Cyclic Expression of Endothelin-converting Enzyme-1 Mediates the Functional Regulation of Seminiferous Tubule Contraction

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Abstract. The potent smooth muscle agonist endothelin-1 (ET-1) is involved in the local control of seminiferous tubule contractility, which results in the forward propulsion of tubular fluid and spermatozoa, through its action on peritubular myoid cells. ET-1, known to be produced in the seminiferous epithelium by Sertoli cells, is derived from the inactive intermediate big endothelin-1 (big ET-1) through a specific cleavage operated by the endothelin-converting enzyme (ECE), a membrane-bound metalloprotease with ectoenzymatic activity. The data presented suggest that the timing of seminiferous tubule contractility is controlled locally by the cyclic interplay between different cell types. We have studied the expression of ECE by Sertoli cells and used myoid cell cultures and seminiferous tubule explants to monitor the biological activity of the enzymatic reaction product. Northern blot analysis showed that ECE-1 (and not ECE-2) is specifically expressed in Sertoli cells; competitive enzyme immunoassay of ET production showed that Sertoli cell monolayers are capable of cleaving big ET-1, an activity inhibited by the ECE inhibitor phosphoramidon. Microfluorimetric analysis of intracellular calcium mobilization in single cells showed that myoid cells do not respond to big endothelin, nor to Sertoli cell plain medium, but to the medium conditioned by Sertoli cells in the presence of big ET-1, resulting in cell contraction and desensitization to further ET-1 stimulation; in situ hybridization analysis shows regional differences in ECE expression, suggesting that pulsatile production of endothelin by Sertoli cells (at specific “stages” of the seminiferous epithelium) may regulate the cyclicity of tubular contraction; when viewed in a scanning electron microscope, segments of seminiferous tubules containing the specific stages characterized by high expression of ECE were observed to contract in response to big ET-1, whereas stages with low ECE expression remained virtually unaffected. These data indicate that endothelin-mediated spatiotemporal control of rhythmic tubular contractility might be operated by Sertoli cells through the cyclic expression of ECE-1, which is, in turn, dependent upon the timing of spermatogenesis.

Key words: endothelin • endothelin-converting enzyme • spermatogenesis • peritubular myoid cells • seminiferous epithelium

1. Abbreviations used in this paper: aa, amino acid; ECE, endothelin-converting enzyme; EIA, enzyme immunoassay; ET, endothelin; FSH, follicle-stimulating hormone; KHH, Krebs-Henseleit-Hepes; PR, phosphoramidon.

ENDOTHELIN-1 (ET-1) is a 21-amino acid (aa) vasoconstrictive peptide originally isolated from the supernatant of cultured porcine aortic endothelial cells (Yanagisawa et al., 1988). Subsequently, three distinct endothelin genes encoding three closely related peptides were identified: ET-1, ET-2, and ET-3 (Inoue et al., 1989). These endothelin isopeptides are each produced from corresponding preproETs of ~200 residues (Inoue et al., 1989) and act on two distinct subtypes of G-protein-coupled receptors termed ET A and ET B (Arai et al., 1990; Sakurai et al., 1990). Longer intermediates termed big endothelins (big ETs, 38–41 aa) are first excised from the preproETs by dibasic pair-specific endopeptidases (Seidah et al., 1993). Big ETs are then further cleaved at Trp21-Val/Ile22 by the endothelin-converting enzyme (ECE) to produce the 21-residue mature peptides (Opgenorth et al., 1992). The fact that the biological activity of big ETs is negligible (Kimura et al., 1989) indicates that ECE is a key enzyme for the production of biologically active ETs.
Complementary DNAs coding for two bovine ECEs have been isolated recently and the corresponding proteins have been termed ECE-1 (Xu et al., 1994) and ECE-2 (Emoto and Y. anagisawa, 1995). Both enzymes are membrane zinc-binding metalloendopeptidases with a single transmembrane domain, a short NH2-terminal cytoplasmic tail and a large extracellular COOH-terminal containing the catalytic domain (Shimada et al., 1996; Turner and Tanzawa, 1997). A analysis of the conversion of big ET-1 into ET-1 by ECE in vivo and in vitro (McMahon et al., 1991; Xu et al., 1994) has demonstrated that the conversion takes place on the cell surface. Recently, the presence of ECE on the plasma membrane has also been confirmed by ultrastructural immunolocalization showing that ECE and angiotensin-converting enzyme colocalize on the luminal membrane of endothelial cells (Barnes et al., 1998).

