

Effects of Endothelin-1 on the Rat Testicular Vasculature

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ABSTRACT: Endothelin-1 (ET-1), a well-known vasoconstrictor substance, is present in the testis but its functional role is unknown. The present study was undertaken to elucidate whether ET-1 may influence testicular blood flow. ET-1 (0.1, 1, 10, 100 ng), an ET_A antagonist (BQ123; 0.01, 1, 100 µg), or saline were administered by intratesticular injections (0.1 ml) in adult rats. The effect on testicular blood flow was monitored using a laser Doppler flowmeter. The localization of immunoreactive ET-1 (irET-1) was studied by immunohistochemistry and the testicular irET-1 concentration was measured in normal and human chorionic gonadotrophin (hCG)-treated rats using a radioimmunoassay. ET-1 injection, in a dose-related way acutely decreased testicular blood flow and this effect was

blocked by an ET_A antagonist. The antagonist itself did not, however, influence testicular blood flow. Accumulation of polymorphonuclear leukocytes was observed in testicular venules 2 hours after ET-1 injection. Immunoreactive ET-1 was observed in Leydig, Sertoli, and endothelial cells. The testicular irET-1 content was increased 2-fold by hCG stimulation but local injection of the ET-1 antagonist did not influence testicular blood flow in hCG-treated rats. The present study suggests that ET-1 could be a hormonally regulated and locally produced modulator of testicular blood flow and microcirculation.

Key words: Endothelin-1, testis, Leydig cell, blood flow.

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Endothelin-1 (ET-1), a 21-amino acid peptide, is one of the most potent vasoconstrictors known (Yanagisawa et al, 1988). It is produced by endothelial cells and by actions mediated through ET_A receptors on vascular smooth muscle cells it induces vasoconstriction and promotes muscle cell growth (Ohlstein and Douglas, 1993). Via binding to endothelial ET_B receptors it may also induce a transient vasodilatation (Takuwa, 1993). ET-1 is also synthesized and may exert diverse actions, outside the vascular system. For example, it may be involved in controlling central nervous activity and renal function (Takuwa, 1993). High concentrations of ET-1 are found in endocrine organs like the pituitary, adrenal, testis, and ovary (Matsumoto et al, 1989; Takuwa, 1993). In the ovary granulosa cells synthesize ET-1 and follicular fluid contains at least 100-fold more ET-1 than plasma (Kamada et al, 1993).

Endothelin-1 is apparently synthesized locally in the testis (Sakurai et al, 1991), principally by Sertoli but also by Leydig cells (Fantoni et al, 1993). The intratesticular ET-1 concentration is reported to be approximately 100-fold higher than in plasma (Matsumoto et al, 1989). Testicular interstitial and myoid cells contain ET_A receptors (Sakaguchi et al, 1992). ET-1 stimulates Leydig cell testosterone secretion (Conte et al, 1993) and it also influences Sertoli and myoid cell function *in vitro* (Filippini et al, 1993; Sharma et al, 1994). It thus appears that ET-1 could be a local regulator of several testicular functions. Against this background we

wanted to examine whether ET-1, in addition, could be involved in the regulation of testicular blood flow.

Materials and Methods

Materials

Endothelin-1, the ET_A-receptor antagonist BQ123, a radioimmunoassay (RIA) kit for ET-1 (RAS 6901), and an ET-1 antiserum for immunohistochemistry (IHC 6901) were purchased from Peninsula Laboratories Europe Ltd., Merseyside, England. Human chorionic gonadotrophin (hCG, Pregnyl) was delivered from Organon (Oss, The Netherlands).

