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# Modulation of cytosolic RNase activity by endogenous RNase inhibitor in rat vaginal epithelial cells on estradiol administration

K. Sridhar Rao, Ravi Sirdeshmukh*, P.D. Gupta

*Centre for Cellular & Molecular Biology, Hyderabad 500 007, India*

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**Abstract**

The cytosolic, alkaline RNase in rat vaginal epithelial cells (VEC) from normal, immature rats was found to be present largely in the free, active form unlike in many other mammalian tissues where it is known to be present in a latent form as a complex with RNase inhibitor (RNasin). Estradiol ($E_2$) administration induced expression of RNasin activity in the VEC from such animals and caused virtually total inhibition of cytosolic RNase activity in these cells by 12 h after the hormone injection. These changes may have metabolic implications in relation to other biochemical events stimulated by estradiol in rat VEC.

*Key words:* Estradiol; RNase; RNase inhibitor; Vaginal epithelial cell

## 1. Introduction

The cytosolic compartment of mammalian cells contains a major alkaline or neutral RNase which is believed to be present in latent form as a complex with a cytosolic protein – the RNase inhibitor (RNasin) and the RNase–RNasin system is implicated in the regulation of cytosolic RNase activity. The level of RNasin, for example, may vary with the metabolic status of the cells or tissue and thus influence RNase activity [1]. Estradiol ($E_2$)-induced modulation of the RNase–RNasin system has been reported earlier [2–5] in uterus which is a major target tissue for $E_2$ action. The hormone has also been shown to regulate the turnover of many mRNAs [6]. These two effects of $E_2$ may imply a regulatory relationship, i.e. $E_2$ may modulate the RNase–RNasin ratio or the complex formation, influence cellular RNase activity which in turn may effect the degradation of cytoplasmic mRNA(s). Earlier studies with the uterus have revealed that in ovariectomised animals, $E_2$ caused some reduction (18–50%) in the levels of uterine cytosolic RNase activity [2,3]. In immature rat uterus on the other hand, the hormone caused an increase in the levels of both RNase and RNasin [4,5] and the net RNase activity was higher than that in the control [5]. Moreover, the increase in the levels of both the RNase and RNasin is not consistent with the expected reciprocal changes in the levels of these two proteins if RNase activity is to be modulated. Vaginal epithelial cells (VEC) are also a target for $E_2$ action and undergo $E_2$-induced growth and differentiation; VEC differ from uterine epithelial cells particularly in the process of terminal differentiation. Rat VEC have, therefore, been used as a system to study $E_2$-induced cellular and biochemical changes [7–9]. In this report, we present data on the cytosolic RNase activity in VEC from rats administered with $E_2$. The hormone treatment was found to cause a profound decrease in the net cytosolic RNase activity (as a result of formation of the RNase–RNasin complex) in these cells in contrast to the observations with uterine cells.

## 2. Materials and methods

### 2.1. $E_2$ treatment and preparation of rat VEC

Immature (30-day-old) female Wistar rats were injected intraperitoneally with $E_2$ in 50% ethanol (10 μg/100 g b.wt.) and were killed at specific time intervals after the hormone injection. Controls received a same volume of vehicle. Vagina from the control and $E_2$ treated animals were dissected out and collected in cold phosphate-buffered saline (PBS). The vagina were slit longitudinally and the epithelial cells were scraped gently with the help of a clean glass slide [10].

### 2.2. Preparation of cytosolic fraction

The VEC were washed three times with cold PBS and resuspended and sonicated in a buffer containing 25 mM Tris, 100 mM NaCl, 10 mM DTT, 1 mM EDTA and 0.25 M sucrose. Sonication was optimized for the minimal damage of nuclei (unpublished results). The sonicate was centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant (S100 fraction or the cytosolic fraction) was collected and stored frozen. Protein estimation was done by Lowry's method [11] after TCA precipitation of the S100 fraction.

*Corresponding author. Fax: (91) (40) 851195.

*Abbreviations*: VEC, vaginal epithelial cells; RNase, ribonuclease; RNasin, RNase inhibitor; $E_2$, estradiol 17-β; pCMB, *p*-chloromercuric benzoate.


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*2.3. Isolation of RNA*

Total RNA from logarithmically growing *E. coli* (used as substrate in RNase assays) was isolated by phenol extraction as described by us earlier [12]. The quality of the RNA was assessed by agarose gel electrophoresis.