The abundance of ECE-1 mRNA in whole testis extracts favors the hypothesis that this enzyme plays an important role by maintaining the ET receptor activation in the testis (Xu et al., 1994). In the mammalian testis, seminiferous tubules are ensheathed by a layer of smooth muscle-like cells, the peritubular myoid cells. In the adult rat, myoid cells are arranged in a squamous epithelial layer in which no major orientation is apparent (Hermo and Clermont, 1976; Palombi et al., 1992). The main biological function of peritubular contractility is the generation of impulses for the progression of spermatozoa (Hargrove et al., 1977). The transport of spermatozoa along the seminiferous tubule lumen towards the rete testis, is thought to result from forces that are not intrinsic to the sperm cells (Ellis et al., 1981; Eddy, 1988). In fact, seminiferous tubules have been reported to undergo rhythmic contraction; in the apparent absence of nerve endings, the fine regulation of contractility is presumably subject to paracrine control.

Recently we demonstrated that ET-1 is specifically able to induce contraction of rat myoid cells both in cell culture and in peritubular tissue (Tripiciano et al., 1996). In addition, we demonstrated the simultaneous presence of ET' and ET' endothelin receptors on individual myoid cells, both of which mediate contraction through distinct regulation of calcium-mediated signaling (Filippini et al., 1993; Tripiciano et al., 1997). The studies of Fantoni et al. (1993) and Maggi et al. (1995) have demonstrated that Sertoli cells produce and secrete ET-1 in rat and human testis. Sertoli cells, somatic cells of the testis that provide the structural framework of the seminiferous tubules and the milieu for germ cell proliferation and differentiation, are targets for the hormones (FSH and testosterone) responsible for the initiation and maintenance of spermatogenesis (Bardin et al., 1988). In the seminiferous epithelium, each Sertoli cell maintains an extensive surface relationship, along its apical sides, with germ cells at various stages of differentiation up to spermatiation while the basal side faces the peritubular myoid cells.

Seminiferous tubule contractility represents a fundamental and potentially critical function in male fertility, controlling testicular output of both fluid and sperm. Therefore, any level of regulation mediating seminiferous tubule contractility may represent a specific control mechanism regulating the timing of the contraction-relaxation cycle. In this study, we have examined the in vivo and in vitro specific expression of ECE in rat Sertoli cells as well as the biological activity of this enzyme from intact cultured Sertoli cells. Furthermore, we highlight the functional relevance of ECE by showing that it can mediate regional seminiferous tubule contraction by converting big ET-1 into fully biologically active ET-1. We provide evidence of differential expression of ECE-1 in the testis during spermatogenesis that underlies a pulsatile production of ET-1, accounting for a novel mechanism controlling contractility.

**Materials and Methods**

**Materials**

Collagenase A from Clostridium histoliticum and DNase-I were obtained from Boehringer Mannheim. Trypsin was purchased from Difco Laboratories. MEM was obtained from GIBCO BRL. Percoll was purchased from Pharmacia. ET-1 and big ET-1 were obtained from Peninsula Laboratories, Inc.; other reagents, when not specified, were purchased from Sigma Chemical Co. Plastic culture dishes and multiwell plates were from Falcon.

**Animals**

The animals used were adult and three-week-old Wistar rats (Charles River), fed ad libitum until killed by CO2 asphyxia or cervical disarticularization. Animals were kept in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Alkaline Phosphatase Cytochemistry**

Selective myoid cell identification through alkaline phosphatase cytochemistry was performed as previously described (Palombi and Di Carlo, 1988), based on the method of Ackermann (Ackermann, 1962). In brief, the fixed cells were incubated in an alkaline solution containing 0.5 mg/ml Fast Blue R R in water and 40 μl/ml α-naphthol phosphate (0.25% solution, pH 8.6). After 30 min incubation in the dark, a purple-blue precipitate appeared specifically on the surface of myoid cells (Palombi and Di Carlo, 1988).

