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STIMULATION OF UTERINE NONHISTONE PROTEIN PHOSPHORYLATION AND NUCLEAR PROTEIN KINASE ACTIVITY BY ESTRADIOL-17β

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Summary

Changes in the phosphorylation of nonhistone chromosomal proteins have been followed in rat uterus stimulated by 17β-estradiol. Isolated uteri were found to incorporate $^{32}P_i$ into nonhistone proteins via an endogenous nuclear protein kinase reaction. The rate of ^{32}P labeling of nonhistone proteins and the activity of nuclear protein kinase(s) were found to be elevated over three- and two-fold respectively in uteri obtained from ovariectomized animals treated with estrogen. A dramatic change was observed in the radioactivity profile of ^{32}P-labeled proteins fractionated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These observations are compatable with the hypothesis that phosphorylation of nonhistone proteins plays a role in the regulation of gene activity in the uterus.

Introduction

Evidence has accumulated in recent years supporting the notion that nonhistone chromosomal proteins play a key role in the regulation of gene expression in eukaryote cells (reviewed in refs. 1—3). They are tissue specific and highly heterogeneous. Alterations in the rates of synthesis of these proteins occur during differentiation and growth of numerous tissues. In vitro they counteract the inhibitory effects of histones on transcription and stimulate RNA synthesis in cell-free systems. In addition, a number of these proteins are phosphorylated, and changes in their state of phosphorylation are thought to play a significant role in their ability to interact with chromatin and function as regulatory proteins [4—6].

Activation of gene transcription appears to be involved in the stimulation of uterine hypertrophy and hyperplasia by 17β-estradiol [7,8]. This stimulation is blocked by inhibitors of RNA and protein synthesis, and synthesis of

RNA and protein, as well as RNA polymerase activity, are increased following administration of the hormone. Chromatin isolated from treated uteri has an enhanced capacity to serve as a template for RNA transcription in a cell-free system. The possibility that nonhistone proteins are involved in this alteration in gene activity is supported by the finding that estradiol stimulates the synthesis of specific proteins in this fraction [8—10]. In order to determine whether changes in their state of phosphorylation might also be occurring, we have investigated the effects of estradiol on ^{32}P incorporation into the nonhistone chromosomal proteins of isolated uteri. It was found that estradiol causes a significant increase in the phosphorylation of particular uterine nonhistone chromosomal proteins, and that these increases are due at least in part to an increase in nuclear protein kinase activity.

Materials and Methods

Preparation of uteri. Female rats (150—180 g) were ovariectomized at least 3 weeks prior to use. 17β-estradiol, 10 μg/animal, was injected intraperitoneally in propylene glycol. Controls received the vehicle alone. At various times after hormone treatment the animals were sacrificed and their uteri removed, stripped of fat and mesentery, slit lengthwise, and rinsed in phosphate-free Eagle's medium. They were then incubated for 1 h at 37°C in Eagle's medium containing 1 mCi/ml $H_3{}^{32}PO_4$ (carrier-free), following which they were rinsed in 0.14 M NaCl and rapidly frozen on solid CO_2.

Isolation of nuclei and nonhistone proteins. The frozen tissue was crushed to a powder in a stainless steel pulverizer chilled to −70°C. 10 vol. of 0.32 M sucrose/5 mM $MgCl_2$ were added to the powder and the tissue was homogenized with a Polytron PT-10 homogenizer at a setting of 3 for 60 s. The homogenate was filtered through two layers of cheesecloth and centrifuged at 700 × *g* for 10 min. The crude nuclear pellet was washed with 0.25 M sucrose/1 mM $MgCl_2$/1% Triton X-100, and then resuspended in 2.3 M sucrose/1 mM $MgCl_2$. The purified nuclear pellet was collected by centrifugation at 22 500 rev./min for 50 min in a Beckman SW 50.1 rotor, and resuspended in 0.25 M sucrose/1 mM $MgCl_2$.

