Brazilian Journal of Medical and Biological Research (2000) 33: 287-293
ISSN 0100-879X

Retinol-induced changes in the phosphorylation levels of histones and high mobility group proteins from Sertoli cells

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Abstract

Chromatin proteins play a role in the organization and functions of DNA. Covalent modifications of nuclear proteins modulate their interactions with DNA sequences and are probably one of the multiple factors involved in the process of switch on/off transcriptionally active regions of DNA. Histones and high mobility group proteins (HMG) are subject to many covalent modifications that may modulate their capacity to bind to DNA. We investigated the changes induced in the phosphorylation pattern of cultured Wistar rat Sertoli cell histones and high mobility group protein subfamilies exposed to 7 µM retinol for up to 48 h. In each experiment, 6 h before the end of the retinol treatment each culture flask received 370 KBq/ml [^{32}P]-phosphate. The histone and HMGs were isolated as previously described [Moreira et al. *Medical Science Research* (1994) 22: 783-784]. The total protein obtained by either method was quantified and electrophoresed as described by Spiker [*Analytical Biochemistry* (1980) 108: 263-265]. The gels were stained with Coomassie brilliant blue R-250 and the stained bands were cut and dissolved in 0.5 ml 30% H_2O_2 at 60°C for 12 h. The vials were chilled and 5.0 ml scintillation liquid was added. The radioactivity in each vial was determined with a liquid scintillation counter. Retinol treatment significantly changed the pattern of each subfamily of histone and high mobility group proteins.

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Research supported by FAPERGS, PROPESQ-UFRGS, FINEP and CAPES.

Received February 4, 1999
Accepted January 14, 2000

Key words
- Sertoli cells
- Phosphorylation
- Histones
- High mobility group proteins
- Retinol

Introduction

Although knowledge about the structure of chromatin is still very limited, rapidly evolving theories suggest that chemical modifications of histones and non-histone chromatin-associated proteins are important in determining changes in chromatin configuration (1). Chemical modifications (e.g., phosphorylation and acetylation) of DNA-associated proteins, in particular non-histones, have been correlated with the regulation of cell growth and development, hormone induction, and cellular transformation (1). Furthermore, some reports show a relatively higher content of phosphorylated non-histone chromosomal proteins in less compact, as opposed to densely organized, chromatin regions (2-4). Chromatin packing must be both specific and reversible to allow the

access of factors to regulatory DNA sequences and the access of the transcription apparatus to coding regions of the genome (5-7). Protein phosphorylation plays an important role in several cellular activities, including the initiation of mitosis and regulation of transcription (6).

In an attempt to better understand the mechanisms of retinoid action in Sertoli cells, our group has been studying the effects of treatment with retinol on N-glycoprotein biosynthesis (7), [methyl-^{3}H]-thymidine incorporation into DNA (8,9) and nuclear protein phosphorylation (10). Treatment with 10 µM retinol for 24 h increased [methyl-^{3}H]-thymidine incorporation into the DNA of cultured rat Sertoli cells, an effect that was not directly related to cell proliferation and was partially inhibited by 3 mM hydroxyurea pre-treatment (9). We have also observed that retinol treatment increases chromatin sensitivity to DNAse I, an effect reversed by pre-treatment with 1,10 phenanthroline (11).

In the present communication, we report studies on the effects of retinol treatment on the phosphorylation of Sertoli cell histones and high mobility group protein (HMG) subfamilies.

Material and Methods

All-trans-retinol, medium 199, DNAse type I and all other chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA). [^{32}P]-Phosphate (7.4 GBq/mmol) was purchased from Amersham International (Amersham Place, Little Chalfont, Buckinghamshire, England).

Sertoli cells were obtained from 15-day-old Wistar rats from our breeding stock. Pregnant rats were housed individually in Plexiglas cages. Litters were culled to eight pups each. The animals were maintained on a 12-h light/dark cycle at a constant temperature of 23^{o}C, with free access to commercial food and water.

The animals were killed by ether asphyxiation, the testes were removed and Sertoli cells were isolated and cultured as previously described (10). After 24 h in culture with medium 199 supplemented with 1% fetal calf serum (FCS) at 34^{o}C under a normal atmosphere, the cells were washed twice with phosphate-buffered saline (PBS) and cultured for another 24 h in medium 199. The plating density was 3.2 x 10^5 cells/cm^2. A small percentage (2-3%) of contaminating peritubular cells was demonstrated histochemically (7).