**Cell Isolation and Culture**

**Sertoli Cells.** Primary Sertoli cell cultures from 18-20-d-old Wistar rats were prepared as previously described (Dorrington et al., 1975). Seminiferous tubules obtained by trypsin dispersion of testicular parenchyma were subjected to collagenase digestion to remove the peritubulum. The resulting fragments of seminiferous epithelium, mainly composed of Sertoli cells, were cultured at 32°C in a humidified atmosphere of 5% CO2 and 95% air in a chemically defined medium (MEM). A fet 3 d in culture, germ cells contaminating the Sertoli cell monolayer were selectively removed through hypotonic shock (Galdieri et al., 1981); the cells were used one day after the treatment.

**Myoid Cell Cultures and Sertoli Cell/Myoid Cell Cocultures.** The supernatant-mixed cell population resulting from the collagenase treatment of seminiferous tubules (see above) was centrifuged at 40 g, yielding mostly minute fragments of tubular wall (Sertoli cells and myoid cells): culturing of this preparation in MEM for 3 d at 37°C results in a mixed monolayer in which myoid cells can be identified (Tripiciano et al., 1996) by differences in their morphology in phase contrast and through alkaline phosphatase cytochemistry after fixation. For pure myoid cell cultures, the tubular wall fragments were digested in trypsin and EDTA to a single cell suspension, subsequently fractionated on a discontinuous Percoll density gradient (Palombi et al., 1988; Filippini et al., 1993). Percoll-purified myoid cells were cultured under serum-free conditions at 37°C. The assessment of myoid cell purity, performed routinely for each preparation on the basis of the presence of alkaline phosphatase activity, was never below 96%.

**Germ Cell Preparations.** Seminiferous tubules from 35-60-d-old rats were freed from interstitial tissue by collagenase treatment and dispersed into single cells, as previously described (Galdieri et al., 1977). The resulting cell suspension, highly enriched in germ cells, was used as such (“mixed germ cells”) or fractionated into several cell classes by velocity sedimentation at unit gravity in an albumin gradient (Lam et al., 1970;...
Boitani et al., 1983). The two cellular fractions, composed, respectively, of middle-late pachytene spermatocytes and of round spermatids (steps 1-8 of spermiogenesis), were found to be ~90% pure; the fraction composed of intermediate spermatids (steps 9-14) was ~60% pure, also containing late spermatids (~10%) and residual bodies (~30%).

**RNA Isolation and Northern Blot Analysis**

RNA was extracted from testicular cells and different organs using the acid guanidine thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). Sertoli cell poly(A)^+ RNA was prepared by means of a Quick Prep mRNA purification kit (Pharmacia Biotech). Total RNA (10 μg) and mRNA (4 μg) were separated in a formaldehyde and 11% agarose gel, transferred to a nitrocellulose membrane Gene Screen Plus, and then hybridized in QuikHyb solution, as recommended by the manufacturer (Stratagene). Random-primer 32P-labeled cDNA inserts (~4.7 kb and ~2 kb) encoding bovine ECE-1 and ECE-2 were used as probes (Xu et al., 1994). The membranes were washed in ×2 SSC and 0.1% SDS at 55°C and were exposed to an x-ray film for 3 d at ~80°C.

**Measurement of [Ca\(^{2+}\)]_i**

[Ca\(^{2+}\)]_i was measured by dual wavelength fluorescence in single cells loaded with the Ca\(^{2+}\)-sensitive indicator fura-2 (Gryniewicz et al., 1985). Testicular myoid cells were plated on coverslips in serum-free MEM. A fter 4 d in culture, the cells were incubated in MEM containing 3 mM fura-2-acetoxyethylster for 1 h at 37°C. The cells were then rinsed with Krebs-Henseleit-Hepes (KHH) buffer (140.7 mM Na^+ , 5.3 mM K^+ , 12.4 mM Cl^− , 0.98 mM PO_4^{−2}, 1.25 mM Ca\(^{2+}\), 0.81 mM Mg\(^{2+}\), 5.5 mM glucose, and 20.3 mM Hepes) supplemented with 0.2% fatty acid-free BSA. Calibration of the signal was obtained at the end of each observation by adding 5 μM ionomycin to the medium. Emission was collected by a photomultiplier carrying a 510-nm cut-off filter and recorded by an ASEM Desk 2010 computer (ASEM SpA), which automatically calculated real-time 340/380 ratios. Calibration of the signal was obtained at the end of each observation. The stages of the seminiferous epithelium were identified from adjacent sections using the criteria of Leblond and Clermont (1952).