The nonhistone chromosomal protein fraction was isolated via a modification of the method of Gershey and Kleinsmith [11]. Nuclei were first extracted with 0.14 NaCl to remove soluble nuclear proteins and ribosomes. Chromatin was then dissolved in 1.0 M NaCl. Histones, DNA, nuclear membranes and nucleoli were removed by lowering the salt concentration to 0.4 M and centrifuging at 95 000 × *g*. The supernatant, containing the nonhistone chromosomal proteins, was dialyzed against 1.0% sodium dodecyl sulfate (SDS)/0.1% mercaptoethanol/0.01 M phosphate buffer pH 7.1, and then concentrated and dialyzed against 0.1% SDS/0.1% mercaptoethanol/10% glycerol/0.01 M phosphate buffer pH 7.1. Proteins were electrophoretically separated on 5% polyacrylamide gels, 12 cm in length, containing 0.1% SDS. The gels were frozen and sliced into 2-mm segments. Radioactivity in the slices was determined in a liquid scintillation counter. Protein molecular weights were calculated by the method of Weber and Osborn [12]. Protein concentrations were determined

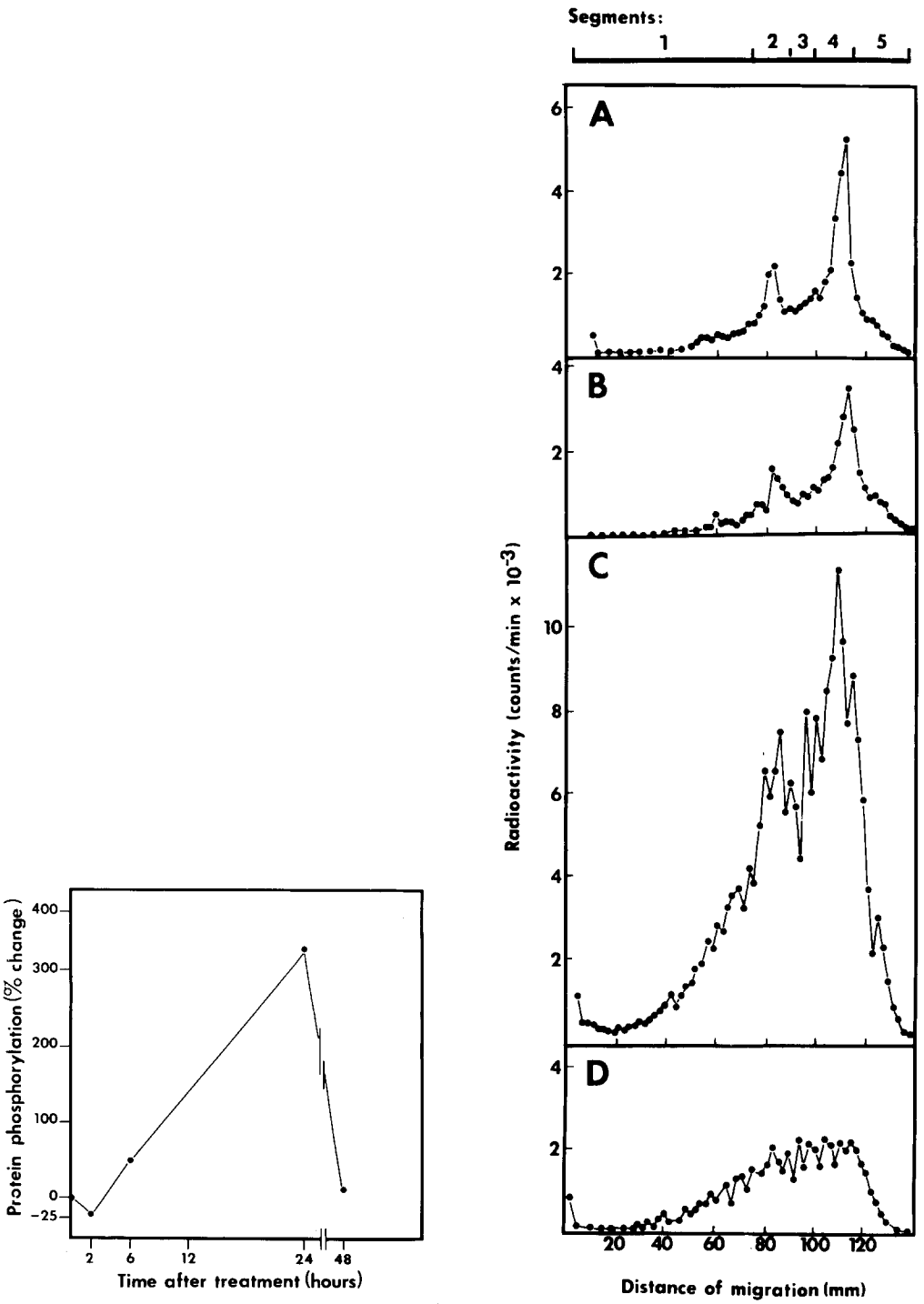

Fig. 1. Effects of estradiol on incorporation of ^{32}P into nonhistone chromosomal proteins of rat uteri. The proteins were labeled and extracted from uteri as described in the text. The data are recorded as percentage change from control values obtained from ovariectomized animals. The average specific activity of nonhistone chromosomal proteins from such controls was 709 cpm $^{32}P/\mu g$ protein.