Animals, Blood Flow, and Vascular Morphology

Adult male Sprague-Dawley rats (300–400 g) were kept in a controlled environment with food and water *ad libitum*. At the day of experiment the animals were anesthetized with pentobarbital (50 mg × kg⁻¹, intraperitoneal injection). During experiments the animals were kept supine on a heating pad (32°C). The scrotal sacs were opened and in order to immobilize the testes they were placed in a small open plastic box containing melted 1.5% agar (E Merck), as earlier described (Hinton et al, 1979). Testicular blood flow was examined in both testes simultaneously using a two-channel laser Doppler flowmeter (PF 4001 master, Perimed AB, Stockholm, Sweden) and two multi-receiver probes (PF 412). The probes were held with micromanipulators approximately 1 mm over the testicular surfaces approximately 5 mm below the cranial pole in an area devoid of large vessels. Blood pressure was measured through a canula inserted in the tail artery using a pressure transducer (SensoNor 840) connected to an isolated pressure amplifier (Lectromed 5290/5291). The laser Doppler flow signals and the blood pressure were continuously monitored and later analyzed by a per-

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sonal computer (Compac 486), using the Perisoft software (Perimed AB, Stockholm, Sweden). Different doses of ET-1 or BQ123 were dissolved in 100 μ l sterile saline and injected into one testis using a 27-gauge hypodermic needle. At least five intact animals were examined for each substance and dose. BQ123 or saline (six rats in each group) was also injected in rats treated subcutaneously with 50 IU hCG 6 hours earlier. The intratesticular injections were placed approximately 10 mm distal to the area where blood flow was measured. In control experiments 100 μ l saline or heat-inactivated ET-1 was injected in the same way. In order to exclude systemic effects, blood flow in the contralateral intact testis was monitored simultaneously.

Endothelin-1 (1 and 100 ng, in 0.1 ml saline) or saline (0.1 ml) was injected into one testis. After 0.5 or 2 hours the testes were removed (five rats in each group), fixed in Bouin's solution, dehydrated, and embedded in paraffin. Testis morphology was then examined in 4- μ m-thick, hematoxylin-eosin-stained, sections. The volume density of polymorphonuclear leukocytes (PMNs) in testicular blood vessels was determined as previously described (Bergh et al, 1986).

Immunohistochemistry and Radioimmunoassay

The testes were removed from adult intact and from rats treated with 50 IU hCG subcutaneously 4, 8, or 24 hours earlier. In anesthetized control animals blood samples were also taken from the inferior caval vein and from veins on the testicular surface. At least six animals were examined in each group.

One testis from each animal was fixed in Bouin's solution and embedded in paraffin. Six-micron-thick sections were deparaffinized, rehydrated, and heated in a microwave oven (600 W) for 2 \times 5 minutes in citrate buffer as earlier described (Taylor et al, 1994). Normal 5% goat serum was used to block unspecific binding. The sections were then incubated overnight at 4°C with a rabbit antiserum (see above) against human and rat ET-1 (diluted 1/500). The immunoreaction was visualized using the supersensitive Multi-link kit (BioGenex, California, USA) using alkaline phosphatase/fast red as detection system. Control sections were incubated with antiserum that had been preincubated with a 100-fold excess of ET-1. In order to evaluate whether interstitial ET-1 immunostaining was localized in Leydig cells or testicular macrophages three adult rats were injected intraperitoneally with ethane dimethane sulfonate (EDS, 75 mg/kg) in order to deplete Leydig cells as earlier described (Collin et al, 1993) and examined 7 days after EDS treatment.

The contralateral testes of the animals described above were homogenized and the intratesticular tissue concentration of immunoreactive endothelin-1 (irET-1) was analyzed using a commercial RIA kit (Peninsula Lab.). According to the supplier of the RIA kit the cross-reactivity was 100% for human, rat, porcine, canine, bovine, and mouse ET-1 and 17–35% for human and porcine Big ET-1. Other tested cross-reactivities, including ET-2 and ET-3, were small (0–7%). The extraction procedure was principally the same as described for prostatic tissue by Langenstroer et al (1993). Approximately 500 mg testicular tissue was placed in 5 ml 0.5 M acetic acid. The tissue was boiled for 10 minutes in a waterbath and thereafter homogenized with a mechanical homogenizer (Ultra-turrax, TP 18/10) for 1 minute. The homogenate was centrifuged at 10,000 \times g for 30 minutes