**Preparation and Treatment of Seminiferous Tubule Segments for Contraction Assay in Scanning Electron Microscopy**

Seminiferous tubules were prepared as previously described (Tricipiano et al., 1996). In brief, testes from 2-mo-old rats were decapsulated and digested under gentle shaking at room temperature in MEM containing 1 mg/ml collagenase. A fter dispersion of the interstitium, the tubular mass was rinsed in MEM, then stretches of tubules were dissected by means of sharp needles and carefully transferred to 35-mm culture dishes in 300 μl of medium. For the dissection of homogeneous samples at precise stages of the seminiferous epithelium, the tubular segments were identified under transillumination (Parvinen and Ruokonen, 1982). The tubules were incubated for 10 min at 32°C in a humidified chamber under an atmosphere containing 5% CO_2. At the end of the incubation time, the medium was replaced by 600 μl of medium to be tested at different experimental conditions. Cultured Sertoli cells were fixed in 2.5% glutaraldehyde, postfixed in 1% OsO_4, dehydrated and critical point dried in ethanol, coated with gold, and then viewed in a Hitachi S-570 scanning electron microscope.

**Evaluation of ECE Activity**

ECE activity in Sertoli cell cultures was assayed through estimation of exogenous big-endothelin conversion (Little et al., 1994). The culture medium, conditioned for 30 min to 3 h in the presence of big ET (with or without ECE inhibitors), was purified on a Sep-Pak C18 solid phase cartridge (Waters). A fter drying by vacuum centrifugation and reconstitution in buffer, the samples were assayed for endothelin content by means of a commercial enzyme immunoassay (ELA) kit (Cayman Chemical Co.) according to the manufacturer’s instructions.

**Preparation of 35S-labeled RNA Probes and In Situ Hybridization**

A dult and 18-d-old Wistar rat testes were fixed in 4% paraformaldehyde in PBS at 4°C overnight. The fixed testes were dehydrated with ethanol and embedded in paraffin by standard procedures. 5-μm-thick paraffin sections were placed on slides pretreated with 3-amino-propyltriethoxysilane. Sections were analyzed by in situ hybridization using the procedure described by Davidson et al. (1988). In brief, before hybridization, sections were deparaffinized, rehydrated, partially digested with proteinase-K (20 μg/ml), and then treated with acetic anhydride. These last two steps were necessary to improve access of the probe to the mRNA and reduce nonspecific binding of the nucleic acid probes. Sections were dehydrated and then incubated at 55°C for ~18 h with 35S-labeled RNA probes. For generation of RNA probes, the 0.5 Kb 5’-PstI-PstI fragment of bovine ECE-1 cDNA, nucleotides 214-751, was subcloned in pBluescript vector and transcribed in vitro with T7 (anti-sense) and T3 (sense) RNA polymerases. Unbound cRNA probe was removed by incubation in RNAase solution (40 μg/ml) for 30 min at 37°C in 0.5 M NaCl, TE buffer and by two 20-min washes at 65°C in 2× saline sodium citrate (SSC). A autoradiography was performed with Ilford K 2 liquid emulsion (Ilford). A fter exposure for the time periods indicated, sections were stained with carmalum and examined under a Zèiss microscope using dark- or bright-field illumination. The stages of the seminiferous epithelium were identified from adjacent sections using the criteria of Lебlond and Clermont (1952).

**Statistical Analysis**

Data are presented as the mean ± SE of results from at least three independent experiments. Student’s t test was used for statistical comparison between means where applicable.

**Results**

**Determination of Expression Levels of ECE-1 and ECE-2 mRNAs in Different Testicular Cells and in Extragonadal Tissue**

RNA blot analysis with the bovine ECE-1 cDNA as probe (Xu et al., 1994) showed that a ~4.7 kb ECE-1 mRNA is expressed abundantly in cultured Sertoli cells (Fig. 1 a). A s shown, the expression of ECE-1 mRNA is much higher in the testis than in the several other tissues (Fig. 1 a). In addition, a 3.1-kb mRNA is expressed at a lower level. Since only the 4.7-kb ECE-1 mRNA is present in Northern blots of poly(A)^+ RNA from cultured rat Sertoli cells, the two different sizes of mRNA are presumably generated by alternative poly(A)^+ addition in the 3’ non-coding region (Fig. 1 b). Conversely, Northern blot analysis of testicular cells with the bovine ECE-2 cDNA as probe (Emoto et al., 1995) revealed no expression of ECE-2 mRNA in the seminiferous epithelial cells, while a 3.3-kb mRNA was detected in the control neural tissue and adrenal gland (not shown).