*2.4. RNase assays*

RNase assays were done using agarose gel electrophoresis essentially as described in [12]. In a typical assay, 1 μg of total RNA (rat spleen or *E. coli*) was incubated with various concentrations of S100 fractions from rat VEC (2–200 ng of total protein) in 10 μl buffer containing 20 mM Tris, pH 7.8, 100 mM NaCl, 10 mM DTT, for 30 min at 37°C (DTT was omitted when assays were done in the presence of *p*-chloromercuric benzoate (pCMB); see Fig. 3). At the end of the incubation, samples were electrophoresed in a 1% agarose gel in 0.09 M Tris-Borate, EDTA buffer (pH 8). RNA in the gel was visualized by ethidium bromide staining. Degradation was assessed by disappearance of the rRNA bands and degradation comparisons were made in terms of the minimum amount of protein required for complete and clear disappearance of the rRNA bands and productions of degradation fragments of the size of 4 S or smaller.

## 3. Results and discussion

For the study of RNase activity in VEC, we routinely used an RNase assay procedure which is based on agarose gel electrophoretic separation of the reaction products from the substrate wherein the extent of degradation is correlated with visually assessed, disappearance of rRNA bands and formation of products with greater electrophoretic mobility. The procedure is adequate for semi-quantitative comparison of relative RNase levels and in this respect, it is more sensitive than other procedures for assaying RNase activity such as the spectrophotometric method or the one based on cCMP hydrolysis [13,14]. These methods detect the activity of ng quantities of bovine pancreatic RNase A but the method as described here can be conveniently used with as low as pg levels of RNase A (80 pg or 0.25 units as in assays reported in [12]). Greater detection sensitivity of this procedure and the use of electrophoretic analysis of the reaction mixture would allow detection of RNase activity even when acid soluble degradation products are not produced (as in assays for angiogenin [15] and as would be expected with very low levels of free cytosolic RNase activity).

When the S100 fraction from rat VEC was assayed for RNase activity at different pHs (Fig. 1A), the activity was observed mostly in the alkaline pH range with a pH maximum around 7.5 and 8. No significant activity could be detected at acidic pH between 4.5 and 6.0. Also, the RNase activity detected in our assays could be completely inhibited in the presence of RNasin (Fig. 1B). Absence of detectable activity at acidic pH and almost total inhibitability of the activity observed in our assays by added RNasin rule out the possibility of any contamination by lysosomal RNases in our S100 preparations; lysosomal fraction of mammalian cells contains acidic RNase insensitive or partially sensitive to RNase inhibitor [16]. Moreover, on electron microscopic examination, the number of lysosomes in rat VEC were found to be very low as compared to other cell types (unpublished results). Thus, we believe that all the activity detected in our assays represents inhibitor-responsive, cytosolic alkaline RNase of the rat VEC. In Fig. 2, a comparison of cytosolic RNase activity from immature rat VEC at specific time periods after estradiol injection is shown. The RNase activity was compared in terms of the minimum amount of cytosolic protein required to cause disappearance of the substrate rRNA bands on electrophoresis. A wide range of S100 protein amounts were tried in pilot experiments and on the basis of those results, the range was chosen for the experiment shown in the Fig. 2. RNase activity in the VEC from the untreated controls could be detected with 4–10 ng of cytosolic protein. The activity levels were found to be somewhat reduced in cells from 3-h $E_2$-treated animals and 10–20 ng of S100 protein were required to observe RNase activity. With 6-h $E_2$-treated samples, RNase activity was apparent with 60–80 ng of S100 protein. Much higher protein amounts were to be used when 12-h and 24-h $E_2$-treated samples were studied. With 12-h $E_2$-treated samples, no detectable RNase activity was observed even with 200 ng (400 ng in another experiment) of protein whereas with 24-h hormone-treated samples RNase activity could be detected above 80 ng of S100 protein. From this and other experiments, we conclude that the cytosolic RNase activity levels drop progressively after $E_2$ injection such that by 12 h it reaches the lowest level and tends to rise thereafter.

Cellular alkaline or neutral RNase exists in cytosol

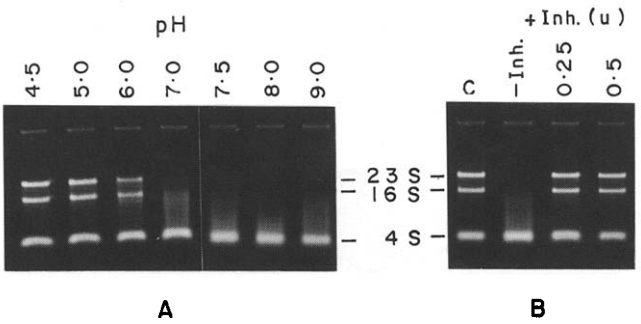


Fig. 1. Characteristics of the total ribonuclease activity of the S100 fraction of the VEC. (A). The effect of pH on nuclease activity. 10 ng of S100 protein of the VEC was incubated with 1 μg of RNA at 37°C for 30 min in a buffer of different pH. For pH 4.5 and 5.0, 20 mM sodium acetate buffer, for pH 6.7, 20 mM phosphate buffer and for pH 7.5 to 9.0, 20 mM Tris-HCl buffer was used. The S100 fraction was diluted at least 100-fold in the respective buffer for the assay. After the reaction, the RNA was run in a 1% agarose gel and stained with EtBr. The nuclease activity was maximum at pH 7.5–8. (B) Effect of placental RNase inhibitor on the nuclease activity. 1 μg of total RNA was incubated as such (lane C) or with 10 ng of S100 protein of the VEC in the absence (−Inh.) or presence (+Inh.) of increasing amounts (units) of human placental RNasin for 30 min at 37°C. At the end of incubation, RNA was analysed as in (A). The nuclease activity was sensitive to added RNase inhibitor.

