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ROLE OF POLYAMINE IN THE REGULATION OF RNA SYNTHESIS IN UTERINE NUCLEOLI

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Summary—Administration of estradiol (E_2) to ovariectomized mature rats has been shown to result in synthesis of uterine polyamines in the same temporal manner as E_2 regulation of nucleolar transcription. Data is presented on the *in vivo* and *in vitro* effects of polyamines on uterine nucleolar RNA synthesis. Transcervical intrauterine administration of putrescine (100 μg), spermidine (100 μg), or spermine (100 μg) resulted in an increased transcriptional activity of 93 and 82% in uterine nucleoli isolated from putrescine and spermidine treated animals, respectively. Spermine administration was without effect on uterine nucleolar transcription. The polyamine-induced increase in transcription was totally accounted for by an increased rate of elongation of previously initiated RNA chains. No effect on the number of nucleolar RNA chains in the act of synthesis was observed.

Preincubation of uterine nucleoli, isolated from control animals (no E_2) with putrescine, spermidine, or spermine in the presence, but not in the absence of ATP, resulted in 44, 83 and 31% increased nucleolar RNA synthesis, respectively. *In vitro* polyamine-induced nucleolar RNA synthesis was correlated with a polyamine activated phosphorylation of nucleolar proteins of 110,000 24,000, 18,000 and 14,000 Da. Results suggest that early E_2 action may result in activation of the polyamine pathway which modulates nucleolar protein kinase activity; initiating an increase in nucleolar transcription.

INTRODUCTION

Estradiol (E_2) stimulation of the uterus results in both hypertrophy and hyperplasia. The response of the uterus to E_2 administration occurs in two phases, initiation and maintenance [1–4]. Certain responses in the uterus are restricted to the early initiation phase of E_2 action [1–3] whereas others are maintained throughout both phases [4]. One early response of E_2 administration is increased uterine ornithine decarboxylase (EC 4.1.1.17) (ODC) activity and subsequent increased polyamine levels, specifically putrescine, spermidine and spermine [5, 6]. It has been shown that polyamine synthesis is required for E_2 induction of cell proliferation in MCF-7 human breast cancer cells but not for E_2 activation of progesterone receptor levels [7]. Therefore, it appears that selective E_2 regulated events might be affected by the polyamine pathway.

Numerous studies have indicated that polyamines are important in cell growth and development [8–12]. Evidence has indicated that polyamine accumulation parallels an increase in RNA content, specifically ribosomal RNA [13–20]. *In vitro* experiments have shown that polyamines can effect the RNA synthesis capacity of isolated nuclei and nucleoli [21–25]. The presence of polyamines during the isolation of rat liver nuclei and nucleoli resulted in structural alterations in the nucleoli and increased transcriptional activity [26, 27].

Administration of E_2 to ovariectomized mature rats resulted in early (4 h) and late (24 h) increases in transcriptional activity of isolated uterine nucleoli [28]. The early stimulation in nucleolar transcription can be totally accounted for by an increased rate of elongation of previously initiated nucleolar RNA chains and is dependent upon synthesis of a short-lived protein(s). Late effects of E_2 treatment resulted in both an increased number of RNA chains (increased initiation) and increased RNA chain elongation, and was independent of the synthesis of a short-lived protein [28, 29].

The aim of this study was to determine whether E_2 regulation of polyamine levels in the uterus is related to increased uterine nucleolar transcriptional activity. Results indicate that *in vivo* administration of polyamines by the transcervical intrauterine route resulted in

stimulation of nucleolar RNA synthesis by an increased elongation rate of previously initiated RNA chains. *In vitro* polyamines in the presence, but not in the absence of ATP, stimulated RNA synthesis in nucleoli isolated from the uterus of control (no E_2) animals. Results suggest that polyamines activate endogenous nucleolar protein kinase(s) that phosphorylate specific nucleolar proteins.

MATERIALS AND METHODS

Preparation of uterine nucleoli, uterine transcriptional assays and determination of the number of RNA chains being synthesized in isolated uterine nucleoli were as described previously [29].

Chemicals

Unlabeled nucleoside triphosphates, polyamines and steroids were obtained from Sigma Chemical Co. (St Louis, MO). Radioactive isotopes were obtained from New England Nuclear (Boston, MA). All other chemicals were reagent grade.