After a 24-h culture period without FCS, half of the cells received medium 199 supplemented with 7 µM retinol for 6, 12, 24 or 48 h. The other half of the cells received only the retinol solvent (ethanol 0.1%) and were used as the control. In each experiment, 6 h before the end of retinol treatment each culture flask received 370 KBq/ml [^{32}P]-phosphate.

The histone and HMGs were extracted as previously described (10). The total protein obtained by either method was quantified (10) and electrophoresed as described by Spiker (12). The gels were stained with Coomassie brilliant blue R-250 and the stained bands were cut and dissolved in 0.5 ml 30% H_2O_2 at 60^{o}C for 12 h. The vials were chilled and 5.0 ml scintillation liquid was added. The radioactivity in each vial was determined with a liquid scintillation counter.

Results are reported as means ± SEM (N = 8). Differences in experimental groups were determined by analysis of variance, followed by the Newman-Keuls test or Student *t*-test when appropriate.

Results

Table 1 shows the effect of 7 µM retinol for different periods of time on histone phosphorylation. Retinol treatment of 12 and 24 h significantly increased total histone phosphorylation and significantly decreased it at 6 and 48 h compared to controls. Table 2 shows an opposite pattern of induced phos-

phorylation in HMGs from retinol-treated cells at 6-, 24- and 48-h time points. Based on these data we decided to investigate the effect of retinol treatment on individual subfamilies of histones and HMGs.

Figure 1 (A-D) and 2 (A-D), respectively, show the effect of different times of exposure to 7 µM retinol on the phosphorylation of histone and HMG subfamilies. For each treatment period, total histone phosphorylation (all subfamilies) for each treated and untreated group was considered to be 100% (for each time, the sum specific activities of all subfamilies of treated and untreated (control) cells = 100%). The objective of this procedure was to compare the phosphorylation pattern, while the intrinsic differences are shown in Tables 1 and 2.

Treatment with 7 µM retinol significantly increased the phosphorylation of the H3 subfamily at 12, 24 and 48 h as compared to H3 isolated from control cells. The H3 phosphorylation level declined during the first 6 h of retinol treatment. Phosphorylated H3 seems to be involved in DNA synthesis activation during the cell cycle (13,14).

H2a also showed a significant decrease in phosphorylation at 48 h. At 6 and 24 h of retinol treatment this subfamily showed an increase in phosphorylation. Phosphorylated H2a seems to be associated with transcriptionally active chromatin (15).

Increased phosphorylation seems to be associated with transcriptionally active genes in the H2b subfamily and with nucleosome stability in the H4 subfamily (15,16). H4 and H2b showed no significant difference in phosphorylation up to 12 h of retinol treatment. There was an increase in phosphorylation at 24 h and a significant decrease at 48 h of retinol treatment compared with the respective histone group from untreated cells.

The linker histone H1 showed a significant rise in phosphorylation level at all times of retinol treatment as compared with H1 from control cells. An increase in H1 phosphorylation is frequently associated with relaxed chromatin (17).

Figure 2 (A-D) shows that there was no significant change in HMG1 phosphorylation with retinol treatment at 6 or 48 h compared with control. However, phosphorylation increased significantly at 12 h and declined significantly at 24 h of retinol treatment, as compared with HMG1 from untreated cells.

HMG2 from retinol-treated Sertoli cells showed a significant increase in phosphorylation compared to control cells at 6 h, but there was a significant decrease at 12 and 24 h of treatment. HMG1 and HMG2 are frequently associated with DNA synthesis in

Table 1 - Incorporation of $^{32}P^{-3}$ into histones isolated from 7 µM retinol-treated rat Sertoli cells.

Histones were isolated from treated and untreated cells at each specified time as described in Material and Methods. Protein was determined by the method of Lowry and radioactivity by liquid scintigraphy. Data are reported as counts x 10^3/mg protein (means ± SEM; N = 8); *P<0.01 compared to control at the same time point (Student *t*-test).

Treatment period	Control	Treated
6 h	350 ± 2.1	298 ± 1.6*
12 h	365 ± 3.4	419 ± 2.2*
24 h	370 ± 1.9	462 ± 3.2*
48 h	525 ± 3.7	456 ± 1.4*

Table 2 - $^{32}P^{-3}$ incorporation into high mobility group protein (HMG) isolated from 7 µM retinol-treated rat Sertoli cells.

