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Changes in Luminal Fluid Protein Composition in the Rat Cauda Epididymidis Following Partial Sympathetic Denervation

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ABSTRACT: Sympathetic denervation of the rat cauda epididymidis by surgical removal of the inferior mesenteric ganglion (IMG) results in an excessive accumulation of sperm in the cauda epididymidis as well as significant changes in cauda sperm motility and cauda epididymal gross histology. The objective of the present study was to determine if the cauda-specific changes in sperm storage, sperm motility, and epididymal histology following the loss of sympathetic innervation were accompanied by changes in the protein composition of epididymal fluid. One and 4 weeks after surgical IMG removal or sham operations, luminal fluid obtained from the caput and cauda epididymidis and cauda epididymal sperm-associated proteins were subjected to two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) and silver-stained proteins were quantitated. One week after IMG removal, two cauda epididymal fluid (CEF) proteins (2 and 13) had increased 43% and 49%, respectively, whereas four CEF proteins (5, 8, 9, and 19) had decreased between 30% and 73% compared to controls. Four weeks after IMG removal, changes in CEF proteins observed 1 week following surgery were no longer present, but the staining intensities of three additional CEF proteins (11, 12, and 18) were reduced an average of 70% compared to control CEF proteins. By obstructing the cauda epididymidis, we confirmed that the changes in CEF protein composition observed following IMG removal were not the result of sperm accumulation but were due directly to the loss of innervation; the staining intensity of CEF protein 2 increased as a result of excessive sperm accumulation in the cauda epididymidis both in the presence and absence of innervation from the IMG. No significant changes in caput epididymal fluid proteins or cauda epididymal sperm-associated proteins were detected following IMG removal. These data show that the protein composition of rat CEF is significantly affected by the loss of sympathetic innervation and suggest that neuronal input may play an important role in the maintenance of epididymal function.

Key words: Innervation, 2-D Polyacrylamide gel electrophoresis, sperm accumulation, spinal cord injury, inferior mesenteric ganglion, spermatozoa.

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Autonomic innervation plays a key role in regulating the functions of the male genitourinary tract (for review see Bell, 1972). Adrenergic, cholinergic, and nonadrenergic noncholinergic (NANC) mechanisms operate in a highly orchestrated fashion to ensure reliable storage and release of urine from the bladder (Anderson, 1993; de Groat et al, 1993), to regulate the transport and storage of spermatozoa in the reproductive tract (Hodson, 1965), and to coordinate the emission and ejaculation of secretions from the sex accessory glands (Farrell and Lyman, 1937; Sjostrand, 1965). Penile erection is also dependent upon vascular and neuromuscular events mediated by the autonomic nervous system (de Groat and Steers, 1988; Anderson, 1993).

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The localization of sympathetic fibers within the epididymis suggests that adrenergic innervation may play a role in mediating epididymal function. Neuroanatomical studies indicate that the adrenergic innervation to the rat cauda epididymidis originates from the inferior mesenteric ganglion (IMG) (Mitchell, 1935; Hodson, 1970). The major pelvic ganglion (MPG) and pelvic accessory ganglion (PAG) also contribute to the innervation of the distal regions of the rat epididymis (unpublished data). Within the epididymis, neuronal fibers are present within peritubular and subepithelial regions (Kuntz, 1953; El-Badawi and Schenk, 1967). Studies employing either chemical (Evans et al, 1972) or surgical (Bacq, 1931; Simeone, 1933) sympathetic denervation have clearly demonstrated that sympathetic innervation within the epididymis is necessary for neuromuscular events required for the transport of spermatozoa through the duct and for seminal emission. Consistent with these earlier studies, we have reported that surgical removal of the IMG and hypogastric nerves significantly alters sperm transport through the rat cauda epididymidis, resulting in the excessive accumu-

lation of spermatozoa and, subsequently, the gross distention of cauda epididymal tubules (Billups et al, 1991a).

Because of the dramatic change in the gross anatomy of the cauda epididymal tubules following the loss of sympathetic innervation (Billups et al, 1991a), we hypothesized that the loss of sympathetic innervation may affect the functions of epididymal epithelial cells, such as the synthesis and secretion of proteins. Proteins synthesized and secreted by epididymal epithelial cells have been shown to associate directly with spermatozoa (Brooks, 1983; Brooks and Tiver, 1983; Iuseum et al, 1989; Ross et al, 1990; Robiltaille et al, 1991; Vreeburg et al, 1992). Moreover, interactions between epididymal fluid proteins and spermatozoa have been shown to be important for both sperm motility and fertilizing ability (Lea et al, 1978; Turner and Reich, 1987).

In the present studies, the hypothesis that sympathetic innervation affects epididymal fluid proteins was explored. Towards this end, the protein composition of rat epididymal fluid was examined at various intervals following the loss of epididymal sympathetic innervation by surgical removal of the IMG. Because epididymal fluid proteins have been shown to interact directly with spermatozoa, we also examined sperm-associated proteins in the presence and absence of sympathetic innervation from the IMG.

Materials and Methods

Study Animals

Adult male Sprague Dawley rats (325–350 g) were purchased from Charles River (Wilmington, MA) and housed in a 14-hour-light: 10-hour-dark cycle. Animals were provided rat chow and water *ad libitum*. All surgical procedures were reviewed and approved by the Johns Hopkins Animal Care and Use Committee.

