Effect of histone acetylation on the formation and removal of B(a)P chromatin adducts

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ABSTRACT

The modification of core histone proteins in mouse 10T1/2 cells and human lung epithelioid (A549) cells by B(a)PDE *in vivo* and *in vitro* was found to be similar. Only histones H2A and H3 were extensively modified. Also other proteins, possibly A24 protein and the minor histone H1 species seem to be binding relatively high levels of this ultimate carcinogen.

Butyrate treatment which causes hyperacetylation of the core histones, did not change the specificity of B(a)PDE binding to core histones, nor did it affect the initial level of DNA modification. The acetylated species of histone H3 were all accessible to B(a)PDE, suggesting that these ϵ -amino-groups of the lysine residues are not the targets of the B(a)PDE. The rate of removal of B(a)P-DNA adducts was not affected by butyrate treatment in either normal human or XP fibroblasts. Furthermore the B(a)P-core histones were not preferentially removed from normal human fibroblast chromatin during a 24 h post-treatment incubation.

INTRODUCTION

Chromatin is a nucleoprotein complex (for reviews see references 1,2 and 3), which characterizes the genomic organization of the higher eukaryotic cell.

The various chromatin components are subject to extensive post-synthetic modification (4-8), which are currently thought to influence the differentiated state and the level of gene expression of the eukaryotic cells (4,9).

In this context post-synthetic modification of chromatin by B(a)PDE, the ultimate carcinogenic form of B(a)P (10-17), may be an important factor in changing the structure of chromatin and thereby in some as yet unknown manner initiating the process of neoplastic transformation. It is therefore important to know precisely the interaction of this hydrocarbon with chromatin.

During the past few years several reports have examined the interaction of B(a)P metabolites and B(a)PDE with chromatin, and the distribution of

B(a)P-DNA adducts in chromatin has been shown to be non-uniform. For instance, the linker DNA, which connects adjacent nucleosomal core particles, contains more covalent B(a)P adducts per unit DNA, than the DNA associated with the nucleosomal core particles (18-21). More recent work suggests that in several cell types (22) as well as under in vitro conditions (19,20), the linker DNA is three times more accessible to B(a)PDE than the nucleosomal core DNA. The binding of B(a)PDE or B(a)P metabolites to histones has been shown to vary (23-26) which may reflect variations in in vitro experimental conditions such as ionic strength (27). Nevertheless, virtually all reports indicate that the nucleosomal core histone H4 seems relatively inaccessible to B(a)PDE.

In recent studies (28,29) we have shown that in hamster embryo cells histones H2A and H3 were modified in vivo and that this B(a)P histone binding represents in situ covalent interaction (29). Furthermore it was shown that other carcinogenic hydrocarbons bind to the core histones with similar specificity (A. Kootstra, M.C. MacLeod, R. Iyer, J.K. Selkirk and T.J. Slaga, manuscript submitted). Since this specificity may be a function of the three dimensional structure of the nucleosome, the binding of B(a)PDE to core histones from other cells was examined, in order to establish whether this selective binding is a general phenomenon. Furthermore, since butyrate is capable, among other things (7,8,30-32) of producing histone hyperacetylation (33-35), we have examined the binding and removal of B(a)P chromatin adducts in the presence and absence of butyrate.

MATERIALS AND METHODS

Chemicals - [^3H]-B(a)PDE was purchased from Midwest Research Institute, Kansas City, USA, and had a specific activity of 3.76 Ci/mmol. [2-C^{14}]-Thymidine (57 mCi/mmol) and [1-C^{14}]-leucine (58 mCi/mmol) were obtained from Amersham, Bucks, England. PMSF, PPO and trypsin inhibitor, type II-S were bought from Sigma Chemical Co., St. Louis, MO, USA. DMSO for fluorography, sodium butyrate, guanidium chloride and ultrapure caesium sulfate were obtained from E. Merk AG, Darmstadt, West-Germany. DMSO used for Cs_2SO_4 gradients and for solubilizing B(a)PDE was obtained from Pierce Chem. Co., Rockford, USA. All materials used for electrophoresis were purchased from Bio-Rad Labs (Suisse) SA, Basel, Switzerland.