at 1°C. The supernatants were stored at –20°C until further analysis. Five hundred microliters of the supernatant was mixed with 500 μ l 20% acetic acid and the mixture was applied to a C18 column (200 mg, Sorbent AB, V. Frölunda, Sweden), which had been pretreated with 3 ml of methanol, 2 ml distilled water, and 2 ml of 10% acetic acid. Tissue extracts were allowed to pass through the column, followed by 2 ml 10% acetic acid and 3 ml ethyl acetate. Endothelin was eluted by passing 1.5 ml of a 1:4 solution of 0.05 M ammonium bicarbonate and methanol and then concentrated under a nitrogen gas stream. The samples were reconstituted with 250 μ l buffer and 100- μ l aliquots were assayed in duplicate using RIA in accordance with the kit description. The sensitivity of the standard curve was 1 pg. When different dilutions of rat plasma were assayed, a good parallelism with the standard curve was obtained.

Statistics

Statistical assessment was carried out by the Kruskal–Wallis non-parametric test, followed by Mann–Whitney *U*-test or Wilcoxon test for paired observations and linear correlation. Values are given as mean \pm SEM. The level of significance was set at 0.05.

Results

Blood Flow

ET-1—Injection of saline into the testis did not significantly influence blood flow in the testicular tissue located approximately 10 mm from the injection site (Fig. 1, Table 1). Local intratesticular injection of ET-1 caused a dose-related decrease in blood flow (Table 1; Fig. 1) compared to the pretreatment level. Low doses caused a slight and transient decrease in blood flow and a moderate reduction in vasomotion frequency (Fig. 1). A high dose (100 ng) caused a pronounced and sustained decrease in blood flow and inhibited vasomotion completely (Table 1; Fig. 1). The magnitude of the reduction in flow and the duration of the response were linearly correlated to ET-1 dose ($r = 0.74$, $P < 0.001$ and $r = 0.77$, $P < 0.001$, respectively). Systemic blood pressure and blood flow in the contralateral noninjected testis were unaffected in all ET-1-treated animals. Injection of heat-inactivated ET-1 (100 ng/0.1 ml) did not significantly influence testicular blood flow (data not shown).

ET_A Antagonist (BQ123)—In testes injected with 1 μ g ET_A antagonist (BQ123) plus 50 ng ET-1 (in 0.1 ml saline) blood flow was not significantly influenced ($1.5 \pm 0.9\%$ change, $n = 8$) demonstrating that the ET-antagonist is able to block ET-1 induced vasoconstriction (Table 1). In animals injected with 0.01, 1 μ g, or 100 μ g BQ123, blood flow was not significantly influenced (-4 ± 2 , -3 ± 2 and $8 \pm 3\%$ change, respectively, $n = 5-8$). In rats treated with 50 IU hCG 6 hours earlier no vasomotion was present as earlier described (Bergh and Damber, 1993). Intratesticular injection of 1 μ g BQ123 or saline