Animals

Mature female albino rats (Small Animal Supply Co., Omaha, NE) weighing 160–180 g were ovariectomized 3–4 weeks prior to use. E_2 (2.5 μg) was given by a single tail vein injection in 0.5 ml of vehicle composed of 5% (v/v) ethanol in isotonic saline for the indicated times. Polyamines were administered by the transcervical intrauterine route in a vehicle composed of 0.3 M NaCl. Treatment vehicles were shown to have no effect on the basal activity of nucleolar transcription being tested.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Nucleolar proteins were separated on SDS–PAGE in a linear gradient of 7.5–20% acrylamide according to the method of Laemmlli [30]. Gels were sliced in 1.5 mm sections and dissolved in 0.5 ml of NCS Tissue Solubilizer (Amersham/Searle, Chicago, IL) and counted after the addition of 10 ml of a toluene-based scintillator.

DNA determinations

DNA was determined by the diphenylamine method of Burton [31].

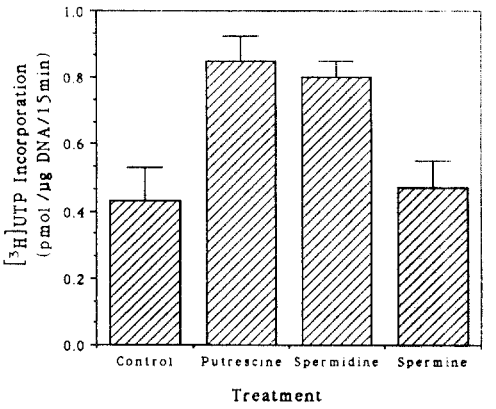

Fig. 1. Effect of *in vivo* administration of polyamines on the transcriptional activity of isolated uterine nucleoli. Groups of 10 identically treated animals were administered either 100 μg of putrescine, spermidine, spermine, or vehicle by the transcervical intrauterine route. Animals were sacrificed 4 h after treatment. Isolated uterine nucleoli were assayed for transcriptional activity [29]. All assays were performed in triplicate. Values are expressed as means ± SD.

RESULTS

Effect of polyamines given in vivo on the rate of RNA synthesis in isolated uterine nucleoli

Since E_2 administration has been shown to stimulate uterine polyamine synthesis in the same temporal manner as the effect of E_2 on nucleolar RNA synthesis [5, 6, 28], we investigated the effect of transcervical intrauterine injections of polyamines (putrescine, spermidine or spermine) on the ability of isolated uterine nucleoli to support RNA synthesis.

Transcervical intrauterine administration of putrescine and spermidine, for 4 h, resulted in a stimulation in the rate of RNA synthesis of 93 and 82%, respectively, over control levels, when measured in isolated uterine nucleoli (Fig. 1). Spermine administration *in vivo* did not effect nucleolar RNA synthesis when measured in the *in vitro* RNA synthesis assay.

In vivo effects of polyamines on stimulation of the rate of RNA synthesis measured in isolated uterine nucleoli could be due to an increased rate of elongation of uterine nucleoli RNA chains and/or to increased numbers of RNA chains being synthesized. *In vivo* administration of a mixture of putrescine, spermidine and spermine to ovariectomized rats resulted in a stimulation of nucleolar RNA synthesis of 112% when compared to nucleoli isolated from the uterus of control animals (Table 1). The number of RNA chains in the act of synthesis determined by the incorporation of [α^{32}P]cordycepin 5′-triphosphate was uneffected by the administration of polyamines. The

Table 1. *In vivo* effect of polyamines on the rate of elongation of nucleolar RNA chain growth and the number of elongating nucleolar RNA chains

Treatment	[5,6-^{3}H]UTP incorporation (pmol/μg DNA)	[α^{32}P]Cordecepin incorporation (pmol/μg DNA)	Elongation rate (^{3}H/^{32}P)
Saline	0.42 ± 0.05	0.03 ± 0.005	13.96
Polyamines	0.89 ± 0.07	0.02 ± 0.004	44.30