**Time-dependent Conversion of Big ET-1 and Big ET-3 by Intact Sertoli Cells**

To analyze ECE activity, we examined whether cultured Sertoli cells can convert synthetic rat big ET-1 exogenously added to the culture medium. We therefore assayed...
the generation of mature ET-1 by means of a competitive enzyme immunoassay (EIA) that does not cross-react with the substrate big ET-1. As shown in Fig. 2, big ET-1 was efficiently converted into ET-1 by intact Sertoli cells in a time-dependent fashion. At a substrate concentration of 1 μM, up to 69% of the added big ET-1 was converted into ET-1. The Sertoli cell ECE was more efficient in converting big ET-1 than big ET-3. When the metalloprotease inhibitor phosphoramidon (PR), known to specifically inhibit ECE activity (Xu et al., 1994), was present during incubation, it completely inhibited the production of mature ET-1 (Fig. 2). The analogue of big ET-1, [D-Val22]big ET-1 [16-38], an inhibitor of ECE (Morita et al., 1994), strongly inhibited ECE activity and was as effective as PR in completely inhibiting the production of ET-1 by Sertoli cells incubated with big ET-1.

**Effect of Big ET-1 or Sertoli Cell–conditioned Medium on Calcium Mobilization in Isolated Myoid Cells**

We have previously demonstrated that ET-1 is able to induce PI turnover and rapidly increase [Ca^{2+}]_i in testicular myoid cells (Tripiciano et al., 1997). Cytofluorimetric analysis of intracellular calcium mobilization, measured by dual wavelength fluorescence in single cells loaded with the Ca^{2+}-sensitive indicator fura-2, indicate that the inactive precursor of ET-1, big ET-1, does not induce calcium mobilization in myoid cells; however, the same cells are able to respond to the addition of ET-1 with an increase in calcium levels (Fig. 3 a), which confirms the total biological inactivity of big ET-1. Conversely, when myoid cells were stimulated with medium conditioned by Sertoli cells for 30 min in the presence of 100 nM big ET-1 (SCMbig), a rapid [Ca^{2+}]_i transient comparable to that induced by ET-1 was observed; subsequent stimulation with ET-1 was ineffective (Fig. 3 b). Therefore, medium conditioned by Sertoli cells in the presence of big ET-1 desensitizes myoid cells to the actions of ET-1, which clearly indicates that the biologically active molecule in SCM big is ET-1 itself, converted from big ET-1 by ECE expressed in Sertoli cells. The observed slow calcium response is comparable to that obtained in response to 0.5–1 nM ET-1 which is below EC_{50}, but sufficient to desensitize to 100 nM ET-1 (not shown). When SCM big were conditioned in presence of phosphoramidon (PR), an inhibitor of ECE, no effect on calcium response was observed even though myoid cells were still responsive to ET-1 (Fig. 3 c). Fig. 3 d shows the levels of ET-1-, SCM big- (treated or untreated with phosphoramidon), and big ET-1-dependent [Ca^{2+}]_i increases (both peak and plateau).

**Morphological Response of Cultured Myoid Cells**

To corroborate the presence of fully functional ECE activity from intact Sertoli cells, we have treated cultured myoid cells with SCM big one day after plating. Treatment at this culture time with SCM big resulted in an immediate
rounding-up with retraction of cytoplasm, which could be directly followed in an inverted microscope (not shown). To assess whether the observed myoid cell contraction was specific for this cell type and whether Sertoli cells express significant activity of ECE, which is able to process big ET-1 into an amount of ET-1 sufficient to determine the contraction of myoid cells, we used mixed cultures containing fragments of seminiferous epithelium and patches of myoid cells. This mixed population of tubular and peritubular tissue was treated with 100 nM big ET-1 and shape changes which occurred 10–20 min after treatment were photographically recorded (Fig. 4). In these cultures, myoid cells patches were observed to undergo contraction in response to big ET-1, while the morphology of adjacent Sertoli cells remained unmodified. We processed the same sample for the detection of alkaline phosphatase activity, a specific marker for testicular myoid cells (Palombi and Di Carlo, 1988), and found that the cells that contracted were stained for alkaline phosphatase (Fig. 4, c and d). Inhibition of ECE activity by 2 mM PR resulted in the block of contractile response to big ET-1 (Fig. 5). Since PR is a metabolically stable phosphorylated sugar derivative and is unlikely to enter cells at an appreciable rate within a short incubation time, our observation indicates that the conversion of big ET into ET is a plasma membrane event that occurs on the extracellular side, analogous to the production of the vasoconstrictor angiotensin II from angiotensin I.