Fig. 2. Comparison of ^{32}P-labeling patterns of nonhistone chromosomal proteins from rat uteri at different times after estradiol administration. Gels were prepared and sliced as described in the text. Radioactivity was normalized for 75 μg protein/gel. (A) ovariectomized control (B) 2 h after estradiol (C) 24 h after estradiol (D) 48 h after estradiol.

by the Lowry et al. procedure [13]. Analysis of recovery of ^{32}P in phosphoserine and phosphothreonine by chromatography on Dowex-50 [14] indicated that 95% of the ^{32}P in the nonhistone chromosomal protein fraction is present in protein (phosphoserine to phosphothreonine ratio = 8/1). This conclusion is reinforced by the stability of the ^{32}P to treatment with hot trichloroacetic acid (5 or 10%), and its lability to boiling in 1.0 M NaOH.

Protein kinase assay. Nuclear protein kinase activity was measured by incubating purified nuclei at 30°C for 10 min in a reaction mixture containing 0.82 mg nuclear protein, 10 μmol NaCl, 5 μCi [γ-^{32}P]ATP (0.23 μmol), 15 μmol Tris · HCl, pH 7.5 in a total volume of 0.5 ml. The reaction was stopped by addition of 10 μmol of unlabeled ATP followed by ice-cold 12% trichloroacetic acid containing 1% sodium pyrophosphate. The precipitates were extracted with 5% trichloroacetic acid at 90°C for 15 min and were then washed with ice-cold 5% trichloroacetic acid/95% ethanol containing 2% sodium acetate, and ethanol/ether (3 : 1). Incorporation of ^{32}P into protein was determined in a liquid scintillation counter.

Results

The ability of isolated uteri obtained from ovariectomized rats to phosphorylate nonhistone chromosomal protein was measured at varying times following administration of estradiol-17β in vivo (Fig. 1). Uteri obtained from animals 2 h after estrogen treatment exhibit a slight depression of protein phosphorylation, but at later times this effect is reversed and a dramatic stimulation occurs. By 24 h after hormone treatment the specific activity of the nonhistone chromosomal protein fraction is more than three-fold higher than that of animals not receiving estrogen. By 48 h, however, the rate of phosphorylation returns to control levels. The dramatic increase in specific activity of the nonhistone chromosomal protein fraction cannot be accounted for by an increased specific activity of the ATP pool, which we have found to increase by no more than 25%. This observation is consistent with other reports of the failure of estradiol to cause significant changes in the specific activity of [^{32}P]ATP [15].

To determine whether the stimulation of nonhistone chromosomal protein phosphorylation by estrogen is selective for individual components of this heterogeneous protein fraction, ^{32}P-labeled protein preparations were fractionated via SDS-polyacrylamide gel electrophoresis. As is shown in Fig. 2, uteri of ovariectomized rats phosphorylate two major proteins of mol. wts. 19 000 and 40 500. There is little change in this pattern 2 h after treatment with estradiol. At 24 h after estradiol administration the phosphorylation pattern has become much more heterogeneous. At 48 h the overall level of phosphorylation has dropped considerably, but the heterogeneity of the labeling pattern has become much more pronounced. To show that the changes in labeling pattern are not due to inherent variability in gel slicing, data from several pooled regions of gel slices were compared from duplicate gels. As is shown in Table I, consistent differences in the labeling patterns can be seen to occur within 24 h after estradiol treatment.

It is interesting to note that the pattern of nonhistone chromosomal protein phosphorylation observed 48 h after estradiol treatment resembles that which

TABLE I

REPRODUCIBILITY OF CHANGES IN NONHISTONE CHROMOSOMAL PROTEIN PHOSPHORYLATION OBSERVED IN SDS-POLYACRYLAMIDE GELS

Gels were divided into five segments (see Fig. 2) and the percent of the total counts in each segment calculated. Two control gels and two gels 24 h after estradiol treatment are compared.