Surgical Removal of the Inferior Mesenteric Ganglion (IMG)

Surgical IMG removal was performed according to the method of Billups et al, (1991a). Briefly, animals (n = 10) were anesthetized, and the IMG and the proximal 1.5–2 cm of each hypogastric nerve were identified and resected. The surgical incision was closed with absorbable suture (Ethicon Coated Vicryl, 4.0; Johnson and Johnson, Inc., Somerville, NJ) and stainless steel wound clips (9 mm; Clay Adams Co., Sparks, MD). Sham-operated control animals (n = 10) underwent similar surgical procedures, except the IMG and hypogastric nerves were left intact.

Induction of Epididymal Sperm Accumulation Using Polyethylene Tubing

Initial studies revealed that the loss of sympathetic innervation following IMG removal resulted in both the abnormal accumulation of spermatozoa within the cauda epididymidis and in changes in the protein composition of epididymal fluid. To determine whether the observed changes in cauda epididymal fluid protein were a consequence of the excessive accumulation of spermatozoa or were directly due to the loss of sympathetic innervation, we examined the protein composition of cauda epididymal fluid from rats in which excessive sperm accumulation was induced without disruption of the sympathetic innervation supplying the epididymis. Epididymal sperm accumulation was induced by partially constricting the vas deferens using 7 mm of polyethylene tubing obtained from a catheter unit (Butterfly ST, catalog no. 4821, tubing inner diameter = 0.040 in.; Abbott Laboratories, North Chicago, IL). The cut ends of the tubing were smoothed by fire-polishing. The 7-mm tubing segment was then cut longitudinally and placed ("cuffed") around the vas deferens 1–2 cm from its junction with the cauda epididymidis (n = 5 rats) (Fig. 1). To prevent occlusion of the deferential artery and vein, a 2-cm piece of 0.0 Prolene suture (Ethicon, Inc., Somerville, NJ) was placed as a stent between the cuff and the vas deferens. The cuff was secured around the vas deferens by tying 4.0 silk suture (Ethicon, Inc.) around each end of the tubing (Fig. 1). Once the cuff was in place, the Prolene stent was removed (Fig. 1). Removal of the stent provided sufficient space between the cuff and the vas deferens to prevent complete occlusion of the deferential artery and vein. This was confirmed visually by observing the return of blood flow in the vessels beneath the cuff once the stent was removed. The contralateral epididymis was visualized through a scrotal incision, but the vas deferens was left unconstricted; this served as sham-operated control tissue. One week following surgery, animals were euthanized by CO_2 overdose and cauda epididymides were excised. Cauda epididymal spermatozoa were obtained as described previously (Billups et al, 1991a) and counted using a hemacytometer to determine the extent of sperm accumulation following cuff constriction. Additionally, cauda epididymal fluid proteins were collected 1 week following surgery and analyzed as described below. Time points beyond 1 week were not examined because of the formation of epididymal granulomas. In addition, tissues that were inflamed, adhesed, eroded, or granulomatous following cuff constriction of the vas deferens were not used for our studies.

Norepinephrine Measurement

To confirm that cuff obstruction of the vas deferens did not affect sympathetic innervation, tissue norepinephrine levels were measured within the epididymis and vas deferens 1 week following surgery using high-performance liquid chromatography (HPLC) according to the methods of Wenk and Greenland (1980). Norepinephrine was expressed as fmol of norepinephrine/mg protein.

Collection of Cauda Epididymal Fluid (CEF)

Excised cauda epididymides were immediately placed in ice-cold phosphate-buffered saline (PBS), pH 7.4, containing the protease inhibitors aprotinin (5 μg/ml), leupeptin (1 μg/ml), and phenylmethylsulfonylfluoride (PMSF) (1 mM). Blood, surrounding fat, and connective tissue were removed from each cauda epididymidis. The cut ends of each cauda were clamped using a hemostat, and the epididymal tubules were pierced several

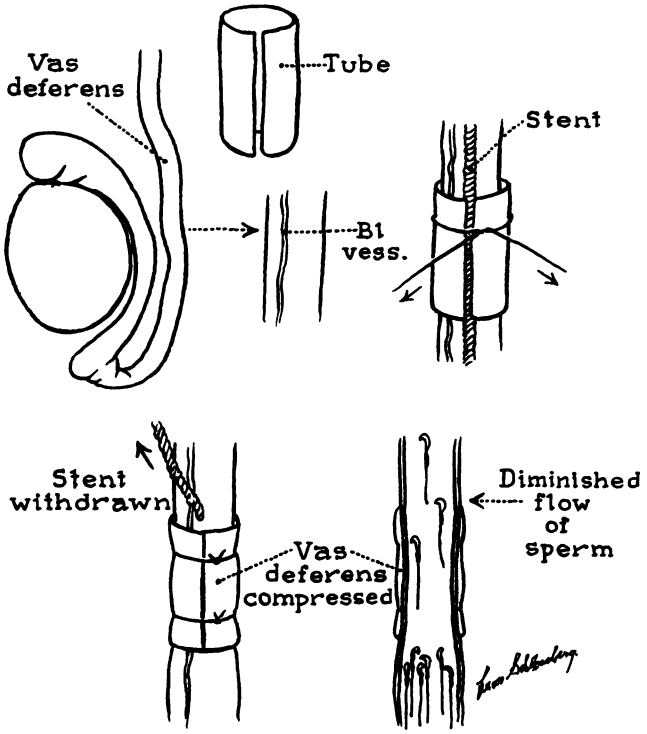

FIG. 1. Illustration of the surgical procedure used to induce sperm accumulation within the rat cauda epididymidis while maintaining intact innervation. A 7-mm segment of polyethylene tubing was placed ("cuffed") around the vas deferens. A stent was used to prevent occlusion of the deferential blood vessels (Bl vess.). The cuff was secured by tying silk suture around each end of the tubing.