Cell Culture and Treatment - Human lung epithelioid cells (A549), normal human skin fibroblasts (strains CRL 1221 and 1121), and XP fibroblasts (CRL 1223), were grown to confluency in either Falcon T175 or T75 flasks

containing 35 or 20 ml respectively Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Mouse fibroblasts (C3H 10T1/2, clone 8) were grown in Basic Essential medium supplemented with 10% fetal calf serum.

For prelabeling the DNA, the cells were treated with [^{14}C]-thymidine as described elsewhere (36). To label the proteins, cells were grown in the presence of [^{14}C]-leucine (50 $\mu\text{Ci/ml}$) for 72 h, and then incubated in non-radioactive medium for 24 h. The cells were then trypsinized, uniformly mixed, reseeded and grown to confluency.

To induce histone hyperacetylation, sodium butyrate (1.0 M sterile solution in phosphate buffered saline, pH 7.5) was added to the cells to make the growth medium 6.0 mM in butyrate. The cells were incubated with butyrate containing medium for at least 24 h prior to treatment with B(a)PDE. [^3H]-B(a)PDE, dissolved in tetrahydrofuran: triethylamine (19:1 v/v) was flash evaporated in a Buchler evapomix (Buchler N.Y. USA) and the residue was dissolved in a known amount of anhydrous DMSO. Aliquots were then added to the medium to a final concentration of 1.3 μM and the cells were incubated for 1 h. For post-treatment incubation the medium was replaced with the appropriate pre-conditioned medium for the indicated times.

Nuclear Isolation - The cells were harvested by trypsinization, and an equal concentration of trypsin inhibitor was added to the cell suspension, to inhibit any further tryptic activity. The cells were pelleted by centrifugation (500 xg) for 10 min, resuspended in 0.25 M Sucrose, 1 mM Tris (pH 7.5), 1 mM MgCl_2 , 0.2 mM CaCl_2 , 0.5% Triton X-100, 0.1 mM PMSF and homogenised with a Dounce homogenizer, using the "A" pestle. The nuclei were pelleted by centrifugation at 2,000 xg for 7 min, gently resuspended in 5-10 ml of the above buffer and centrifuged three more times to yield a clean nuclear preparation. The nuclei were routinely monitored by light microscopy.

In order to estimate the nucleic acid content of the nuclear suspensions, 0.1 ml of the suspension was mixed with 0.9 ml 0.01% SDS (in water) solution in triplicate, and the absorbance at 260 nm was determined.

DNA purification - DNA was purified by isopycnic banding using Cs_2SO_4 gradients (37). Aliquots of the nuclear pellets were solubilized in 6M guanidium chloride solution, sonicated to reduce its viscosity and 1.45 ml of the nuclear solution was layered on 1.85 ml of 2.2 M Cs_2SO_4 solution. Centrifugation was carried out for 40-44 h in an SW60 rotor at 17°C at 32,000 rpm. The gradients were fractionated by collecting 8 drop fractions. The fractions containing DNA were pooled and dialysed against 0.2 mM EDTA (pH

7.2) for 24 h with 4 buffer changes. The radioactivity was measured in a Beckman LS 9000 scintillation counter and the $[^3\text{H}] : [^{14}\text{C}]$ ratios were determined.

Acid Extraction of Nuclear Proteins - The nuclear suspensions were pelleted by centrifugation, resuspended in 2.5 ml 0.4 N H_2SO_4 and centrifuged at 10,000 rpm for 15 min in a Beckman J-21 C centrifuge. The pellets were washed twice with 1.5 ml 0.4 N H_2SO_4 and the acid soluble proteins were precipitated from the combined supernatants by addition of 4 volumes of ethanol. After 24 h at -20°C the proteins were pelleted by centrifugation at 10,000 rpm for 20 min. The precipitate was dried at room temperature, solubilized in 3M urea, 0.9 N acetic acid, and electrophoresed in acid-urea polyacrylamide gels (38).

For analysis of core histones, nuclei were first extracted (3x) with 0.65 M NaCl, 3mM Tris pH 7.5, 0.1 mM PMSF, to remove the bulk of the non-histone proteins and histone H1. The residual acid soluble nuclear proteins, mainly core histones H2A, H2B, H3 and H4, were then isolated from the nuclear pellets with 0.4 N H_2SO_4 as described above.