**Myoid Cell Contraction Induced by Big ET-1 in Peritubular Tissue**

Fig. 6 a shows the surface of a seminiferous tubule as viewed in the scanning electron microscope. In the adult testis, the myoid cells appear arranged in a continuous monolayer of epithelioid polygonal cells, particularly flat and wide and with bulging central nucleus. Addition of either 100 nM ET-1 (Fig. 6 b) or 100 nM big ET-1 (Fig. 6 c) results in dramatic contraction of the myoid peritubular cells, which display enhanced bulging of the central area and reduced distance between cell centers in most areas. From a morphological point of view treatment with either ET-1 or with its inactive precursor, big ET-1, induces a basically equal contraction of myoid cells; the only difference between these two treatments is in the timing required to achieve this effect. In fact, we observed myoid cell contraction within 15 s of ET-1 treatment, but only ∼10 min after big ET-1 addition, presumably because more time is required for a sufficient amount of big ET-1 to be converted into biologically active ET-1 by ECE-1 expressed by adjacent Sertoli cells in the seminiferous tubule. When seminiferous tubules were challenged with big ET-1 in the presence of PR, we did not observe any contraction of myoid cells, which appeared as flat as in the control sample (Fig. 6 d). Furthermore, as a further control, we challenged seminiferous tubules with SCM big. In this case, strong contraction of the myoid peritubular cells was observed within a few seconds (Fig. 7 a). When the seminiferous tubules were stimulated only with Sertoli cell-conditioned medium, the surface of myoid cells appeared to be unaffected, as in the control samples (Fig. 7 b).

**In Situ Hybridization of ECE-1 mRNA in the Rat Testis**

To explore the possibility that the production of ET within the seminiferous epithelium is a discontinuous, cyclically regulated process, we studied the transcription of ECE-1 by in situ hybridization. Sense and antisense RNA for ECE-1 mRNA was prepared as detailed in Materials and Methods. Interestingly, the ECE-1 probe showed striking regional differences in the level of signal (Fig. 8, a and c). The density of the grains was maximal at stages IX-X of the cycle and at the background level in all other stages. The control samples, hybridized with sense ECE-1 probe, dis-
played a low level of background labeling, with no appreciable differences in grain density between seminiferous tubule profiles and interstitium, thus confirming the specificity of the hybridization signals. In tubules at stages IX-X, the bovine ECE-1 probe hybridized to a basal columnar region surrounding the germ cells (Fig. 8 c, left tubule). This indicates that the ECE-1 gene is expressed above all in Sertoli cells prevalently in the basal region. The findings are in agreement with and extend the above Northern blot analysis, indicating that ECE-1 is expressed in Sertoli cells from adult animals in a cyclical fashion during the seminiferous cycle in stages IX-X soon after spermiation. By contrast, testis from 20-d-old rats exhibits homogeneous labeling intensity in all seminiferous tubules (not shown).

Peritubular Myoid Cell Contractility Is Controlled by ECE Expression at Specific Stages of the Seminiferous Epithelium Cycle