Groups of 8 animals were administered a mixture of putrescine, spermidine, and spermine (100 μg each) or 1.8% NaCl carrier by the transcervical intrauterine route. Animals were sacrificed 4 h following injection of polyamine. Uterine nucleoli were isolated and assayed for transcriptional activity ([5,6-^{3}H]UTP incorporation) or number of nucleolar RNA chains in the act of synthesis ([α^{32}P]cordecepin 5′-triphosphate incorporation). Elongation rates were determined by the ratio of total nucleolar RNA synthesis to number of nucleolar RNA chains ([5,6-^{3}H]UTP/[α^{32}P]cordecepin 5′-triphosphate). All assays were performed in triplicate and values are expressed as means ± SD.

rate of elongation of nucleolar RNA chains, determined from the ratio of total RNA synthesis capacity (pmol[5,6-^{3}H]UTP/μg DNA) to number of nucleolar RNA chains (pmol[α^{32}P]cordycepin 5′-triphosphate/μg DNA) is increased by 127% above controls. Increased nucleolar RNA synthesis from polyamine treated animals could be totally accounted for by an increased rate of elongation of previously initiated RNA chains.

In vitro effect of polyamines on the rate of uterine nucleolar RNA synthesis

The question of whether *in vivo* administration of polyamines results in a direct effect on nucleolar RNA synthesis cannot be definitely determined from the above experiment. Polyamines are known to have effects on numerous biochemical pathways [7–27] any of which could be the ultimate effector of nucleolar RNA synthesis. Administration of difluoromethylornithinine (DMFO), an irreversible inhibitor of ODC to control or 4 h E_2 treated animals (given just prior to E_2 treatment) partially eliminated the subsequent increase in *in vitro* nucleolar RNA synthesis due to *in vivo* E_2 treatment (data not shown). Experiments were therefore conducted to determine whether polyamines were capable of directly stimulating uterine nucleolar RNA synthesis. Isolated nucleoli from control animals (no E_2) were incubated at 37°C for 15 min in the presence or absence of individual polyamines and with or without ATP. Following preincubation, uterine nucleoli were re-isolated by centrifugation and the rate of uterine nucleolar RNA synthesis determined. Preincubation of uterine nucleoli in the presences of polyamines did not effect the ability of the subsequently isolated nucleoli to synthesize RNA (Fig. 2). Addition of ATP to the polyamine–nucleolar preincubation reaction resulted in an increase in the rate of nucleolar RNA synthesis when compared to nucleoli preincubated in the absence of polyamines and ATP. Putrescine, spermidine and spermine in the presence of ATP increased nucleolar RNA synthesis by 44, 83 and 31%, respectively, when compared to nucleoli incubated in their absence. ATP alone during the preincubation had no effect. Preincubations in the presence of a combination of polyamines and ATP resulted in stimulation of nucleolar RNA synthesis, which was not seen in the absence of ATP.

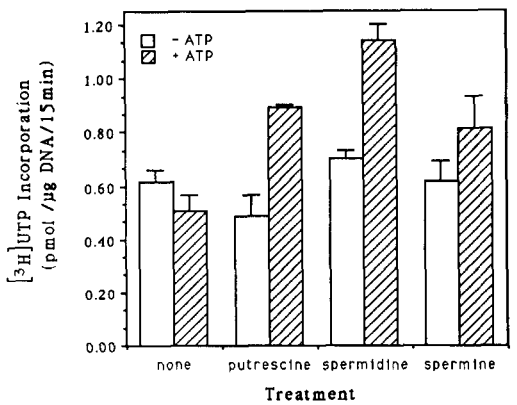

Fig. 2. *In vitro* effect of polyamines on the transcriptional activity of uterine nucleoli isolated from control animals. Uterine nucleoli isolated from 20 ovariectomized mature rats were preincubated at 30°C for 15 min with or without putrescine (1 mM), spermidine (1 mM) or spermine (1 mM) in the presence or absence of 1 mM ATP. Following preincubation the nucleoli were reisolated by centrifugation and assayed for transcriptional activity [29]. All assays were performed in triplicate and values are expressed as means ± SD.