Electrophoresis and fluorography - Acid extracted nuclear proteins were analysed on acid-urea polyacrylamide slab gels (38). The gels were pre-electrophoresed at 4°C for 18 h and the proteins were then electrophoresed at constant voltage (190V) for 8 h.

Analysis of total nuclear proteins was carried out using SDS-polyacrylamide (15%) slab gel electrophoresis (39). For this aliquots of nuclear pellets were solubilized in 100-200 μl SDS-sample application buffer and briefly sonicated to reduce the viscosity. Samples, up to 15 μl /slot were electrophoresed at constant voltage (80V) for 6-7 h until the tracking dye, bromophenol blue, had reached the bottom of the gel.

The proteins were visualised by staining the gels for 15 min in 20% acetic acid, 32% isopropanol and 0.05 - 0.1% Coomassie Blue R250. The gels were once rinsed in water and left in 10% acetic acid overnight. The stained gels were photographed using a polaroid type 665 film. For fluorography, the gels were treated with DMSO and PPO as described elsewhere (40). The Kodak X-ray film was exposed at -80°C for up to 5 months to visualize the radioactivity that was associated with the carcinogen. Alternatively the gels were sliced with a Mickle gel slicer after staining. The polyacrylamide was solubilised in 0.2 ml H_2O_2 (75°C for 18 h) and subjected to scintillation counting.

RESULTS

Specificity of protein binding - Intact human lung epithelial (A549) cells and mouse 10T1/2 cells were incubated for 1 h with B(a)PDE, and the nuclear proteins were analysed by gel electrophoresis. The distribution of the carcinogen was determined by fluorography and is shown in Fig. 1. In both cell types the core histones H2A and H3 were modified by B(a)PDE, while binding to histones H2B and H4 was not detected. Furthermore the fluorograms of the nuclear proteins from 10T1/2 and A549 cells, which were exposed for 4 1/2 - 5 months, showed clearly that other nuclear proteins were also labeled by B(a)PDE. In particular a protein X that migrated between H1 and H3 on SDS gels and above histone H1 on acid-urea gels (Fig. 5), which is tentatively identified as A24 protein (31,41,42), seems extremely susceptible to modification by B(a)PDE. The major species of histone H1 appear also to be

Fig. 1 Binding of B(a)PDE to nuclear proteins from mouse 10T1/2 cells and human lung A549 cell. Cells and isolated nuclei were incubated in the presence of B(a)PDE for 1 h, and the nuclear proteins were separated on SDS-polyacrylamide (15%) gels. A, represents stained protein gels from 10T1/2 nuclei. B, is the corresponding fluorogram indicating the position of the carcinogen. C and D represent the stained protein pattern and E and F are the corresponding fluorograms of the A549 nuclei labeled by B(a)PDE in vitro and in vivo respectively. X marks the position where A24 protein migrates in this system. Lanes marked L and CE are low molecular weight standards (Bio Rad) and chicken erythrocyte histones respectively.

labeled by the carcinogen, although to a minor extent, while the two bands on the fluorogram just below H1 suggest that these could be histone H1⁰. The correct identification of these proteins must await further analysis, as is the case with a considerable number of the high molecular weight non-histone proteins which also bind B(a)PDE.

When isolated nuclei from A549 cells were reacted in vitro for 1 h with B(a)PDE, analysis of the nuclear proteins showed that the modifications were very similar (Fig.1). Thus treatment of intact cells and isolated nuclei with B(a)PDE gives rise to the same type of specificity. We have shown elsewhere that this binding represents covalent modifications of the nuclear proteins (29).