To investigate whether the restricted expression of ECE-1 could be functionally related to the regulation of peritubular contractility induced by big ET-1, seminiferous tubule
segments from adult testis were microdissected to isolate specific “stages” of the seminiferous tubules (Parvinen and Ruokonen, 1982) and their ability to respond to either ET-1 or big ET-1 was studied at the scanning electron microscope. Fig. 9 a shows a transilluminated tubular segment in which the transition from stage VIII to stage IX is very apparent. The hatched line indicates the level at which the tubules were dissected. Two groups of specific stages of the seminiferous tubule were tested: VII-VIII and IX-XI, which showed low and high ECE expression, respectively. Fig. 9 shows that treatment with big ET-1 is able to induce a strong contraction of seminiferous tubules at stages IX-X in 10 min (Fig. 9 g); by contrast, the seminiferous tubule fragments containing stages VII-VIII are totally unaffected by the treatment with big ET-1 (Fig. 9 f). Furthermore, ET-1 was still active in inducing an immediate contraction of the myoid peritubular cells in both groups of seminiferous tubule fragments (Fig. 9, d and e).
Figure 6. Scanning electron micrograph showing the peritubular surface of adult seminiferous tubules. (a) Control condition. Myoid cell contraction immediately after treatment with 100 nM ET-1 (b) and after 10 min treatment with 100 nM big ET-1 (c). (d) Lack of response after treatment with 100 nM big ET-1 plus 2 mM phosphoramidon. Bar, 10 μm.
In this report we describe the distribution of ECE-1 during the seminiferous epithelium cycle and present evidence that differential expression of ECE-1 in the Sertoli cells during spermatogenesis results in specific and regional seminiferous tubule contraction.

It has been shown that cultured Sertoli cells exhibit a basal production of ET-1 in the media (Fantoni et al., 1993). Preliminary observations, which showed that Sertoli cells incubated with ECE-1 specific inhibitors strongly reduced the secretion of ET-1 while increasing the accumulation of big ET-1 (not shown), prompted us to hypothesize a role for ECE-1 as a local regulator of ET-1 actions. Furthermore, the occurrence of phosphoramidon-sensitive ECE activity on Sertoli cells suggests that some processing of secreted big ET-1 may occur on the surface of ET-1-producing cells, adjacent to myoid cells. Since big ET-1 appears to be much more stable than ET-1 to generic proteolytic degradation (Murphy et al., 1994), this targeted conversion may allow more effective delivery of the active product in intact form to its receptors on myoid cells.

Since the prediction of its existence (Yanagisawa et al., 1988), ECE has been considered to be a potential site of regulation of endothelin production as well as a plausible target for therapeutic intervention in the endothelin system. Recently, the existence of three distinct ECE-1 isoforms has been demonstrated (Shimada et al., 1995; Valdenaire et al., 1995; Schweizer et al., 1997). These three isoforms (ECE-1a, ECE-1b, and ECE-1c) differ only in their N-terminal regions and are derived from a single gene through the use of alternative promoters. The three isoforms show similar kinetic rate constants, processing big ETs with similar velocities and have all been found to cleave the three big endothelins, but with a clear preference for big ET-1, which is in agreement with our results showing that intact Sertoli cell ECE-1 converts big ET-1 more efficiently than big ET-2 or big ET-3.

Recently, Yanagisawa et al. (1998) clearly demonstrated that the activity of ECE-1 is essential and that a physiologically relevant endothelin-converting enzyme exists for both big ET-1 and big ET-3 in vivo. In fact, ECE-1−/− mice (which all died within 30 min of birth) reproduced the phenotype resulting from the defects in both ET-1/ETA− and ET-3/ETB− mediated signaling pathways, which clearly shows that mature ET-1 and ET-3 are not synthesized in the relevant microenvironments without ECE-1 activity. Furthermore, a significant amount of mature ET-1/ET-2 still existed in the serum of ECE-1−/− embryos despite the absence of ECE-1, which suggests that other peptidases are responsible for the production of mature ETs. Intriguingly however, these remaining mature ETs completely failed to rescue the developmental phenotype of ECE-1−/− mice, which indicates that defined mature ETs must be produced at specific microenvironments in order to achieve a biological effect. The present study provides evidence that the restricted expression of ECE-1 might play a pivotal role in the control of peritubular contractility by providing a fine local modulation of biologically active ET levels.

If ET acts as a local regulator of seminiferous tubule contractility, it is conceivable that ECE is localized on the basal side of the Sertoli cells. In fact, Northern blot analy-
sis showed ECE-1 mRNA in cell extracts from purified Sertoli cells. Our in situ hybridization studies indicate that ECE-1 is predominantly localized in tubular areas where Sertoli cell bodies reside, particularly in the basal region. Sertoli cells are the only somatic cell type in the seminiferous epithelium; along the side of these elongated perennial elements, it is possible to observe, at any given time, several generations of germ cells, which flow radially to be eventually released as mature sperm into the tubular lumen. It has long been known that activities of the Sertoli cell, among which FSH responsiveness, vary according to the specific subset of differentiating germ cells with which it is associated ("stages" of the seminiferous epithelium) (Parvinen, 1982). In the prepuberal rat, in which the cyclicality of the epithelium has not been established yet, uniform expression of ECE was observed; in the adult, by contrast, ECE expression appears to be regulated in a temporal and spatial manner during spermatogenesis and the seminiferous epithelium cycle. Interestingly, expression of ECE-1 is exclusively restricted, in the adult rat, to stages IX-X of the cycle. These stages are characterized by the fact that they immediately follow spermatiation and represent ~5% of the entire cycle length, which may explain why ECE expression was overlooked in a previous study (Takahashi et al., 1995).