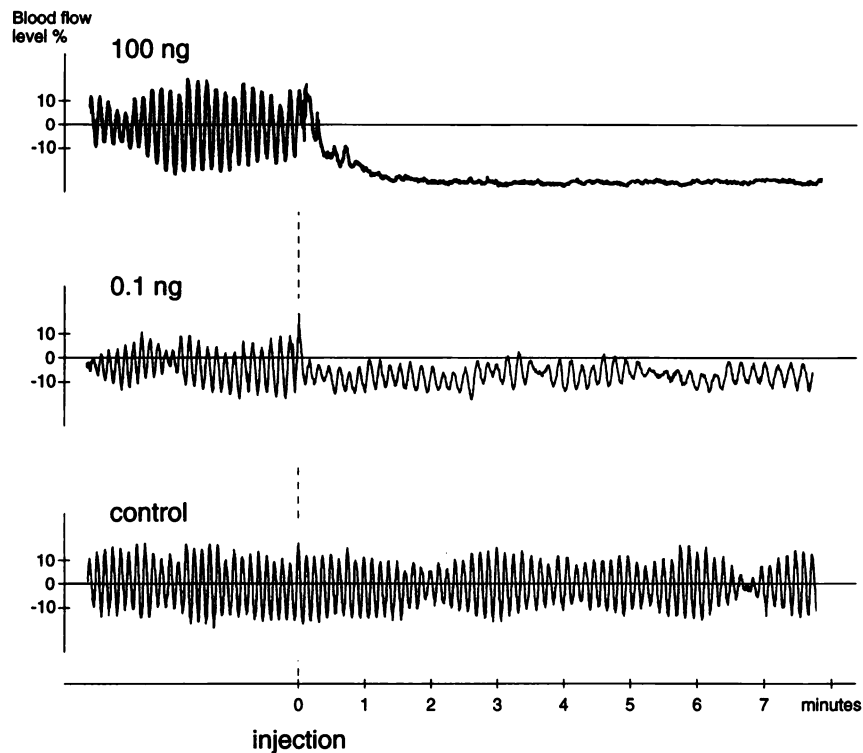


FIG. 1. Recordings of testicular blood flow in individual representative rats after intratesticular injections (0.1 ml) of saline and different doses of ET-1. ET-1 induced a decrease in blood flow level compared to the pretreatment level (marked 0).

did not significantly influence testicular blood flow in such rats (the change in flow was -2 ± 2 and $2 \pm 2\%$, respectively, $n = 5-8$).

Vascular Morphology

In testes injected with a high dose of ET-1 (100 ng) 2 hours earlier, but not 0.5 hours, PMNs accumulated in testicular venules and migrated into the interstitial space (Fig. 2). PMN accumulation was not observed in testes injected with 1 ng ET-1 or saline (Fig. 2). The volume density of PMNs in testicular blood vessels 2 hours after injection of saline, 1 ng, or 100 ng ET-1 was 1.3 ± 0.3 , 1.6 ± 0.2 , and $6.6 \pm 0.7\%$, respectively ($n = 5$ in each group). The increase seen after 100 ng ET-1 was statistically significant ($P < 0.05$).

Table 1. Effects of intratesticular injection of ET-1 in different doses on testicular blood flow (values are means \pm SEM, $n = 5-8$)

Dose ET-1 (ng)	Effect (%)	Duration of response (minutes)
0	-2 ± 2	6 ± 1
0.1	$-7 \pm 1^*$	$5 \pm 1^*$
1	$-9 \pm 1^*$	$6 \pm 1^*$
10	$-14 \pm 3^*$	$10 \pm 2^*$
100	$-36 \pm 7^*$	$>60^*$

* Significantly different from pretreatment level $P < 0.05$, Wilcoxon test for paired observations and Mann-Whitney U-test.

Immunohistochemistry

Staining for irET-1 was observed in most but not all interstitial cells, in Sertoli cells, and in endothelial cells in intratesticular arterioles (Fig. 3). In EDS-treated Leydig cell-depleted testes the remaining interstitial cells (principally macrophages) were unstained (not shown), suggesting that Leydig cells but not testicular macrophages contain irET-1. Control sections incubated with antiserum neutralized with excess of ET-1 were unstained (Fig. 3). The ET-1 staining pattern was not changed by hCG treatment.

Radioimmunoassay

The irET-1 content in control and hCG-treated testes is shown in Figure 4. The irET-1 concentration in peripheral plasma and in the testicular vein in intact untreated rats was 12 ± 2 and 53 ± 10 (pg/ml, $n = 7$), respectively. The difference between peripheral and testicular venous plasma was statistically significant ($P < 0.01$, according to Wilcoxon's test for paired observations). The hCG treatment cause an approximately twofold increase in testicular irET-1 content.