Effect of histone hyperacetylation. - Since butyrate inhibits histone deacetylation and thus causes the accumulation of acetylated histone species (33-35), the effect of butyrate with respect to B(a)PDE binding to chromatin was examined. Fig. 2 shows that when human lung (A549) cells were incubated in the presence of 6 mM butyrate for 24 h, the acetylated species of H4, in particular, accumulated. When the A549 cells were grown in the presence of 0, 2, 4 and 6 mM butyrate for 24 h and then treated with B(a)PDE for 1 h, the labeling pattern of the core histones showed no dramatic change in specificity (Fig.3). Thus hyperacetylation of the core histones did not affect the specificity of the carcinogen and only histones H2A and H3 were predominantly labeled. Acid extraction of the nuclear proteins from control and 6 mM butyrate treated cells did show as in Fig. 1 extensive acetylation of H4. To examine whether the histone acetylation had any influence on the extent of DNA modification by B(a)PDE, the residual nuclear DNA after acid extraction of the histones was neutralised, dissolved in 6M guanidium chloride and isolated from Cs₂SO₄ gradients (37). Fig. 4 shows the corresponding absorbance profiles and the relative amount of [³H]-B(a)PDE bound to the DNA fractions. The amount of carcinogen bound per unit DNA was similar when comparing the DNA from 0 and 6 mM butyrate treated cells.

In order to examine whether the acetylated species of the core histones were capable of binding B(a)PDE, the acid extracted nuclear proteins from untreated controls and 6 mM butyrate treated cells (A549) were separated on acid urea gels, stained (Fig. 5A) and treated for fluorography (Fig. 5B).

The stained gels Fig. 5A, lane 1 and 2 show the acid extracted nuclear proteins from control cells (not treated with either butyrate or B(a)PDE) and B(a)PDE treated cells respectively. The pattern is identical and thus shows that treatment with B(a)PDE alone does not change the relative levels of

Fig. 2 Effect of butyrate on histone acetylation. Acid extracted nuclear proteins from A549 cells were separated on acid-urea gels. A, proteins from control cells. B, proteins from A549 cells treated with 6.0 mM butyrate for 24 h; migration was from top to bottom. Purified chicken erythrocyte histones were used as markers to identify the histone bands. Gels were stained and photographed as described in Materials and Methods.

histone acetylation. The last two lanes in Fig. 5A and B are duplicates, which show the nuclear protein patterns of butyrate treated cells that were labeled with B(a)PDE for 1 h.

The corresponding fluorogram shows that the carcinogen is able to bind with the acetylated species of the core histones. In particular this is clearly shown for H3 (Fig. 5B lane 4) where the mono, di, and although not so clearly visible, the tri and tetra acetylated forms of H3 are all capable of interacting with B(a)PDE (anti). Furthermore, the cells not treated with butyrate contain a low level of the mono-acetylated H3 which also binds the

Fig. 3 Effect of butyrate on B(a)PDE protein binding. A, stained total nuclear proteins from human lung (A549) cells after 24 h treatment with 0, 2, 4 and 6 mM butyrate. Lane marked L represents low molecular weight standards (Bio Rad). B, corresponding fluorogram, showing the position of the $[^3\text{H}]$ -B(a)PDE labeled proteins. Proteins were separated on SDS-polyacrylamide (15%) gels. Migration was from top to bottom. No significant changes in B(a)PDE histone binding were observed.

carcinogen (Fig. 5 A and B, lane 2).

Although histones H2A and H2B run together in this gel system, it is clear from the SDS gels (Fig. 3) that the labeling in this region on the acid urea gels (Fig. 5) is due to histone H2A. In butyrate treated cells it appears that the mono-acetylated H2A also can interact with B(a)PDE. Fig. 5 also shows that the histones H1 are labeled to a minor extent when compared with the core histones. The protein marked as X, which is tentatively identified as A24 protein seems also subject to reversible acetylation, since the relative intensity of the two bands in the stained gel shifts towards the mono-acetylated species after butyrate treatment. The corresponding fluorogram shows that this protein was also extensively modified by B(a)PDE.

Fig. 4 Isopicnic DNA isolation of Cs_2SO_4 gradients. Aliquots of the fractions from the gradients were diluted with water and the absorbance at 260 nm and the radioactivity were determined. Both control and 6.0 mM butyrate treated A549 cells have similar levels of carcinogen bound per unit DNA. Identical results were obtained from A549 cells treated with 2 and 4 mM butyrate (data not shown).