Administration of polyamines *in vivo* was capable of stimulating the rate of elongation of nucleolar RNA synthesis similar to the early E_2 (4 h) stimulated increase in nucleolar transcription. We therefore investigated the effect of preincubating uterine nucleoli isolated from control or 4 h E_2 treated ovariectomized rats with or without spermidine in the presence or absence of ATP on the capacity of the nucleoli to synthesise RNA. E_2 treatment *in vivo* resulted

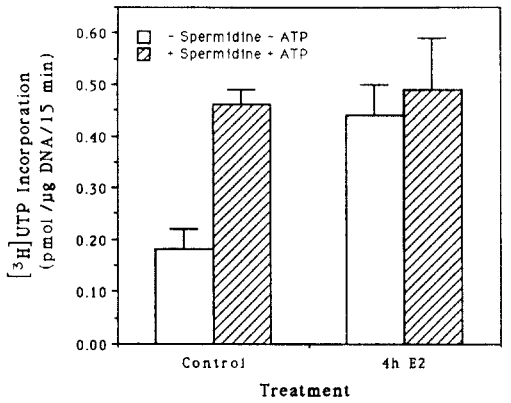

Fig. 3. *In vitro* effect of spermidine on the transcriptional activity of uterine nucleoli isolated from control or 4 h E_2 treated animals. Groups of 8 identically treated animals were administered E_2 (2.5 μg) or vehicle i.v., then sacrificed 4 h later. Uterine nucleoli were isolated and preincubated with or without spermidine (1 mM) at 30°C for 25 min in the presence or absence of 1 mM ATP. Nucleoli were then reisolated by centrifugation and assayed for transcriptional activity [29]. All assays were performed in triplicate and reported as means ± SD.

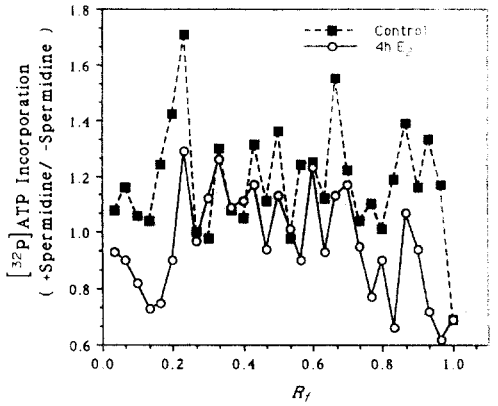

Fig. 4. SDS–PAGE of uterine nucleolar proteins phosphorylated in the presence or absence of spermidine. Groups of 10 identically treated animals were administered E_2 (2.5 μg) or vehicle i.v., then sacrificed 4 h later. Uterine nucleoli were isolated and nucleoli equivalent to 200 μg DNA incubated at 37°C for 30 min with or without 1 mM spermidine in the presence of [γ-32P]ATP. The samples were acid precipitated, washed free of unincorporated [γ-32P]ATP, solubilized and separated by SDS–PAGE. The radioactivity present in 1.5 mm slices was determined and results expressed as the ratio of incorporated [γ-32P]ATP in the presence vs absence of spermidine. M_w standards of *E. coli* B galactosidase (116,250 Da), rabbit muscle phosphorylase b (97,400 Da), bovine serum albumin (66,200 Da), hen egg white ovalbumin (42,699 Da), bovine carbonic anhydrase (31,000 Da), soybean trypsin inhibitor (21,500 Da), and hen egg white lysozyme (14,400 Da) correspond to an R_f of 0.17, 0.30, 0.35, 0.46, 0.62, 0.75 and 0.93, respectively.

in a 144% increase in transcriptional activity in isolated uterine nucleoli when compared to nucleoli isolated from control animals (Fig. 3). Differences in transcriptional activity of nucleoli isolated from control animals compared with E_2 treated animals were eliminated following preincubation of nucleoli from control animals in the presence of spermidine and ATP. Preincubation of nucleoli from 4 h E_2 treated animals with spermidine plus ATP had no additional effect on RNA synthesis.