HMGs were isolated from treated and untreated cells at each specified time as described in Material and Methods. Protein was determined by the method of Lowry and radioactivity by liquid scintigraphy. Data are reported as counts x 10^3/mg protein (means ± SEM; N = 8). *P<0.01 compared to control at the same time point (Student *t*-test)

Treatment period	Control	Treated
6 h	125 ± 1.1	176 ± 3.5*
12 h	137 ± 4.4	143 ± 2.2*
24 h	150 ± 2.9	112 ± 4.8*
48 h	275 ± 3.4	619 ± 2.6*

proliferative undifferentiated cells (18). At 48 h there was no significant difference between HMG2 from treated and untreated cells.

Dephosphorylated HMG14 and HMG17 seem to be associated with transcriptionally active chromatin (16,19). HMG14 from retinol-treated Sertoli cells showed significantly higher phosphorylation after 6 or 48 h and a significantly lower level at 24 h of treatment as compared with control.

HMG17 from retinol-treated cells showed a decrease in phosphorylation at 6, 12 and 24 h and an increase at 48 h as compared with HMG17 from untreated cells.

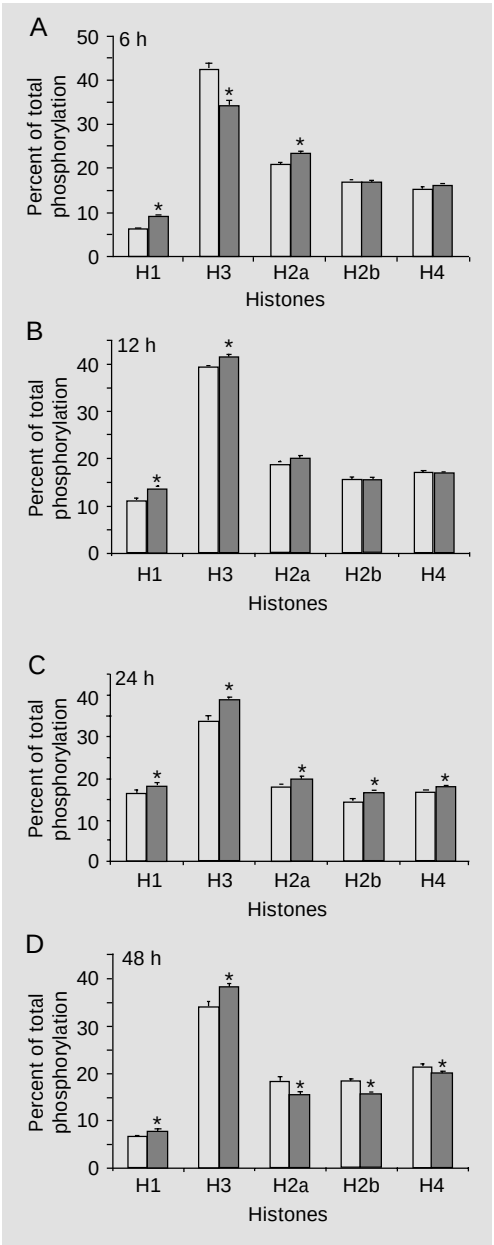

Figure 1 - Incorporation of ^{32}P-3 into histone subfamilies isolated from 7 µM retinol-treated rat Sertoli cells. Sertoli cells were treated with retinol (7 µM) for 6, 12, 24 and 48 h and a 10 µCi/ml of ^{32}P-3 pulse was applied 6 h before the end of each treatment. Treated cells are indicated by striped columns and controls by open columns. Cells were scraped and washed twice with cold PBS. Histones were isolated, quantified and electrophoresed. The gel was scanned by densitometry, each band was cut and dissolved, and radioactivity was counted by liquid scintigraphy. Percent phosphorylation for each subfamily was related to total histone phosphorylation. For each treatment period, total histone phosphorylation for each treated or untreated group was considered to be 100% (for each time, the sum of the specific activities of all subfamilies of treated or untreated (control) cells = 100%). The objective of this procedure was to compare the phosphorylation pattern, while the intrinsic differences are shown in Tables 1 and 2. Data are reported as means ± SEM; N = 8. *P<0.01 compared to untreated control cells (Newman-Keuls test). *A*, 6-h retinol treatment. *B*, 12-h retinol treatment. *C*, 24-h retinol treatment. *D*, 48-h retinol treatment.

Discussion

A number of observations on nucleosome rearrangements and transitions in transcription- and replication-related events suggest that chromatin is a structure capable of considerably dynamic behavior (20-22). Our previous results demonstrated that retinol treatment for 6, 12, 24 or 48 h increased the sensitivity of chromatin to DNAse I (11).