Discussion

The cellular origin, regulation and physiological role of testicular ET-1 synthesis is largely unknown, but ET-1

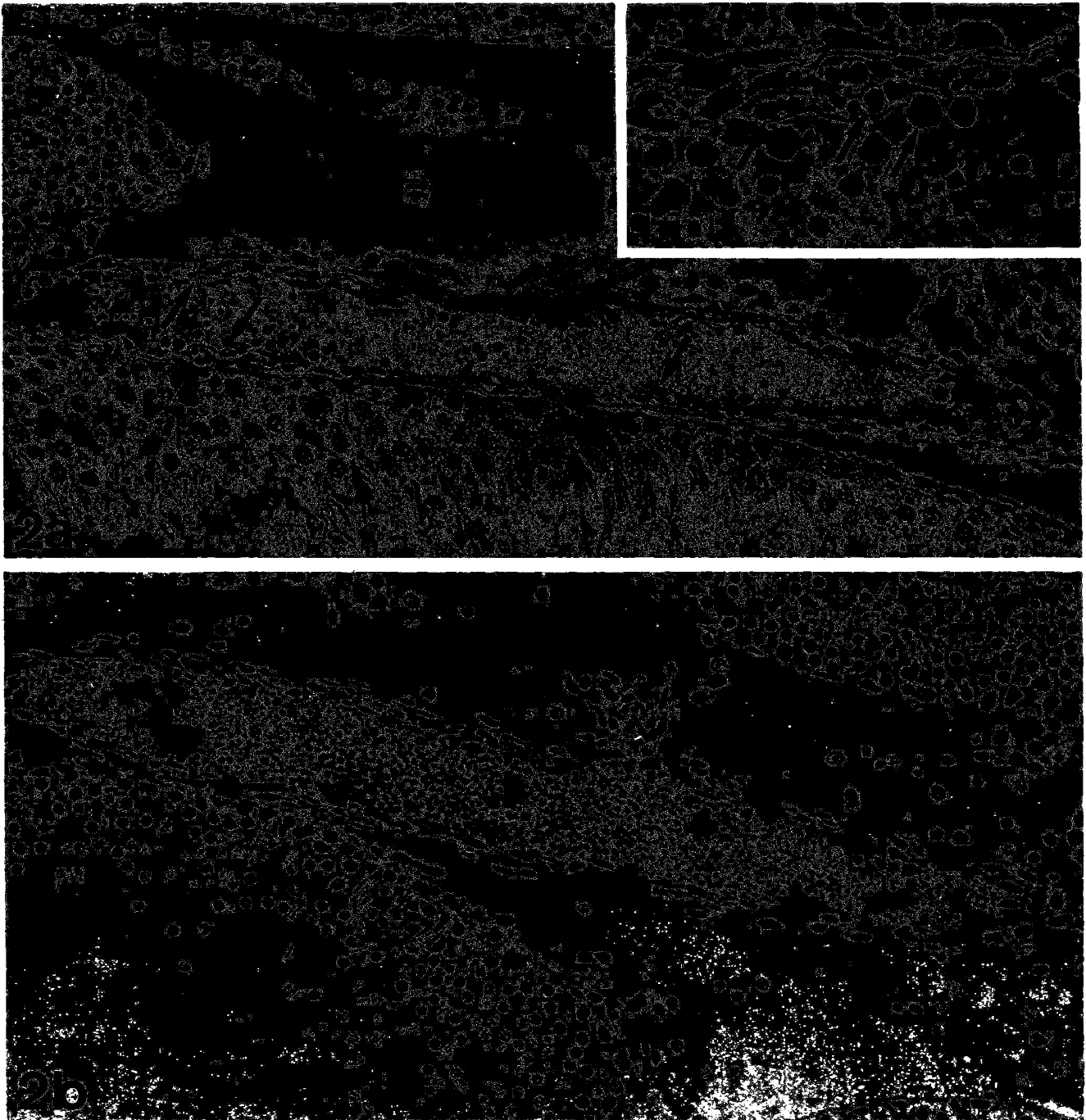


FIG. 2. Testicular sections from testes injected with 100 ng ET-1 (a) or saline (b) 2 hours prior to examination; 300 \times magnification. Intravascular and migrating PMNs (arrows) are observed after ET-1 but not after saline injection. Insert (i, detail of area marked with asterisk) shows adhering and migrating PMNs (arrows) in higher magnification (1,000 \times).