times using a sterile scalpel blade. The luminal contents that extruded from each cauda epididymis were collected in a microfuge tube and centrifuged (12,400 × *g*) at 4°C for 30 minutes to sediment sperm. The clear, viscous CEF overlying the sperm pellet was aspirated, assayed for protein content as described for norepinephrine measurement, and diluted (1:1) in two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) sample buffer containing urea (8 M), CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; 4.0%), dithiothreitol (DTT; 140 mM), Ampholine 3-10 (80%), Ampholine 5-8 (20%), and the protease inhibitors aprotinin (5 μg/ml), leupeptin (1 μg/ml), and PMSF (1 mM). All samples were stored at −70°C prior to electrophoresis. CEF proteins collected using the method described above exhibited a staining pattern similar to CEF proteins recovered using micropuncture techniques (Olson and Hinton, 1985).

Collection of Caput Epididymal Fluid

Previously established micropuncture techniques (Hinton et al, 1979) were utilized to collect caput epididymal luminal fluid from sham-operated control rats and from rats 1 and 4 weeks following surgical removal of the IMG. Micropuncture samples of caput epididymal luminal fluid were centrifuged at 4°C at 9,000 × *g* for 10 minutes to sediment spermatozoa, and the supernatants were collected and stored at −70°C. Within 5–7 days, samples were assayed for protein content and diluted in 2-D PAGE sample buffer containing protease inhibitors as indicated above for CEF. Diluted samples were stored at −70°C prior to electrophoresis.

Cauda Epididymal Sperm-associated Proteins

Following centrifugation to remove CEF, cauda epididymal spermatozoa present in each pellet were gently resuspended in cold Tris buffer (pH 7.8) containing 1 mM magnesium chloride (Tris-$MgCl_2$) and centrifuged (12,400 × *g*, 4°C) to remove residual epididymal fluid. Sperm pellets were again suspended in cold Tris-$MgCl_2$ buffer and centrifuged for 10 minutes at 12,400 × *g* at 4°C. Supernatants were discarded, sperm pellets were resuspended in 0.1 M Tris (pH 6.8) containing 10 mM $MgCl_2$, and sperm suspensions were sonicated at 4°C for approximately 1 minute (four 15-second bursts) using a Sonifier Cell Disrupter equipped with a 0.5-in. solid horn tip (Heat Systems-Ultrasonics, Inc., New York, NY). Sonication-resistant sperm cell nuclei in each sample were counted using a hemacytometer. Sodium dodecyl sulfate (SDS; 2% final concentration) and DTT (1 mM final concentration) were added to each sperm sample. After incubation at 75°C for 45–60 minutes, samples were centrifuged for 60 minutes at 10,000 × *g*. To remove DNA and RNA from the resulting supernatant, DNase (5 μg/ml) (catalog no. D-4513; Sigma, St. Louis, MO) and RNase (2 μg/ml) (catalog no. R-7003; Sigma) were added to each sample. Acetone (85% final concentration, room temperature) was added to the supernatant to precipitate proteins from the SDS solution. After 10 minutes, samples were centrifuged for 2 minutes at 20°C, and the resulting protein pellets were air dried, diluted in 2-D PAGE sample buffer (described above), and assayed for protein content using methods indicated previously. Samples were stored at −70°C prior to electrophoresis.

2-D PAGE

Two-dimensional PAGE was performed according to the methods of O'Farrell (1975) with the following modifications. A GT tube gel electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA) was used for isoelectric focusing in the first dimension in the range of pH 4.7–8.0. The cathode and anode buffers consisted of 0.2 M NaOH and 0.1 M phosphoric acid, respectively. Freshly polymerized tube gels devoid of protein were electrophoresed 15 minutes at 200 V, 30 minutes at 300 V, and 30 minutes at 400 V. For electrophoresis of epididymal fluid from sham-operated control and IMG-denervated rats, 25 μg of protein from either caput or cauda epididymal fluid was loaded onto prefocused tube gels. For electrophoresis of cauda epididymal sperm-associated proteins, approximately 30 μg of protein isolated from 1.5 to 2 × 10^6 cauda epididymal spermatozoa were loaded onto prefocused tube gels. All proteins were electrophoresed at 400 V for 5 hours, then at 450 V for 18 hours.

A vertical slab gel unit (SE 600, Hoefer Scientific Instruments) was used for the electrophoresis of CEF proteins in the second dimension. Second-dimensional electrophoresis of caput epididymal fluid proteins as well as sperm-associated proteins was performed using a Protean II xi Multi-Cell system (Bio-Rad Laboratories, Hercules, CA). For caput epididymal fluid proteins, CEF proteins and cauda sperm-associated proteins, isoelectrically focused tube gels were placed onto 5% acrylamide stacking gels overlying 12% polyacrylamide–SDS denaturing-

reducing gels. Equivalent amounts of molecular weight markers (catalog no. 1050s; Diversified Biotech, Boston, MA) were loaded on all second-dimension SDS gels and used as standards for protein quantitation described below. Second-dimension gels were run at 10–20 mA/gel until proteins passed through the stacking gel. The current was then increased to 25–30 mA/gel for electrophoresis in the resolving gel. Running buffer for second-dimensional electrophoresis consisted of Tris (0.3%), glycine (1.6%), and SDS (0.1%). To control the temperature of the resolving gels and running buffer when using the Protean II xi system, the electrophoresis apparatus was cooled to 18–20°C using a refrigerated circulation bath (RCB 300; Hoefer Scientific Instruments).