Removal of B(a)P chromatin adducts. - To examine the rate of removal of B(a)P adducts from chromosomal DNA and core histones, normal human fibroblasts were treated with 6 mM butyrate for 24 h to induce histone hyperacetylation, which was verified by gel electrophoresis (data not shown), and then treated with [^3H]-B(a)PDE (1.3 μM) for 1 h. Post-treatment incubation with conditioned medium containing butyrate was continued for 8 h and 24 h. The nuclear DNA was purified from Cs_2SO_4 gradients. The DNA which was uniformly labeled with [^{14}C]-thymidine was isolated after dialysis and the relative ratios of [^3H]-carcinogen to [^{14}C]-thymidine labeled DNA were calculated. Fig. 6 shows that in the butyrate treated cells and the control cells the B(a)P DNA adducts were removed at a similar rate during a 24 h period.

To examine the fate of the carcinogen histone complex as a function of post-treatment incubation, normal human fibroblast proteins were uniformly labeled with [^{14}C]-leucine as described in materials and methods, and treated with butyrate and B(a)PDE as described above. The core histones were extracted with H_2SO_4 after the nuclei had been washed with 0.65 M NaCl. The precipitated acid soluble proteins were resolubilized and aliquots were counted to determine the [^3H] : [^{14}C] ratios. This ratio did not change as a function of post-treatment incubation in either butyrate treated or control cells, indicating that the carcinogen-modified core histones were not

Fig. 5 Binding of B(a)PDE to acetylated core histones. A, stained total acid extracted nuclear proteins from A549 cells on an acid-urea gel. Lane 1 shows untreated control. Lane 2 B(a)PDE treated, and lanes 3 and 4 represent butyrate treatment for 24 h followed by incubation with B(a)PDE for 1 h. Lanes 3 and 4 are duplicates except that lane 4 contains slightly more protein. X marks the approximate position of A24 protein. B, corresponding fluorogram of the stained gel shown in A. The bands represent the position of the $[^3\text{H}]$ -B(a)PDE modified proteins. The positions of the acetylated histone species are indicated.

selectively removed during post-treatment incubation (Fig. 6). To check whether these cells were viable in terms of DNA repair capacity, the nuclear DNA after acid extraction was washed to neutralize the pH and purified on Cs_2SO_4 gradients. The amount of carcinogen per unit DNA (using 20 A260 = 1 mg/ml DNA) was calculated, and 39% and 55% of the initial B(a)P-DNA adducts had been removed after 8 h and 24 h post-treatment incubation respectively.

Finally, since it had been suggested (43,44) that the inability to repair DNA damage in the XP fibroblasts may in part be a function of the chromatin structure, XP fibroblasts were treated with 6.0 mM butyrate and B(a)PDE as described for normal fibroblasts. The amount of carcinogen bound to $[^{14}\text{C}]$ -thymidine labeled DNA as a function of post-treatment incubation was determined (Fig. 6). The control XP cells and the butyrate treated cells showed similar $[^3\text{H}] : [^{14}\text{C}]$ ratios at 0 h, 8 h and 24 h, indicating that

Fig. 6 Comparisons of the rate of removal of B(a)P adducts from DNA and nuclear core histone proteins from normal human fibroblasts and XP fibroblasts, in the presence and absence of 6.0 mM butyrate. Histones or DNA were uniformly labeled with [^{14}C]-leucine or [^{14}C]-thymidine respectively and isolated from purified nuclei. DNA was further purified on Cs_2SO_4 gradients while the core histone proteins were isolated from nuclei that were washed with 0.65 M NaCl as described in Materials and Methods. The carcinogen bound per unit DNA or associated with the histones, was obtained by plotting the ratios of [^3H] : [^{14}C] as a percentage of the initial ratio. Each point represents the average of two or three experiments and the vertical bars indicate the maximum variation from the mean. Control (●) and butyrate treated (▲) DNA from C1221 cells. Control (□) and butyrate treated (■) core histone proteins from C1221 cells. Control (○) and butyrate treated (◐) DNA from XP fibroblasts. Note that butyrate had no effect on the rate of removal of B(a)P adducts from either DNA or histones.

butyrate did not change the inability of XP cells to remove B(a)P-DNA adducts.