Role of ATP in the polyamine stimulation of nucleolar transcription

Activation of transcription in nucleoli isolated from the uterus of control animals by *in vitro* preincubation in the presence of polyamines could only be attained if ATP was present during the preincubation. It has previously been suggested that polyamines regulate certain biochemical events by activating specific protein kinases [32–39]. Experiments were conducted to determine whether putrescine, spermidine or spermine were capable of activating protein kinases present in uterine nucleoli isolated from control or 4 h E_2 treated animals. Uterine nucleoli were incubated in the presence of [γ-32P]ATP with or without putrescine, spermidine, or spermine at 37°C for 30 min. The

Table 2. Effect of polyamines on the *in vitro* phosphorylation of uterine nucleolar proteins

Treatment *in vivo*	Addition *in vitro*	[γ-32P]ATP incorporation (pmol/mg DNA)	Percent increased with polyamines
Control	None	1.67 ± 0.11	100
Control	Putrescine	1.67 ± 0.06	100
Control	Spermidine	2.10 ± 0.17	126
Control	Spermine	1.92 ± 0.10	115
E_2	None	2.46 ± 0.26	100
E_2	Putrescine	2.06 ± 0.17	84
E_2	Spermidine	2.65 ± 0.48	108
E_2	Spermine	2.73 ± 0.13	111

Groups of 10 identically treated animals were administered 2.5 μg of E_2 or hormone carrier by the i.v. route. Animals were sacrificed 4 h following treatment. Uterine nucleoli were isolated and nucleoli equivalent to 1.5 μg DNA incubated with or without 1 mM polyamines (putrescine, spermidine, or spermine) in the presence of [γ-32P]ATP at 37°C for 30 min. Samples were acid precipitated and the amount of radioactivity present in the pellet was determined. All assays were performed in triplicate and values are expressed as means ± SD. Controls and E_2 treatment without polyamines = 100%.

samples were precipitated and the radioactivity incorporated into protein was determined. Putrescine was without effect on the activation of protein kinases present in nucleoli isolated from the uterus of either control or 4 h E_2 treated animals (Table 2). Spermine had a modest effect (15 and 11%) on phosphorylation of protein(s) present in nucleoli isolated from control or 4 h E_2 treated animals, respectively. Incubations in the presence of spermidine, resulted in a 26% increase in phosphorylation of protein(s) present in uterine nucleoli isolated from control animals, while incubations of uterine nucleoli isolated from 4 h E_2 treated animals in the presence of spermidine demonstrated no increase in protein phosphorylation. *In vivo* treatment with E_2 for 4 h stimulated an increase of 47% in the phosphorylation of uterine nucleolar proteins compared to nucleoli isolated from control animals, suggesting that E_2 may stimulate a general increase in phosphorylation of nucleolar proteins.

Selective nucleolar proteins could be substrates for the spermidine activated protein kinase, therefore investigation into the phosphorylation of specific nucleolar proteins in the presence or absence of spermidine was undertaken. Uterine nucleoli isolated from control or 4 h E_2 treated animals were incubated with [γ^{32}P]ATP in the presence or absence of spermidine. Nucleolar proteins were isolated and separated by SDS–PAGE and the radioactivity in gel slices determined. The results were expressed as the ratio of phosphorylation in the presence vs absence of spermidine (Fig. 4) and demonstrate that spermidine stimulated phosphorylation by 70, 55, 39 and 33% in proteins determined to be 110,000 and 24,000, 18,000 and 14,000 Da, respectively, in uterine nucleoli isolated from control animals. Similar peaks appeared in uterine nucleoli isolated from 4 h E_2 treated animals but treatment with spermidine increased phosphorylation of the 110,000 and 24,000 Da proteins by only 30 and 17%, respectively. These results indicate that polyamine activated protein kinases exist in uterine nucleoli and are capable of phosphorylating specific nucleolar proteins. The *in vitro* polyamine activation is greater in nucleoli isolated from control animals compared to nucleoli isolated from 4 h E_2 treated animals, where *in vivo* polyamine activated phosphorylation may have already occurred.