may function as a local regulator of Sertoli, myoid, and Leydig cells (see introduction). In this study irET-1 was observed in Leydig, Sertoli, and endothelial cells. Previous studies have suggested that ET-1 is produced principally in Sertoli cells but to some extent also in Leydig cells (Fantoni et al, 1993). In this study we show that irET-1 is increased after hCG treatment. It should how-

ever be noted that our ET-1 RIA probably cross-reacts with big-endothelin and we can therefore not exclude the possibility that changes in testicular irET-1 content could be caused by changes in big-endothelin. Recent studies do however demonstrate that the testis contains exceptionally high levels of endothelin-converting enzyme (Xu et al, 1994), and that this enzyme is localized in Leydig

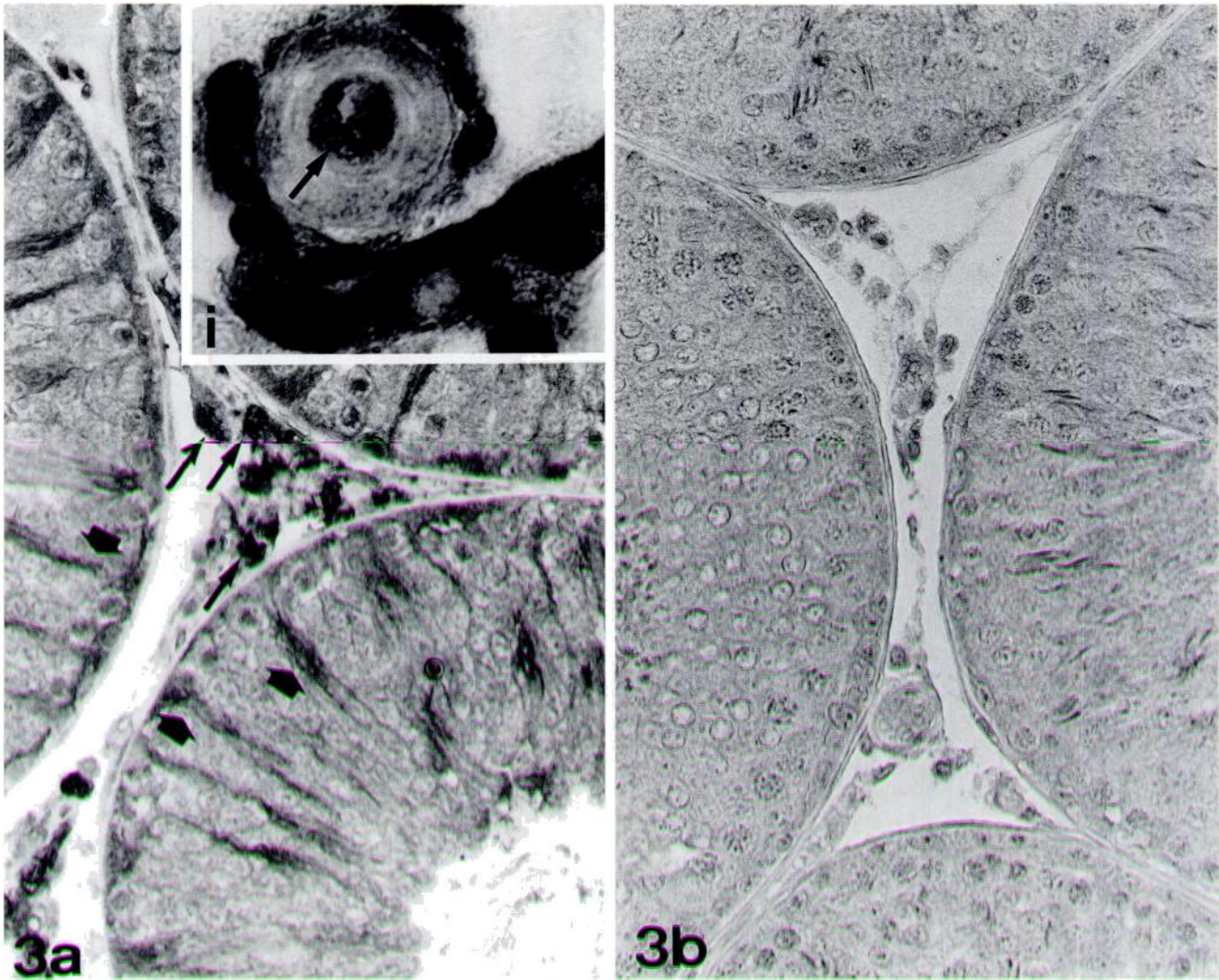


FIG. 3. Immunolocalization of ET-1 in the adult rat testis (a, 400 \times magnification). Immunostaining is present in Leydig cells (thin arrows) and in Sertoli cells (thick arrows), but not in capillaries. Insert (i, 1,000 \times magnification) shows heavily stained Leydig cells close to a small artery with moderately immunostained endothelium (arrow). In a control section incubated with ET-1 antiserum and an excess of ET-1 (b, 400 \times magnification) no immunostaining is observed.

cells and not in the seminiferous tubules (Takahashi et al, 1995). These observations suggest that Leydig cells could be the principal site of testicular ET-1 synthesis. As hCG treatment increases irET-1 *in vivo* and FSH inhibits Sertoli cell