When segments of seminiferous tubule at precise stages of the seminiferous epithelium cycle were dissected and individually exposed to the inactive precursor big ET-1 to test their ability to induce myoid cell contraction through the generation of active ET-1, fragments containing stages preceding IX were found to be unresponsive to the precursor. By contrast, in segments from stages after spermatiation, normal contraction of myoid cells was observed in response to the inactive precursor, which indicates efficient processing of big ET-1. In parallel samples, directly stimulated with ET, no difference in responsiveness to the active peptide was observed, which suggests that myoid cells are constantly capable of responding. These experiments demonstrate a direct correlation between a restricted expression of ECE-1 and its biological function.

A perspective that warrants exploration is the mechanisms that regulate the expression of ECE-1 and the developmental transition from the diffuse to the restricted pattern of distribution of ECE-1, which may be connected to the known cyclic (Parvinen, 1982) and developmental changes in hormonal sensitivity Sertoli cells undergo (reviewed in Gondos and Berndston, 1993). Moreover, alterations in the pattern of ECE-1 and ET production might be involved in the pathogenesis of peritubular hyalinization, given the well-known role played by ET in fibrosis and matrix overproduction in a number of tissues (Hahn et al., 1993; Hocher et al., 1999).

In conclusion, our data could be used to outline a simplified model concerning the regulation of seminiferous tubule contractility, according to which the restricted expression pattern of ECE-1 would finely modulate local endothelin levels. In this model, ET-1 precursors produced by Sertoli cells are processed to biologically active ET-1 only in restricted areas of seminiferous tubule according to the spatiotemporal control of ECE-1 expression on the Sertoli cells (in turn, presumably dependent upon the spermatogenic cycle). Thus, seminiferous tubule contraction may originate in the specific tubular segments adjacent to those at which spermatiation has just occurred, to be propagated as effective peristaltic waves by additional mechanisms that have yet to be identified.

We wish to thank Dr. Elisabetta Dejana for her valuable discussion and critical reading of the manuscript. The skillful technical assistance of Mr. Quinto Giustiniani is gratefully acknowledged.

Research supported by grants from the Italian Ministero dell’Università e della Ricerca Scientifica e Tecnologica (M.U.R.S.T.).

Received for publication 1 December 1998 and in revised form 22 March 1999.

Figure 8. Localization of ECE-1 transcripts in testicular sections hybridized with 32P-labeled antisense (a and c) and sense (b and d) RNA probes. Autoradiographies were exposed for 3 wk. (a and b) Dark field, (c and d) carmalum counterstain. (c) Stage IX-X (left side tubule) shows intense basal labeling, while in the adjacent tubule (at stage IV-V, right side) the labeling is not above background. Bar: (a and b) 200 μm; (c and d) 40 μm.
Figure 9. Different distribution of ECE activity along the seminiferous tubule. (a) Transilluminated tubular segment in which the transition from stage VIII (left side, dark) to stage IX (right side, light) is apparent. The hatched line indicates the level at which the tubules were dissected to yield segments with expected different ECE activity shown in d–g. (b and c) Toluidine blue-stained Epon sections showing the seminiferous epithelium composition at stage VII-VIII (b) and IX-X (c). Scanning electron micrograph of peritubular surface of seminiferous tubule segments isolated at precise stages of the seminiferous epithelium and treated as follows: (d) stage VII-VIII and (e) stage IX-XI, 10 nM ET-1 followed by immediate fixation; (f) stage VII-VIII and (g) stage IX-XI: 100 nM big ET-1, fixed after 15 min. Only stage IX-XI seminiferous epithelium shows an intrinsic ability to stimulate myoid cell contraction through conversion of big ET-1. Bars: (a) 100 μm; (b and c) 30 μm; (d–g) 25 μm.