H1, the linker histone that closes two turns of DNA around the nucleosome, plays an essential role in the formation and stabilization of the 30-nm high-order chromatin fiber (23). Pennings et al. (6) suggest the probable importance of dephosphorylated H1 as a general repressor of nucleosome mobility. Because of its pivotal role in packaging the genome in the high-order chromatin structure, histone H1 dephosphorylation/phosphorylation turnover seems to play a role in a general reversible repression mechanism (24-26).

Histone H1 (Figure 1) from retinol-treated cells was more phosphorylated than H1 from untreated cells at each time studied. Our results are in agreement with those of Takada et al. (27) which indicate an increase in DNA primase activity with an increase in the number of phosphate molecules bound to histone H1. In his review, Lewin (14) suggested that the addition of dephosphorylated H1 to transcriptional active chromatin causes a rapid decline in transcription. In mobile nucleosomes the restriction of mobility by linker histones (dephosphorylated H1) might be expected to exert a major influence on the accessibility of chromatin to regulatory molecules (28).

H3 is a very important marker of the cell cycle. Its phosphorylation level determines

the control of the entry of the cell into phase S of the cell cycle (6). This subfamily is one of the static components of the nucleosome core. The H2a-H2b dyad from retinol-treated cells showed a change in phosphorylation level from 12 to 24 h as compared with that from untreated cells. This covalent modification of the dyad (phosphorylation, acetylation or ubiquitination) is frequently associated with transcriptionally active chromatin (4,17). These data, taken together with the increased sensitivity to DNAse I (11) in retinol-treated cells during the same period of time, suggest that chromatin may be more relaxed in retinol-treated cells.

The pattern of HMG subfamily phosphorylation in treated Sertoli cells was different from that in untreated cells, in agreement with our previous data (10).

HMG1 and HMG2 from retinol-treated cells showed decreased levels of phosphorylation at 6 h compared to control, and this difference persisted until 24 h. At 48 h there was an inversion in the phosphorylation pattern of this HMG subfamily. HMG1 and HMG2 are associated with linker histones and Kohlstaedt and Cole (3) have reported a strong competition between HMG1 and H1 for the same interaction site in chromatin (4,29,30).

Although the cellular function of the HMG14/17 proteins is not fully understood, the functions of these proteins very probably depend on proper interaction with nucleosomes in chromatin. HMG14/17 are the only known nuclear proteins which specifically recognize the 146-base pair nucleosomal core particle (31). Chromosomal proteins HMG14/17 may play a role in the generation of structure features which are unique to transcriptionally active genes (19).

HMG14 and 17 specifically bind to nucleosome core particles. Therefore they can be considered an integral part of the chromatin fiber. Multiple specific interactions between the protein and nucleic acid components in chromatin facilitate complex processes such as replication, DNA repair, recombination, and transcription. These proteins probably may still act as "architectural" elements and facilitate transcription in the context of chromatin, perhaps by negating the repressive effects of histones and inducing structural changes that ease the binding of other factors to their targets. Their modular structure may reflect a requirement for interaction with both the DNA and histone in nucleosomes and may facilitate mul-