Silver Staining Procedure

Upon completion of electrophoresis, gels were stored at 4°C in an aqueous solution of methanol (50%) and acetic acid (10%). Prior to staining, gels were rehydrated in water for 1 hour, then washed in 50 mM phosphate buffer (pH 7.2), followed by 5% glutaraldehyde containing 5 mM DTT. After six to eight thorough rinses with water, gels were silver stained using the method of Wray et al (1981). Once a suitable staining intensity had been achieved, a 5% acetic acid solution was used to stop the staining reaction. After 20 minutes in the acetic acid solution, gels were stored in the dark at 4°C in a solution of glycerol (7%) and methanol (10%). Prior to quantitation of protein staining intensities, gels were placed between two sheets of transparent cellophane (catalog no. SE1202; Hoefer Scientific Instruments) and dried using warm air (Easy Breeze Gel Drier; Hoefer Scientific Instruments).

Quantitation of Proteins on Silver-stained Gels

Images of dried 2-D SDS-PAGE gels were scanned into a Sun Sparc LX workstation database using a Howtek Scanmaster 3+ scanner at a resolution of 300 dots per inch. The staining intensities of individual proteins were calculated using the VISAGE 2-D electrophoresis gel analysis system software (VISAGE version 4.6Q; Millipore, Bedford, MA). Protein staining intensities were compared between control and treatment groups. To control for variations in staining intensity from gel to gel, protein staining intensities on each gel were expressed relative to the staining intensities of equivalent amounts of molecular weight standards loaded on the same gels. Only those proteins that were clearly and consistently resolved on triplicate gels were selected for quantitation. In addition, the linearity of protein staining intensities was determined using a standard curve of the protein used as an internal molecular weight standard; all experimental values fell within the linear range of this standard curve.

Caput epididymal fluid proteins, CEF proteins, and sperm-associated proteins were identified numerically, and a protein corresponding to serum albumin was identified by the letter A. Twenty CEF proteins, 8 caput epididymal fluid proteins, and 20 sperm associated proteins were quantitated.

Statistical Analyses

Protein staining intensities between experimental and control groups were compared using analysis of variance (ANOVA; for comparison of epididymal proteins following IMG removal, and for comparison of cauda epididymal sperm numbers) and Student's *t*-test (for comparison of epididymal proteins following the induction of cauda epididymal sperm accumulation using a polyethylene cuff and tissue norepinephrine content). Data that did not conform to a normal Gaussian distribution were subjected to non-parametric Kruskal Wallis ANOVA and Mann-Whitney rank sum test for independent samples. A P value of less than 0.05 was accepted as significant.

Results

Changes in CEF Proteins Following Surgical Removal of Sympathetic Innervation from the IMG

Two-dimensional polyacrylamide gels of CEF proteins from sham-operated control rats exhibited a silver-stained pattern, shown in Figure 2a. Nineteen CEF proteins were identified numerically (1–19) and quantitated. A CEF protein with an isoelectric point of 6.2–6.4 and a molecular weight of 66 kDa that corresponded to serum albumin was labeled protein A according to the nomenclature of Olson and Hinton (1985). Based on molecular weight and isoelectric point, CEF proteins 13 and 17 in the present study are similar to proteins D-E and proteins B-C, respectively, described previously by Brooks (1983). In CEF from sham-operated control rats, proteins 17 and A (Fig. 2a) exhibited the highest staining intensities when examined using computer-assisted image analysis.

One and 4 weeks following the loss of sympathetic innervation from the IMG, all CEF proteins were still present and no new proteins were detected. The staining intensities of individual CEF proteins following sympathectomy were, however, significantly altered compared to controls. One week following IMG removal, the staining intensities of proteins 2 and 13 were significantly increased ($P < 0.05$) compared to the intensities of the same proteins in sham-operated control CEF (Fig. 2b). Conversely, the staining intensities of proteins 5, 8, 9, and 19 were significantly reduced ($P < 0.05$) compared to sham-operated controls 1 week following IMG removal (Fig. 2b). Four weeks following IMG removal, the staining intensities of CEF proteins 11, 12, and 18 were significantly reduced ($P < 0.05$) compared with sham-operated controls (Fig. 2b). Interestingly, at 4 weeks after sympathectomy the staining intensities of CEF proteins 2, 5, 8, 9, 13, and 19 were no longer significantly different from sham-operated controls, as was observed 1 week following IMG removal (Fig. 2b). The total CEF protein concentration was not different from control CEF 1 or 4 weeks following IMG removal.