ET-1 secretion *in vitro* (Fantoni et al, 1993), it is possible that testicular ET-1 synthesis could be regulated by gonadotrophins. Our own unpublished observations also suggest that the testicular irET-1 content is highly increased in pathological conditions such as cryptorchidism. These observations suggest that the intratesticular irET-1 concentration may fluctuate in both physiological and pathophysiological conditions.

In this study we demonstrate that ET-1 could be involved in the regulation of testicular blood flow. Local injection of ET-1 causes a dose-related decrease in testicular blood flow, and the effect could be blocked by con-

comitant injection of an ET_A-receptor antagonist. An intact rat testis (approximately 2 g) apparently contains about 0.2 ng ET-1. Local injection of 0.1 or 1 ng into the testis resulted in a slight but significant reduction in testicular blood flow during approximately 5 minutes. This suggests that physiological variations in testicular ET-1 content could influence testicular blood flow. Pharmacological ET-1 doses (100 ng) caused a more pronounced (~33%) and sustained decrease (for at least 60 minutes) in testicular blood flow. Such high doses, in contrast to lower doses, also inhibited vasomotion. In other vascular beds, local injection of similar doses of ET-1 apparently cause a considerably larger drop in blood flow than in the testis (Lawrence et al, 1995; Mayhan and Rubinstein, 1995), suggesting a lower sensitivity in this organ. Local injection of BQ123 in different doses did not significantly