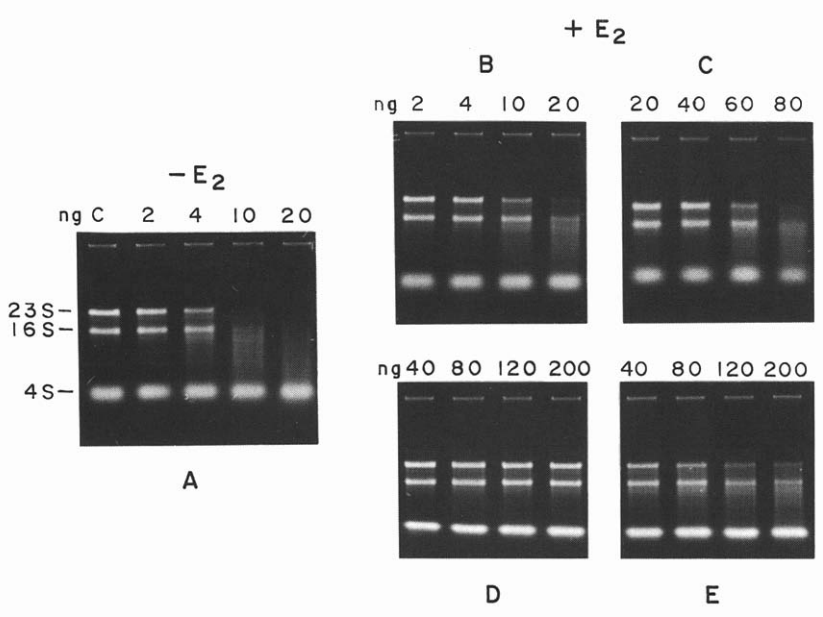


Fig. 2. Levels of alkaline RNase in S100 fraction of VEC from control and $E_2$ injected rats. S100 fractions were prepared from VEC from control and $E_2$ injected rats as described in section 2 and assayed for RNase activity as follows. 1 $\mu$g of total RNA was incubated with different amounts of S100 fraction protein (2–200 ng) for 30 min at 37°C. RNA was analysed as in Fig. 1. In $E_2$ treated animals, cytosolic alkaline RNase activity of the VEC reduced several fold by 12 h after hormone injection.

largely in a latent form as a complex with RNasin. The main experimental evidence in support of this is that no RNase activity could be detected in the mammalian cytosolic fraction unless the inhibitor is inactivated by agents like pCMB [1]. With the use of the assay procedure described here even with 10 $\mu$g of the cytosolic protein fraction from rat liver, no detectable RNase activity could be observed when the assay did not include pCMB (data not shown). The situation with the rat VEC appears to be different in that the enzyme activity could be detected even in the absence of pCMB (Fig. 2) suggesting the presence of significant amounts of free, active RNase in these cells. Furthermore, Fig. 3 shows the levels of the free and total cytosolic RNase activity of VEC from $E_2$-treated and untreated rats when the assays were done in the absence or presence of 0.2 mM pCMB. The results indicated: (i) the RNase activity level detected in the S100 fraction of VEC from the untreated control was similar in the assays done in presence or absence of pCMB (left sections) suggesting that virtually all the RNase in the control rat VEC exists in the free, active form and not as the latent, RNase–RNasin complex; (ii) the RNase activity in the S100 fraction of the VEC from $E_2$-treated animals could be released by pCMB treatment (right sections) and no significant difference was observed between the total RNase activity (lower sections; + pCMB assays) of $E_2$-treated and untreated samples. Therefore, reduced levels of RNase activity in 12-h $E_2$-treated samples (Figs. 2 and 3; −pCMB assays) appears not to be due to reduction in the amount of the RNase protein but due to induction of latency on account of complexing of the enzyme with RNasin; and (iii) the total activity level, i.e. the activity released by pCMB treatment (+pCMB assay), in $E_2$-treated samples was equivalent to the activity level in the control VEC (in which almost all the RNase is present in free active form; see above). Therefore, on $E_2$ treatment (12 h), all the RNase in the VEC seemed to be complexed and inactivated by the inhibitor. Complex formation could occur either on activation of the pre-existing cytosolic inhibitor or as a result of induction of its expression by $E_2$. We are presently working on these two possibilities. The possibility of any property of the enzyme being responsible for it's inhibitor-insensitivity in normal VEC is unlikely because the enzyme from the control tissue samples was potentially sensitive to added RNasin (Fig. 1).