CEF Proteins Following Constriction of the Vas Deferens in the Presence of Intact Sympathetic Innervation

To examine the effects of only sperm accumulation on the protein composition of CEF, a polyethylene cuff was

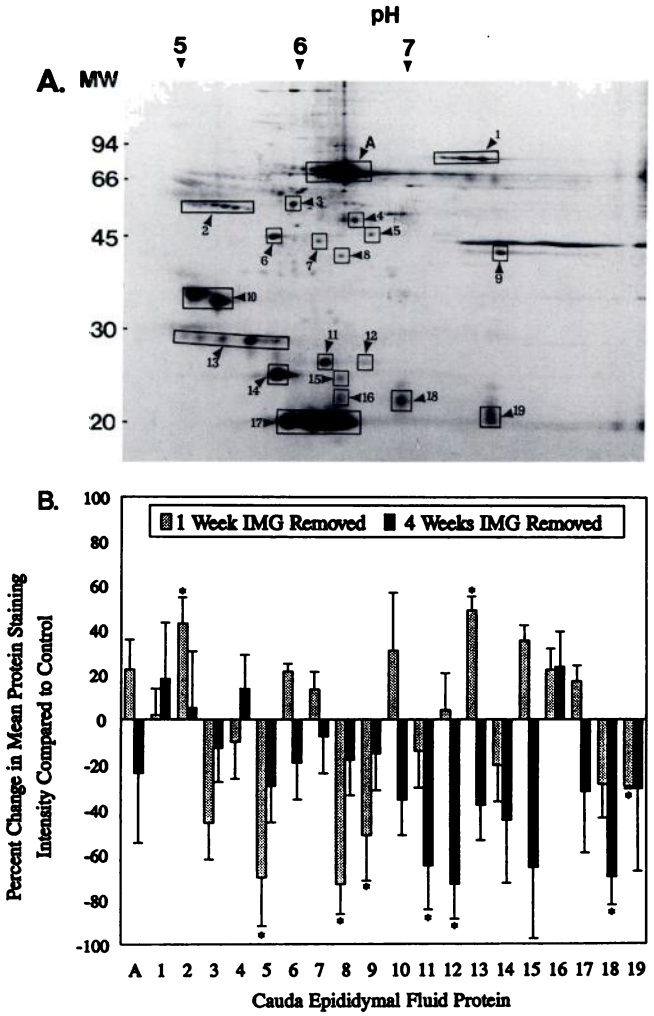

FIG. 2. Changes in rat cauda epididymal fluid (CEF) proteins following IMG removal. **(A)** Silver-stained 2-D SDS denaturing gel of CEF collected from sham-operated control rats showing the localization of CEF proteins A and 1–19. Molecular weight (kDa) and isoelectric standards (pH) are indicated. **(B)** Quantitative changes in the staining intensity of CEF proteins 1 (gray bar) and 4 (dark bar) weeks following IMG removal. Bars reflect the percent change in mean protein staining intensity ± standard error of the mean (SEM). Significant changes in mean protein staining intensity from controls are indicated (*) for CEF proteins 2, 5, 8, 9, 11, 12, 13, 18, and 19.

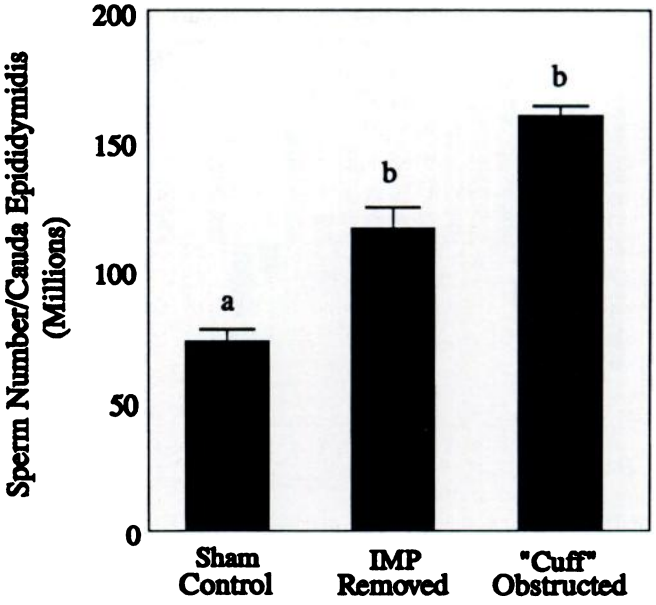

FIG. 3. Sperm numbers in the rat cauda epididymidis 1 week following sham operations, IMG removal, or cuff obstruction of the vas deferens. All surgical procedures are described in the Methods and Materials section. Data are mean ± standard error. Bars with different letters are statistically different ($P < 0.05$).

used to induce sperm accumulation within the cauda epididymidis in the presence of sympathetic innervation (Fig. 1). One week after polyethylene "cuffs" were used to induce epididymal sperm accumulation, sperm numbers within cauda epididymides were similar to those reported following IMG removal (Fig. 3). Norepinephrine content in the cauda epididymides and vasa deferentia was not significantly altered following constriction of the vas using the cuff, as would be expected had sympathetic innervation been lost (Table 1). Therefore, with this experimental paradigm, CEF protein composition could be examined in the presence of sperm accumulation and undisturbed sympathetic innervation within the cauda epididymidis.