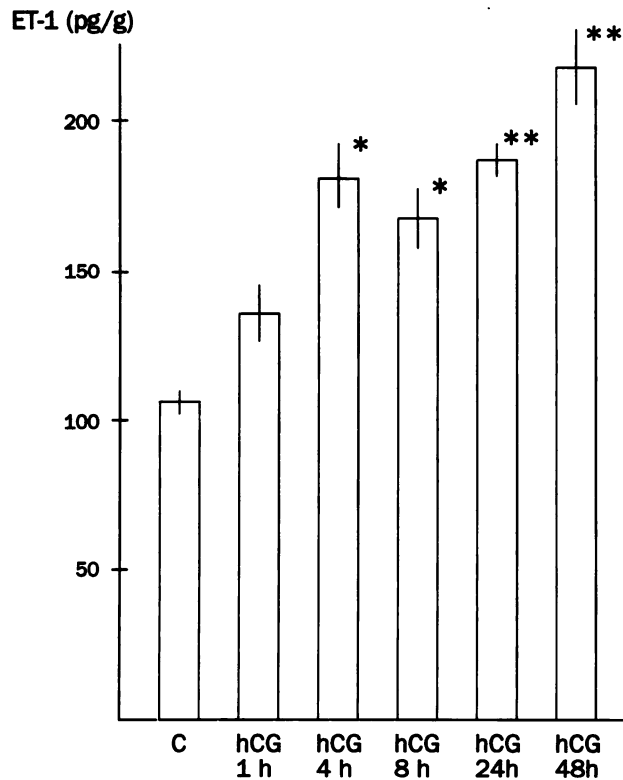


FIG. 4. Histogram showing the intratesticular irET-1 concentration (pg/g) in intact testes, in testes various times after hCG treatment. Values are means of six observations and bars indicate the SEM. Value significantly different control testes, * $P < 0.02$, ** $P < 0.002$ according to Mann-Whitney U -test.

influence testicular blood flow (although a slight increase was observed with the highest dose), suggesting that testicular blood vessels, such as those in the rat skin (Lawrence et al, 1995), may not be tonically influenced by ET-1.

In several tissues endothelial-derived ET-1, by influencing the adjacent vascular muscle cells, is involved in blood flow regulation (Haynes and Webb, 1993; Takuwa, 1993). It could therefore be argued that although testicular parenchymal cells may produce ET-1, the smooth muscle cells in testicular arteries are principally influenced by endothelial ET-1. However, local injection of ET-1 into the testicular interstitium and consequently influencing the testicular arteries from the outside altered testicular blood flow. It is therefore likely that Leydig cell- and possibly Sertoli cell-derived ET-1 could be of importance in the local control of testicular blood flow. The physiological and/or pathophysiological significance of ET-1 in the regulation of testicular blood flow is, however, unknown. Interestingly the testicular vasculature is particularly sensitive to the vasoconstrictor effects of serotonin (Free, 1977; Collin et al, 1996) and in other vascular beds ET-1, in concentrations present in the testis, is known to potentiate the vasoconstrictor effects of serotonin (Yang

et al, 1990, 1992; Masaki, 1995). This may suggest that testicular ET-1 may act in combination with other vasoactive substances.

Human chorionic gonadotrophin treatment in high doses (50 IU or more) inhibits vasomotion, induces PMN accumulation in testicular venules, and causes a major decrease in testicular blood flow at 4–6 hours after treatment, a decrease that may result in damage to the seminiferous tubules (van Vliet et al, 1988; Bergh and Damber, 1993). In contrast, FSH treatment does not influence total testicular blood flow or microcirculation (Bergh and Damber, 1993). As the levels of immunoreactive ET-1 are increased after hCG treatment it is possible that ET-1 could be a mediator of the vascular effects of hCG. Local injection of BQ123 did not significantly influence blood flow in hCG-treated rats suggesting that ET-1 is probably not the principal mediator of hCG-induced changes in blood flow. ET-1 in a high dose did, however, cause PMN accumulation in testicular venules of the same magnitude as after hCG treatment (Bergh et al, 1986), suggesting that ET-1 could be involved in the hCG induced inflammatory response. The finding that ET-1 may have proinflammatory effects in the testis is in line with the observation that it causes PMN accumulation in the lungs (Filep et al, 1995) and a breakdown of the blood–spinal cord barrier (Westmark et al, 1995). ET-1-induced testicular PMN accumulation is probably not related to reduced blood flow as serotonin, which dramatically reduces flow (Collin et al, 1996), does not induce PMN accumulation in the testis (Bergh and Damber, 1993). We have previously suggested that the testis, due to its special vascular anatomy and physiology with remarkably low microvascular pressure and tissue oxygen concentration, could be particularly susceptible to reductions in testicular blood flow (Bergh and Damber, 1993). The present and previous observations that the testis produces and responds to vasoconstrictors like ET-1 and serotonin (Collin et al, 1996) suggest that disturbances in the local secretion of such substances could be involved in testicular pathophysiology. Further studies are thus needed in order to understand the role of ET-1 and its relation to other locally produced vasoactive substances, in the control of the testicular vasculature.

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