column (see Fig. 4b of Gurley, et al., 1990) and an elution gradient of acetonitrile in water containing 0.2% trifluoroacetic acid running from 0% to 20% acetonitrile in 10 min and then from 20% to 50% acetonitrile in 180 min (see fig. 9a of Gurley, et al., 1990). With this method histone H3 was subfractionated into two variants, the less hydrophobic (lhp) H3 and the more hydrophobic (mhp) H3.

The histones eluting from the column were monitored by UV absorption using a Waters model 481 flow variable wavelength spectrophotometer set at 215 nm. The radioactivity of the ^{32}P incorporated into the proteins was measured by flow liquid scintillation counting using a Berthold LB504 HPLC Radioactivity Monitor and Flo-Scint A liquid scintillation cocktail (Packard). The UV absorption and radioactivity data were collected in a Berthod LB510 Chromatography Data System computer which calculated the specific activity of each HPLC peak as cts/volt from the radioactivity counts in each peak and the area of the UV peak measured in volts.

RESULTS

The cell cycle distribution of the synchronized cultures used in these experiments was determined by flow cyctometry as shown in Fig. 1. The percentage G1 cells was determined from the first Gaussian peak, the percentage S cells was determined from the following polynomial area, and the percentage G2+M cells was determined from the second Gaussian peak (Fig. 1A). With the exception of the M culture (Fig. 1F), there were no significant numbers of mitotic cells in the synchronized cultures (Fig. 1, B-D). Fluorescence microscopy indicated that all the G2+M cells in the Colcemid-arrested M culture were M cells (Fig. 1F) and less that 2% of the G2+M cells in the Hoechst-arrested G2 culture were M cells (Fig. 1E).

The fractionation of histones by HPLC is demonstrated in Fig. 2A. Histone H1 was eluted first at 90 min followed by a group of acid soluble nonhistone proteins between 90 and 130 min. These were followed by H2B, H2A and H4 between 130 and 145 min. Finally, the two varients of histone H3 were eluted, lhp-H3 at 160 min and mhp-H3 at 170 min.

The ^{32}P-phosphate incorporated into these proteins in 2h is shown in Fig. 2B. In these exponential cells histone H1 is significantly phosphorylated as are two varients of H2A which are not competely resolved by this chromatography. Histone H4 is phosphorylated to a lesser extent and the two H3's contain only a trace of phosphorylation. H3 is only phosphorylated in mitosis (Gurley, et al. 1978a) and exponential cells contain only 1-2% mitotic cells which account for its trace in Fig. 2B. Previous work has demonstrated that histone H2B is not phosphoryated in CHO cells (Gurley, et al., 1973). Four peaks of phosphorylation are observed amoung the unidentified acid soluble nonhistone proteins (u1, u2, u3, and u4).

The 2h incorporation of ^{32}P-phosphate into the four phosphorylated histones was measured from such data in synchronized cultures either with a 2h treatment with 10μM $NaAsO_2$, or without such treatment. Comparisons of the specific activities of these histones is shown in Fig. 3. The phosphorylation of H1 (Fig. 3A) was found to be absent in G1 cells, active in cells at the G1/S boundary and throughout S and G2, and greatest in M as previously observed (Gurley, et al., 1978b). $NaAsO_2$ inhibited only the mitotic H1 phosphorylation, reducing the phosphorylation rate to 38.1% of the control level; that is, to the level of interphase phosphorylation.

The phosphorylations of histones H2A and H4 were constitutive, being independent of cell cycle phase as prevously observed (Gurley, et al., 1978b) and their phosphorylations were unaffected by $NaAsO_2$ (Figs. 3B and 3E). In contrast, the two H3 variants displayed only trace levels of phosphorylation in G1 and S phases and only low levels in G2 which was not greatly effected by $NaAsO_2$ (Figs. 3C and 3D). However, the phosphorylation of H3 was greatly elevated in M and this M-specific H3

phosphorylation was greatly inhibited by $NaAsO_2$ to 19% of control in lhp-H3 (Fig. 3C) and to 24% of control in mhp-H3 (Fig. 3D).