In 2-D polyacrylamide gels containing control CEF, 20 proteins (1–19 and protein A) were identified, as described in the previous section. One week following cuff induction of sperm accumulation within the cauda epididymidis, the 20 proteins identified within control CEF were still present (see Fig. 2a), and no new proteins were detected. Compared to proteins from sham-operated controls, however, significant changes in the staining intensities of individual CEF proteins were observed 1 week following cuff obstruction. The staining intensities of CEF proteins A, 1, 2, 4, 12, and 14 were significantly increased compared to the intensities of the same proteins from sham-operated controls (Fig. 4). Although the increased staining intensity of CEF protein 2 following cuff was similar to the increase in this protein observed 1 week following IMG removal (Fig. 2b), the increased staining intensities of CEF proteins A, 1, 4, 12, and 14 were observed following cuff obstruction of the vas deferens (Fig. 4), but

Table 1. *Tissue norepinephrine content (ng/g tissue) following constriction of the vas deferens using polyethylene tubing*

	Cauda epididymidis	Vas deferens
Sham control	1,410.30 ± 214.50	17,595.30 ± 1,429.70
"Cuff" obstructed	1,861.70 ± 104.70	16,808.30 ± 1,522.20
P value	0.095	0.716

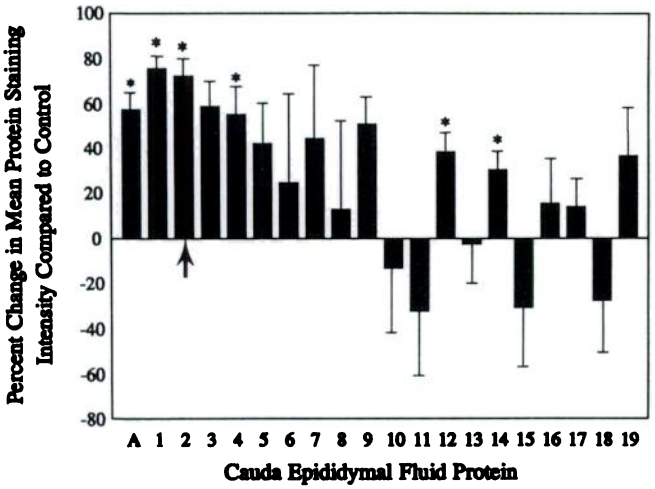

FIG. 4. Changes in CEF protein staining intensity in the presence of neuronal input from the IMG and sperm accumulation induced for 1 week by constricting the vas using polyethylene tubing as described in the Materials and Methods Sections. Data are the percent change in mean protein staining intensity ± SEM. Significant changes in mean protein staining intensities compared to controls are indicated (*) for CEF proteins A, 1, 2, 4, 12, and 14 ($P < 0.05$). The significant protein changes observed following cuff obstruction were different than those CEF protein changed following IMG removal (see Fig. 2b) except for CEF protein 2 (arrow), which was similarly affected by both cuff obstruction and IMG removal.

not after surgical removal of sympathetic innervation from the IMG (Figure 2b).

Other CEF proteins, including 3, 5–11, 13, and 15–19, were not significantly altered 1 week following cuff obstruction of the vas deferens (Fig. 4). In contrast, the staining intensities of CEF proteins 5, 8, 9, 13, and 19 were significantly altered 1 week following the loss of sympathetic innervation from the IMG.

Cauda Epididymal Sperm-associated Proteins in the Presence and Absence of Sympathetic Innervation from the IMG

Silver-stained 2-D PAGE of detergent-extracted proteins associated with cauda epididymal spermatozoa from sham-operated control rats exhibited a pattern shown in Figure 5a. Twenty sperm-associated proteins were identified numerically (1–20). Sperm proteins 10, 11 (CEF protein 10), and 16 (CEF protein 18) were found to comigrate with CEF proteins. Quantitation of the staining intensities of sperm-associated proteins revealed no significant changes 4 weeks following IMP removal, compared with sham-operated controls (Fig. 5b).

Caput Epididymal Fluid Proteins in the Presence and Absence of Sympathetic Innervation from the IMG

Samples of luminal fluid obtained by micropuncture from the caput epididymides of sham-operated control animals

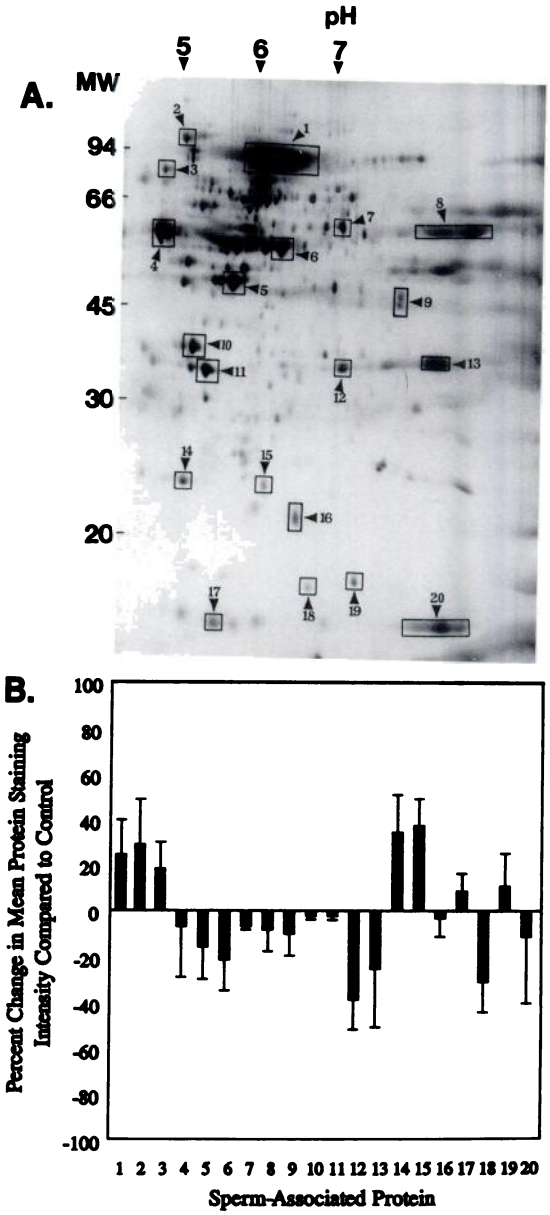

FIG. 5. Cauda epididymal sperm-associated proteins extracted using SDS detergent 4 weeks following IMG removal. (A) Silver-stained 2-D gel of detergent-extracted cauda sperm proteins (1–20) from sham-operated control rats. (B) Percent change in mean protein staining intensity from control following IMG removal ± SEM. No significant alterations in sperm-associated proteins were observed following IMG removal.