DISCUSSION

Previous results from this laboratory have shown that early E_2 treatment (4 h) increased transcriptional activity in isolated uterine nucleoli and this increase was dependent upon the synthesis of a protein(s) with a short half-life [28]. ODC, the rate limiting enzyme in polyamine synthesis, is induced in the uterus by E_2 [5, 6]. The reported half life for ODC in eucaryotic cells is between 15–45 min [11, 12]. Induction of polyamine synthesis has long been known to play an important role in cell proliferation and development [8–12]. Polyamines are thought to exert their effects on a number of biochemical parameters such as DNA, protein and RNA synthesis, specifically ribosomal RNA [8–27]. However, the molecular mechanism by which polyamines regulate these processes is not known. Polyamine levels in the uterus of ovariectomized mature rats increased in a time dependent manner following E_2 treatment which correlated with the induction of nucleolar RNA synthesis [5, 6, 29]. It is plausible that the short-lived protein required for E_2 induced nucleolar transcription is ODC, and the end product of this enzyme, polyamines, are the molecular effectors which activate nucleolar RNA synthesis.

The hypothesis stated above is supported by data presented in this paper demonstrating that *in vivo* injection of polyamines (putrescine or spermidine) into the uterine lumen of ovariectomized rats for 4 h induced increased transcriptional activity of isolated uterine nucleoli when compared to nucleoli isolated from vehicle-injected animals. Whether putrescine directly effects the nucleolar RNA synthesis capacity or is converted to spermidine which elicits the stimulatory effect on the nucleolus is not known. Nucleoli isolated in the presence of spermidine exhibit morphological changes and increased transcription [26, 27]. Anucleolated mutant embryos of *Xenopus laevis* do not synthesize and accumulate ribosomal RNA or spermidine [40]. Our hypothesis is further supported by *in vitro* experiments which show that putrescine and spermidine in the presence, but not in the absence of ATP, increased transcription in nucleoli isolated from control animals. Polyamine stimulation was not observed if nucleoli were isolated from the uterus of 4 h E_2 treated animals. The increase in nucleolar transcription induced by polyamines *in vivo* could be totally accounted for by an increased rate of

elongation of nucleolar RNA chains, the same site where *in vivo* E_2 (4 h) treatment exerted the stimulatory effect on nucleolar RNA synthesis [29].

The molecular mechanisms by which polyamines regulate the rate of nucleolar transcription is not known. Numerous studies have demonstrated that polyamines effect chromatin structure and transcription [3–27]. Structural changes due to a number of physiological agents can be correlated with alterations in sensitivity to nucleases [41–46], transition of DNA from B to Z form [46] or supercoil relaxation activity of both procaryotic and eucaryotic Topoisomerase I [47–49]. Any one or a combination of these changes might account for the increased rate of uterine nucleolar RNA synthesis observed with *in vivo* E_2 or polyamine treatment of ovariectomized rats; or *in vitro* polyamine stimulation, in the presence of ATP, of uterine nucleoli isolated from control animals. The necessity for the presence of ATP for the induction by polyamines in nucleolar transcriptional activity suggests that polyamines may influence cellular processes such as nucleolar RNA synthesis by altering (increasing) phosphorylation of nucleolar proteins. Studies have demonstrated that specific protein kinase activities are regulated by polyamines [32–39]. Nuclear and nucleolar proteins have been identified as substrates for polyamine activated nuclear protein kinases including certain subunits for RNA polymerase I and II [50–52], high mobility-group proteins 14 and 17 [53, 54], neutral and basic non-histone proteins [39], and salt soluble acidic proteins [32]. Polyamine activated phosphorylation has been correlated with increased transcription [16, 18, 32, 34]. Our results suggest that incubation of uterine nucleoli from control animals, in the presence of spermidine, activated a nucleolar protein kinase(s) that phosphorylated several nucleolar proteins (M_ws 110,000, 24,000, 18,000 and 15,000) above that seen with nucleoli from the uterus of 4 h E_2 treated animals. This difference suggest that phosphorylation may have already occurred in E_2 treated animals. *In vivo* phosphorylation of uterine nucleolar proteins with M_ws of 110,000 and 24,000 have been observed following 4 h of E_2 treatment (unpublished observation). Whether the phosphorylation of any of these proteins is responsible for the increased rate in elongation of nucleolar RNA synthesis is not known, but the increased phosphorylation does correlate with activation of transcriptional activity. Isolation and/or purification of the phosphorylated nucleolar proteins and protein kinase(s) may help resolve the question of how polyamines mediate increased nucleolar RNA synthesis.

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