Gel segment	% Total radioactivity			
	Controls		Estradiol-treated	
1	18.0	18.7	19.8	23.8
2	18.0	15.9	13.4	14.6
3	16.0	15.8	22.1	20.0
4	35.5	36.1	23.2	21.1
5	12.5	13.5	21.4	20.5

occurs in intact adult females (Fig. 3), but it is quite different from that of ovariectomized animals (Fig. 2a). In spite of this dramatic difference in phosphorylation pattern between ovariectomized and intact rats, their overall rate of nonhistone chromosomal protein phosphorylation is the same. Hence, it is clear that similarities in overall rate of protein phosphorylation may mask underlying differences of great magnitude in the types of polypeptides being phosphorylated.

To ascertain whether the decrease in ^{32}P labeling of high molecular weight nonhistone chromosomal protein observed in the absence of estradiol is due to the presence of protease activity, a reciprocal set of mixing experiments was performed. In one case ^{32}P-labeled uteri from ovariectomized rats were mixed with unlabeled uteri from intact rats prior to isolation of nonhistone chromosomal protein. In the other case, the opposite combination was utilized. As is shown in Fig. 4, in neither case was the labeling pattern altered from what is normally observed for either ovariectomized or intact rats, suggesting that protease activity is not responsible for the differences in the ^{32}P labeling of nonhistone proteins in these two states.

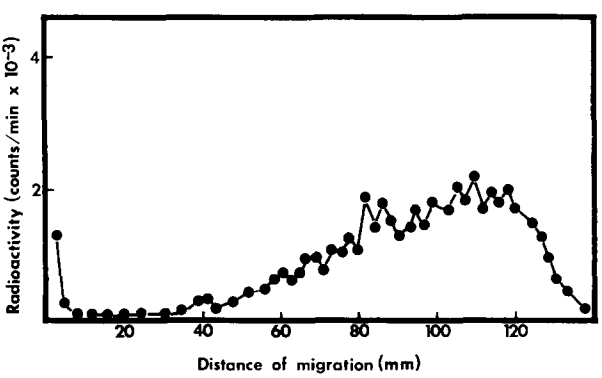

Fig. 3. ^{32}P-labeling pattern of nonhistone chromosomal proteins of uteri from noncastrate animals in estrus. The stage of the estrus cycle was determined from vaginal smears. Radioactivity was normalized for 75 μg protein/gel. Note the similarity to the results from ovariectomized animals 48 h after estrogen administration (Fig. 2D).

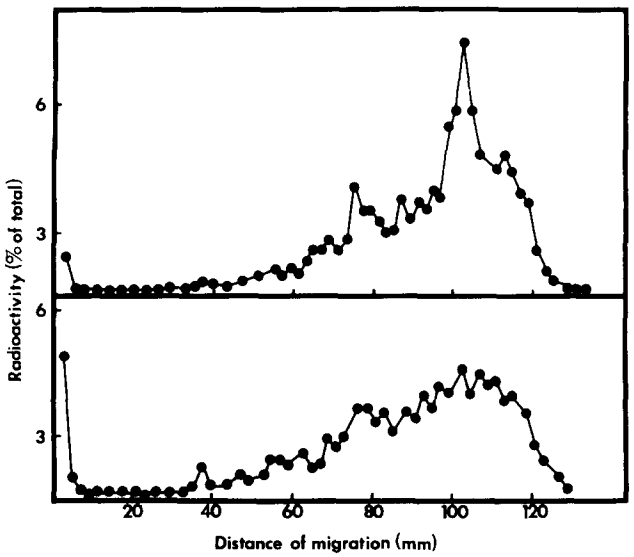

Fig. 4. Comparison of ^{32}P-labeling patterns of nonhistone chromosomal proteins from a mixture of radioactive uteri from noncastrate rats and nonradioactive uteri from ovariectomized rats (bottom), and nonhistone chromosomal proteins prepared from a mixture of radioactive uteri from ovariectomized rats and nonradioactive uteri from noncastrate rats (top). Gels were prepared and sliced as described in the text. Note similarity to Figs. 2A and 3, respectively.

Experiments were also carried out to determine whether the increase in protein phosphorylation observed in uteri of estrogen-treated animals was due to an increase in the activity of nuclear protein kinase(s). As is shown in Fig. 5, changes in enzyme activity closely parallel the changes in specific activity of the ^{32}P-labeled nonhistone chromosomal protein fraction, although the changes in the level of protein kinase activity are not quite as great. The only exception to this correlation occurs at 48 h, where protein kinase activity remains elevated