The exact role of the cytosolic RNase-RNasin system in mammalian tissue is not yet clear. There are some earlier reports about changes in the cytosolic RNase and inhibitor activity levels which may have metabolic implications [1,17]. The effect of $E_2$ on cytosolic, RNase levels in rat uterine tissue has also been reported earlier [2–5]. $E_2$ treatment was found to result in concomitant increase in the levels of both RNase and the inhibitor in this tissue by 2–4 days after the hormone injection and the net RNase activity was higher than that in the control [5]. In view of its putative role in the regulation of RNase activity, increase in the inhibitor levels is expected to correlate with decrease in cellular RNase activity [1], however the above observation with rat uterine tissue from immature animals was found to be inconsistent with this expectation. These observations could not be accounted for by impurities in the cytosolic fractions used. It is unlikely that under the experimental conditions used by these researches, lysosomal RNase activity (RNase which is active at acidic pH and is partially sensitive to the inhib-

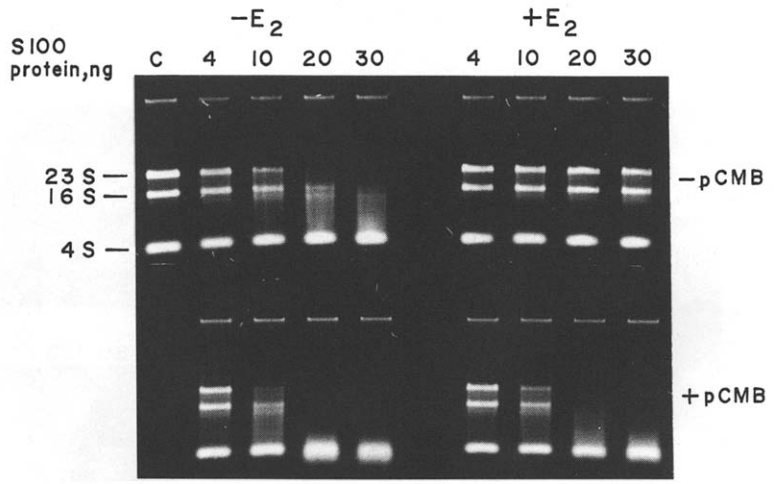


Fig. 3. Comparison of free (−pCMB) and total (+pCMB) RNase activity of VEC from control and $E_2$ treated rats. RNase assays were done as in Fig. 2 but in the presence or absence of 0.2 mM pCMB (which inactivates the RNase inhibitor and releases RNase from the RNase – inhibitor complex). The amount of S100 protein used for the assay was as indicated. There was virtually no latent RNase in control VEC whereas it was present in 12-h $E_2$-treated samples.

itor) could contaminate and be detected in the cytosolic fraction ('high-speed supernate'). However, these authors could not rule out the possibility that their observation could be attributed to the heterogeneity of the cell types in the rat uterus. In this investigation, early effects (0–24 h) of $E_2$ administration were not studied. Therefore, our present results with rat VEC are difficult to compare with the above study using uterine tissue from immature rats. However, in an independent study, RNase levels in the uterine tissue from ovariectomised animals were studied [2,3] and were found to be low at 6 h after the hormone treatment as compared with the control. Although the latter study is consistent with our observation with rat VEC, the decrease in the RNase activity level in rat VEC at 12 h after $E_2$ injection observed by us, is more distinct (Fig. 2) when compared with the uterine tissue (18–50% decrease). Thus, our observations with rat VEC indicate very strong modulation in the levels of the cytosolic RNase activity and this appears to be due to changes in the RNase–RNasin system triggered by an external signal like $E_2$. The findings may be useful not only to further study the mechanism of this process but also to explore the metabolic role of the RNase–RNasin system.

$E_2$ is also known to regulate the expression of several genes [18–20]. In rat VEC, our earlier studies [8,9] have shown that the hormone stimulated several biochemical processes related to growth and differentiation. Other experiments (unpublished) in our lab have indicated that $E_2$-treatment stimulated DNA synthesis in these cells and synthesis of a major proliferation-specific protein keratin and accumulation of its mRNA. Coincidently, $E_2$-induced changes in the activity of cytosolic RNase and RNasin happen in parallel with both these biochemical events. A more extensive and independent study is required to investigate whether all these changes have any metabolic relationship and rat VEC would be a very useful system for this purpose.

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