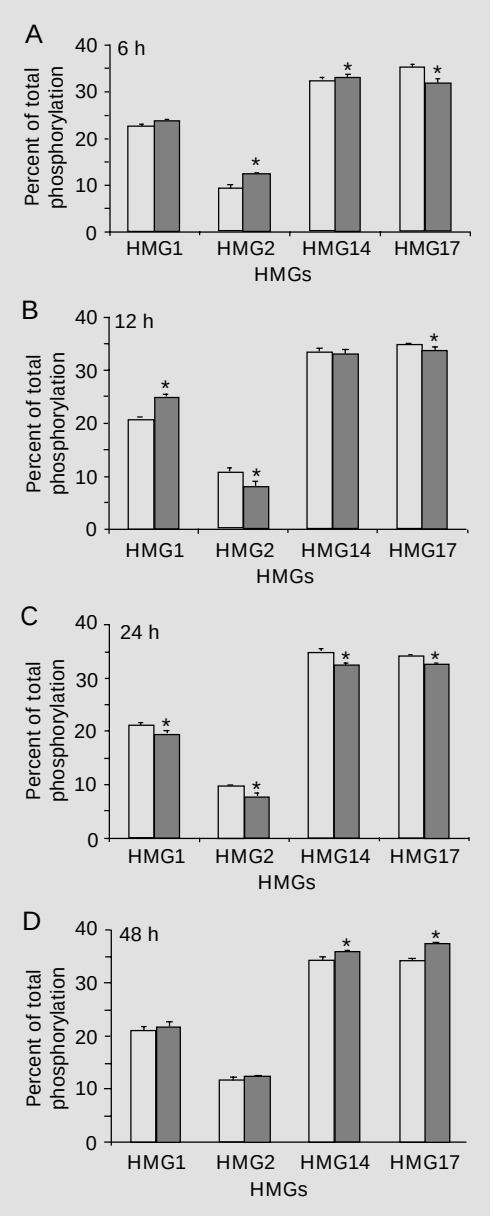

Figure 2 - $^{32}P^{-3}$ incorporation into high mobility group protein (HMG) subfamilies isolated from 7 µM retinol-treated rat Sertoli cells. Sertoli cells were treated with retinol (7 µM) for 6, 12, 24 and 48 h; 6 h before the end of each treatment a pulse with 10 µCi/ml of $^{32}P^{-3}$ was applied. Cells were scraped and washed twice with cold PBS. HMGs were isolated, quantified and electrophoresed. The gel was scanned by densitometry, each band was cut and dissolved, and radioactivity was counted by liquid scintigraphy. The percentage of each subfamily phosphorylation is reported in relation to total HMG phosphorylation. For each treatment period, total HMG phosphorylation for each treated or untreated group was considered to be 100% (for each time, the sum of the specific activities of all subfamilies of treated and untreated (control) cells = 100%). The objective of this calculation was to compare the phosphorylation pattern, while the intrinsic differences are shown in Tables 1 and 2. Data are reported as means ± SEM; N = 8. *P<0.01 compared to untreated control cells (Newman-Keuls test). *A*, 6-h retinol treatment. *B*, 12-h retinol treatment. *C*, 24-h retinol treatment. *D*, 48-h retinol treatment.

tiple cooperative interactions which increase the overall flexibility of multiple component structures such as active chromatin (20).

Tremethick et al. (15,16) proposed that H2a and H2b were replaced in active chromatin by HMG14 and HMG17 and we observed an opposite pattern of phosphorylation between these groups of HMGs and histones (Figures 1 and 2 B,C). Taking into account our previously published results that chromatin from treated cells is more sensitive to DNAse I digestion (11), and the results presented in this paper that chromatin from treated cells also has a higher percentage of phosphorylated histone H2a-H2b and a lower percentage of phosphorylated HMG14 and HMG17 (30), we believe our data are in agreement with the proposals of Tremethick et al. (15,16).

Hansen and Wolffe (32) showed that removal of histone H2a-H2b from nucleosomal assays has a major influence on chromatin compactation and in the repression of RNA polymerase III transcription initiation and elongation. Thus, they propose that one consequence of depletion of H2a-H2b from active chromatin is to stabilize a more extended chromatin structure that will facilitate the entry of RNA polymerase (21). This work suggests that the increased relaxation of chromatin we observed at 24-h retinol treatment was probably due to chromatin activation.

Weisbrod and Weintraub (33) described experiments that define a group of proteins which are easily eluted from chromatin and are responsible in part for increasing the sensitivity of chromatin DNAse I. These proteins correspond to HMG14/17 (30).

The introduction of negatively charged phosphate groups on chromatin-associated proteins is likely to loosen the chromatin structure, and this looseness in chromatin structure could be highly transient. Thus, histone and HMG phosphorylation/dephosphorylation turnover may allow temporary access to DNA during replication, repair, recombination and transcription (23,34-36). Accumulated evidence strongly indicates that one cannot explain gene regulation merely by the interaction between DNA and nuclear proteins. Removal of histone-controlled repression is the necessary step in the turning off of a eukaryotic gene (23). If this conclusion is correct, it becomes important to explain how the ties between histone core and the TATA box are relaxed (14,17).

One may speculate that an increase in the level of histone phosphorylation is necessary but not sufficient to promote all the biological events in chromatin regions engaged in chromatin activation.

The process of HMG and histone phosphorylation/dephosphorylation is dynamic and amino acid residues are continuously phosphorylated/dephosphorylated enzymatically. Chadee et al. (2) observed increased phosphorylation of histone in mouse fibroblasts transformed with an active mitogen-activated protein kinase K. Since retinol alters the pattern of histone and HMG phosphorylation (9), one may ask whether histone and HMG kinases or phosphatases are more active in retinol-treated cells or whether their substrates are more available to enzymes themselves (32,37).

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