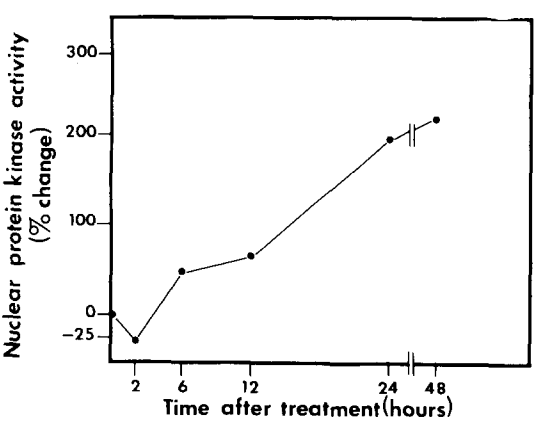

Fig. 5. Effects of estradiol on nuclear protein kinase activity. Enzymatic activity was assayed as described in the text. The changes are recorded as percentage change from control values obtained from ovariectomized animals. The average incorporation for such control nuclei was 55 000 cpm ^{32}P/mg nuclear protein.

while the rate of phosphorylation of nonhistone chromosomal proteins has dropped back to control levels (Fig. 1).

Discussion

The present experiments demonstrate the presence of nuclear protein kinase activity and nonhistone phosphoproteins in nuclei of rat uterus. Administration of 17β-estradiol has been shown to cause a three-fold increase in the phosphorylation rate of these proteins, and to enhance specifically the phosphorylation of many polypeptides which are barely labeled in the absence of hormone. Although the results were obtained by incubating isolated uteri with ^{32}P, the assumption that similar changes occur in vivo is supported by other findings that patterns of radioisotope incorporation into uterine proteins are the same whether the isotope is administered in vitro or in vivo (refs. 16 and 17 and Cohen, M., unpublished observations).

The fact that the current results show the estradiol-induced increase in the chromosomal protein phosphorylation to be accounted for at least in part by an increase in activity of nuclear protein kinase(s) does not exclude the possibility that alterations in the rates of synthesis and migration of nonhistone proteins into the nucleus are also involved. Others have shown in fact an estradiol-stimulated accumulation of specific chromosomal proteins in uterine chromatin [8,9]. Our own finding that the increase in protein kinase activity extends beyond the time of enhanced nonhistone chromosomal protein phosphorylation also argues that factors other than kinase activity are involved in regulating the phosphorylation of these proteins.

Since cyclic AMP is widely known to be involved in the regulation of protein phosphorylation reactions [18], the present results raise the question of whether cyclic AMP is elevated in estrogen-stimulated uteri. Because there are conflicting reports in the literature on this point [19—21], it is difficult to come to a firm conclusion. Although the presently available data make it appear unlikely that estrogen causes an acute elevation of cyclic AMP as an immediate, primary effect, modest increases in cyclic AMP may occur several hours later [20,21]. This timing correlates with the present observation that nonhistone chromosomal protein phosphorylation is not altered until several hours after estrogen administration. Since the presence of multiple nuclear protein kinases that are both stimulated and inhibited by cyclic AMP have been reported in other tissues [22], it is conceivable that the present effects are mediated in part by such changes in cyclic AMP. It is also possible, however, that regulation via cyclic GMP is involved, since it has been reported that this nucleotide is elevated by estrogen treatment [23], and effects of cyclic GMP on nonhistone chromosomal protein phosphorylation have been observed in other cell types [24].

It is obvious from the present experiments that gross changes in the phosphorylation of nonhistone chromosomal proteins are not among the initial events involved in the stimulation of uterine gene transcription by estradiol. Increases in histone acetylation [25] and stimulation of IP protein synthesis [26] clearly occur prior to the observed increase in nuclear protein phosphorylation. However, it should be emphasized that the techniques employed in

the current studies were not sensitive enough to detect changes in the phosphorylation of minor protein components.

Although changes in nonhistone chromosomal protein phosphorylation may not be involved in the initial response of the uterus to estrogen stimulation, the overall uterine response to estrogen is known to be biphasic. Although there is a small early stimulation of RNA synthesis, the rate of accumulation of RNA is greatest during the interval between 6 and 24 h after hormone treatment [27]. Increases in the synthesis of ribosomal RNA, and its 45 S and 32 S precursors, and 4 S and 5 S RNA, are not striking until 2—4 h after estradiol treatment [28]. Also, the high-salt-dependent RNA polymerase, which synthesized DNA-like RNA, does not increase until 12—24 h after estradiol administration [7,8]. Therefore, even though phosphorylation of nonhistone chromosomal proteins may not be involved in the initial phase of gene activation, it could play a role in the second phase of the uterine response when there is a more massive increase in the rate of RNA and protein synthesis.

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