contained eight proteins (Fig. 6a) that were consistently resolved in control caput epididymal fluid. These proteins were identified numerically (1–7), as previously described (Olson and Hinton, 1985); albumin was identified by the letter A (Olson and Hinton, 1985). Proteins 3 and 7 were the most abundant proteins present in caput epididymal luminal fluid. Protein 3 contained multiple isoforms (a–f) corresponding in molecular weights and isoelectric points to the protein clusterin, also referred to as sulfated

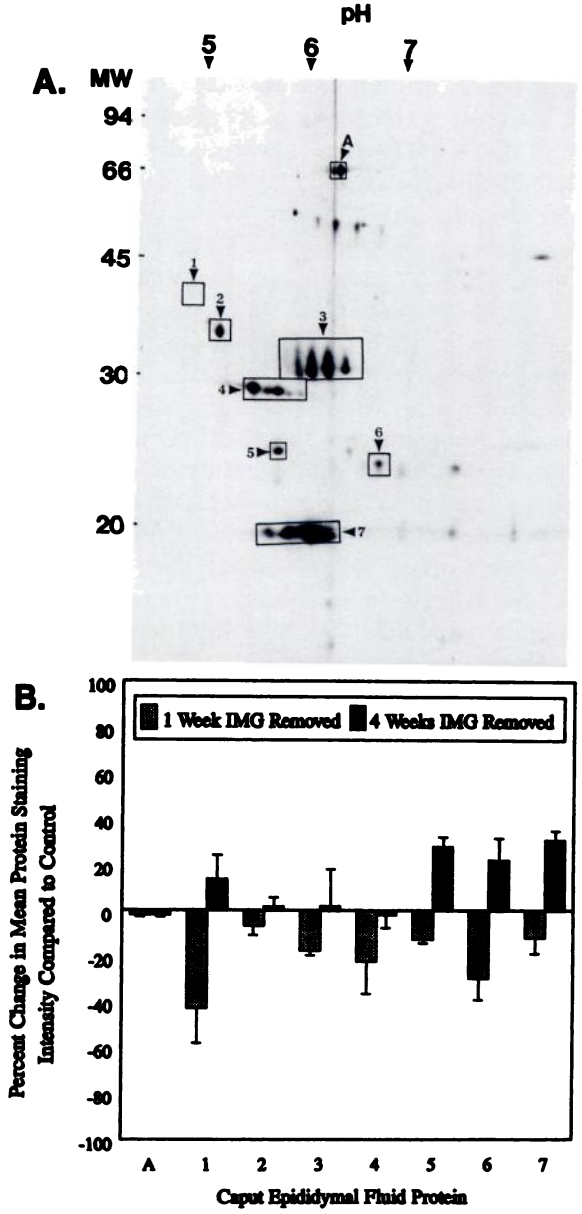

FIG. 6. Caput epididymal fluid proteins 1 and 4 weeks following IMG removal. **(A)** Silver-stained 2-D SDS denaturing gel of caput epididymal fluid proteins collected by micropuncture from sham-operated control animals showing the localization of proteins A and 1–7. **(B)** Quantitation of silver-staining intensities of caput epididymal fluid proteins 1 (gray bars) and 4 weeks (dark bars) following IMG removal. Data are the percent change in mean protein staining intensity ± SEM. No statistical differences in mean protein staining intensities were observed 1 or 4 weeks following IMG removal compared to controls.

glycoprotein-2 (SGP-2) (Olson and Hinton, 1985). One and 4 weeks following IMG removal, no significant changes were detected in the number or staining intensities of caput epididymal fluid proteins when compared with the staining intensities of proteins from sham-operated controls (Fig. 6b).

Discussion

The present study demonstrates clearly that significant but temporal alterations occur in the protein composition of fluid within the lumen of the rat epididymis following the loss of sympathetic innervation. Moreover, these protein changes were observed exclusively within the caudal region of the epididymis. These findings strongly support the principle that adrenergic innervation is important for the maintenance of a normal protein microenvironment within the rat cauda epididymidis.

The regional specificity of the sympathectomy-induced changes in luminal fluid protein composition within the rat epididymidis is noteworthy. To date, alterations in neurotransmitter content, sperm transport (Billups et al, 1991a), sperm motility (Billups et al, 1991b), and, in the present study, luminal fluid protein composition have been observed only within the cauda epididymidis following IMG removal. Two observations may explain why the caput epididymidis is not affected by the loss of innervation following IMG removal. First, the caput epididymidis is innervated primarily by neuronal sources other than the IMG (Mitchell, 1935). Second, the density of innervation within the caput epididymidis is much less than that in the cauda (Norberg et al, 1967; Baumgarten et al, 1968). Accordingly, it is not unexpected that the structural and functional alterations in epididymal function following IMG removal occur exclusively within the cauda epididymidis.