DISCUSSION

These experiments demonstrate that 10μM $NaAsO_2$ inhibits only the mitotic-specific H1 and H3 phosphorylations. Interphase histone phosphorylations were not significantly affected. We have prevously demonstrated that this low dose of $NaAsO_2$ does not affect ATP pool levels (Gurley, et al., 1980). The observations that interphase, as well as mitotic, H2A and H4 phosphorylations are essentially unaffected by this treatment confirms that the mitotic-specific inhibition of H1 and H3 phosphorylation is not the result of a non-specific affect on ATP synthesis.

Our laboratory has previously proposed an involvement of mitotic-specific H1 and H3 phosphorylations in the control of cell proliferation (Gurley, et al., 1978a; 1978b). The blockade of cell division with $NaAsO_2$ (Gurley, et al., 1980) and the simultaneous inhibition of mitotic-specific H1 and H3 phosphorplations with $NaAsO_2$ support this hypothesis. Furthermore, this work implies that $NaAsO_2$ may induce this division delay by specifically inhibiting one or two specific histone kinases from among the several histone kinases known to be operating during the cell cycle (Sherr, 1993).

One likely histone kinase candidate for $NaAsO_2$ inhibition is the cyclin B/$p34^{cdc2}$ complex. In proliferating cells the catalytic activity of this histone kinase is confined to M phase (reviewed by Norbury and Nurse, 1992). $NaAsO_2$ may inhibit this kinase by disrupting the interaction between the cyclin B and the $p34^{cdc2}$ or it may prevent removal of the inhibitory phosphates from threonine-14 and tyrosine-15 within the ATP-binding site of $p34^{cdc2}$ (Sherr, 1993). Other possibilities are that the arsenite might inhibit the phosphorylation of the cycin B which is required for activation of this M-specific histone kinase, or it might interfere with the binding of the cdc25 phosphatase to the cyclin B/$p34^{cdc2}$ complex (Zheng and Ruderman, 1993). Little is known about the H3 kinases so it is not possible to select a candidate for $NaAsO_2$ inhibition of H3 phosphorylation at this time.

In our previous work we have domonstrated a correlation between histone phosphorylation and chromatin condensation (Gurley, et al., 1981). Those observations lead to the hypothesis that the mitotic phosphorylation of H1 and H3 play a role in the restructuring of interphase chromatin into metaphase chromosomes. Interference of this restructuring process through $NaAsO_2$ inhibition of H1 and H3 phosphorylation may lead to structurally damaged chromosomes resulting in the increased risks of cancer discussed at the beginning of this report. These speculations provide ideas for new hypotheses concerning the relationships between arsenic carcinogenicity, histone phosphorylation and chromatin structure. The data in this report indicate that $NaAsO_2$ may be a useful probe into the mechanisms controlling the cell cycle and their relationships to carcinogenesis.

Acknowledgements

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FIGURE LEGENDS

Figure 1. Flow cytometry of synchronized CHO cultures. The cell cycle distribution of cells in G1, S, and G2 + M was determined by computer analysis of these histograms and by fluorescence microscopy to determine the mitotic index.

Figure 2. Reversed-phase HPLC of histones from control (no arsenite treatment) exponential CHO cells labeled 2h with ^{32}P-phosphate. (A) Elution of histones detected by UV absorption at 215 nm. (B) Radioactivity of ^{32}P incorporated into proteins detected by flow liquid scintillation counting. The bars under the peaks denote the areas integrated. The first and last bars indicate the levels used for background subtraction.

Figure 3. Phosphorylation of histone fractions in various phases of the cell cycle in untreated control cells and in cells treated with 10μM sodium arsenite. Histone phosphorylation was measured as the specific activity (counts/volt) obtained from HPLC fractions such as seen in Figure 2.

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Arsenite Effects On Histone Phosphorylation