DISCUSSION

The nucleosomal core particle, which contains two of each of the core histones H2A, H2B, H3 and H4, and 146 base pairs of DNA has been shown to represent the fundamental unit of eukaryotic chromatin in all higher eukaryotes (1-3). The extreme conservatism of the core histones (3,45) indicates that precise stereo-chemical interactions take place to allow the formation of the nucleosomal core particles.

The experiments described here show that B(a)PDE interacts selectively with the core histone proteins in human A549 and mouse 10T1/2 cells. As was

shown in hamster embryo cells (28,29), the core histones H2A and H3 were the main targets for B(a)PDE, which suggests that similar three dimensional constraints, with respect to B(a)PDE-histone binding, operate in the nucleosomes of hamster, mouse and human cells. Furthermore, under carefully controlled in vitro conditions, in the absence of excess organic solvents, this specificity was maintained in the case of A549 core histones.

Both SDS-polyacrylamide gels, where proteins migrate according to their molecular weight, and the acid-urea polyacrylamide gels, where proteins migrate according to their net charge show that the same core histones, H2A and H3, are preferentially modified by B(a)PDE. We can thus exclude some minor contaminant of the histones that would migrate on SDS polyacrylamide gels with the same molecular weight or with the same net charge in acid-urea polyacrylamide gels.

The monoacetylated species of H3 under normal conditions also bound B(a)PDE, which suggests that the active fraction of the eukaryotic genome, which is thought to contain acetylated histones (2,46,47), also binds the carcinogen to a similar extent as the inactive chromatin fraction.

Using butyrate to generate hyperacetylated core histones, it was shown that all acetylated forms of H3 were labeled by B(a)PDE. This implies that those ϵ -aminogroups of lysine residues which are subject to acetylation are not the targets of B(a)PDE. Furthermore, in the acid-urea gels the position of the stained and B(a)PDE labeled H3 proteins coincided, which means that the binding of B(a)PDE to the polypeptide has not affected the net charge of the protein. Consequently the binding of B(a)PDE to core histone proteins probably does not involve any charged amino acids. The binding of B(a)PDE to a protein which migrated like A24 protein (31,41,42) was noted in both mouse 10T1/2 and A549 cells (Fig. 1). Current experimental evidence suggests that A24 protein is associated with the inactive chromatin fraction (41,42) and that it may play a role in higher order structure of chromatin (41). The binding of B(a)PDE to this protein may influence its relationship with chromatin.

As shown elsewhere (25,29), and in this report the major histone H1 species appear to have a low affinity for binding B(a)PDE, although these proteins did bind considerable amounts of the carcinogen when they were dissociated from chromatin (48). Therefore, the interaction of the major histone H1 species with chromatin seems to block some of the potential B(a)PDE binding sites on H1. There are however two bands just below the major H1 molecule that also bind the carcinogen (Fig. 1). The position of these

bands is similar to H1⁰. Since it has been shown that the accumulation of H1⁰ molecules is correlated with growth inhibition (48), the binding of B(a)PDE to these proteins could have important consequences.

Thus, the binding of an ultimate carcinogen to A24 protein, H1 and H1⁰, together with B(a)PDE modifications of the DNA and core histones, may facilitate changes in the structure-function relationship of chromatin which could be an important aspect in the initiation process that eventually leads to neoplastic transformation (49). Hence the interaction of carcinogens with all chromatin components must be characterized in order to evaluate the individual and cumulative effects of chromatin-carcinogen interactions.

Finally these studies have shown that butyrate treatment does not influence the initial levels of DNA modification, nor does it change the rate of removal of B(a)P-DNA adducts from chromatin in either normal human fibroblasts or XP fibroblasts. This suggest that if the structure of chromatin influences the rate of removal of B(a)P adducts, or the lack of removal in the case of XP cells (44,50), the subtle changes in chromatin, brought about by histone acetylation have apparently no influence on this process.

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ABBREVIATIONS

B(a)PDE	: (±) 7 α ,8 β -dihydroxy-9 β ,10 β -epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene
PMSF	: phenylmethylsulfonylfluoride
PPO	: 2,5-diphenyloxazole
DMSO	: dimethylsulfoxide
Cs ₂ SO ₄	: Caesium sulfate
XP	: xeroderma pigmentosum
SDS	: sodium dodecyl sulfate.

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