The staining intensities of nine proteins present within cauda epididymal luminal fluid were significantly altered following the loss of sympathetic innervation by surgical removal of the IMG. Although the functions of these proteins are yet unknown, it is conceivable that these CEF proteins may be involved in the functional maintenance of spermatozoa during storage within the cauda epididymidis. Studies by Cuasnicu and Bedford (1989) have shown that the storage of spermatozoa within the cauda epididymidis is a dynamic process that is compromised if spermatozoa are stored for extended periods of time. Therefore, it is possible that, with the observed changes in luminal fluid proteins, the ability of the cauda epididymidis to maintain spermatozoa is severely compromised following the loss of sympathetic innervation from the IMG.

The effect of the loss of sympathetic innervation on CEF proteins is temporal; distinct but consistent changes in CEF protein staining intensities were observed 1 and 4 weeks following IMG removal. The temporal nature of these changes following the loss of cauda epididymal sympathetic innervation suggests that nerves from the IMG influence CEF proteins through a complex mechanism that is poorly understood and requires further study.

The alterations in CEF protein staining intensities following IMG removal may be attributed to denervation-induced changes in epididymal protein synthesis, secretion, or absorption. Changes in protein synthesis are unlikely because *de novo* protein synthesis occurs primarily in the proximal regions of the epididymis (Brooks and Higgins, 1980; Kohane et al, 1980; Vreeburg et al, 1990), and, to date, no significant changes in the proximal epididymal regions have been detected following IMG removal. Consistent with these findings, the present study revealed no significant changes in caput epididymal luminal fluid proteins 1 or 4 weeks following IMG removal. Beyond the micropuncture site within the caput epididymidis, however, altered protein synthesis and/or secretion could have affected luminal fluid proteins present in cauda epididymal luminal fluid. The degree to which proteins beyond the micropuncture site contributed to cauda epididymal luminal fluid proteins was not estimated in the present study. An alternative explanation for protein changes following denervation is that the absorption of luminal fluid proteins by cauda epididymal epithelial cells is altered following IMG removal. Clearly, however, the precise mechanisms by which the loss of innervation affects the protein composition of cauda epididymal luminal fluid is yet unclear and merits further examination.

The results of the present study indicate that, unlike proteins found in cauda epididymal luminal fluid, the composition of proteins associated with cauda epididymal spermatozoa is not significantly altered following the loss of innervation from the IMG. These data suggest that the changes in the motility of cauda epididymal spermatozoa that are associated with the loss of innervation (Billups et al, 1991b) may be related to changes in composition/concentration of other non-proteinaceous luminal fluid constituents within the epididymis. This hypothesis requires further investigation.

Abnormal sperm accumulation within the cauda epididymidis may also be responsible for the alterations in the protein composition of CEF following IMG removal. We addressed this possibility by mechanically inducing sperm accumulation within the cauda epididymidis by using polyethylene tubing to constrict the vas deferens while maintaining sympathetic innervation. With this approach, we showed that only one CEF protein was reduced following sperm accumulation both in the presence and absence of neuronal input from the IMG. Consequently, the majority of protein changes in CEF observed following IMG removal are a direct result of the loss of sympathetic innervation and not the consequence of sperm accumulation within the cauda epididymidis.

It is possible that CEF protein composition may be affected by temperature changes induced by surgical removal of the IMG. Previous pharmacological studies have shown that chemical sympathectomy results in systemic vasodilatation and increased blood flow; this could potentially result in increases in basal body temperature. Bedford (1978) has reported that elevated temperature alters the protein composition of cauda epididymal luminal fluid. In our studies, however, it is unlikely that the changes in cauda epididymal luminal proteins following the loss of innervation are due to an elevation of temperature. First, proteins in CEF that are temperature sensitive include those having molecular weights of 18, 23, 52, 48, 34–38, and 100–200 kDa (Esponda and Bedford, 1986). Only one CEF protein (18) that is altered following IMG removal has a molecular weight similar to temperature-sensitive CEF proteins previously reported. Second, sympathectomy and temperature elevation have opposing effects on cauda epididymal sperm storage capacity; denervation results in increased cauda epididymal sperm storage, whereas elevated temperature reduces sperm storage. Taken together, these observations suggest that alterations in the protein composition of rat cauda epididymal fluid following IMG removal are not the result of temperature elevation.

The results of the present study suggest that innervation originating from the IMG mediates epithelial cell function required for maintaining the protein milieu of luminal fluid within the rat cauda epididymidis. El-Badawi and Schenk (1967) and Kuntz (1953) have reported that neuronal fibers terminate within the peritubular smooth muscle and subepithelial regions of the epididymis. It is reasonable to speculate that neurochemicals and neuropeptides arising from these nerves may act not only to support neuromotor activities within the epididymis but also affect epithelial cell function. Studies by Wong (1988a,b) have shown that adrenergic agonists such as isoproterenol, adrenaline, and phenylephrine stimulate epithelial cell secretion by receptor-mediated mechanisms. Accordingly, it is reasonable to speculate that the loss of adrenergic innervation within the cauda epididymidis may compromise epithelial cell function and thereby affect the protein composition of cauda epididymal luminal fluid. This hypothesis is consistent with the results of the present study that demonstrated significant alterations in the staining intensities of nine CEF proteins following the loss of sympathetic innervation from the IMG.

The loss of innervation has been shown to affect the protein composition of other tissues. In the prostate, denervation following MPG ablation significantly modifies the protein composition of prostatic fluid (Wang et al, 1991; Martinez-Pineiro et al, 1993). Additionally, there is a reduction in protein content in the pancreas following extrinsic denervation (Becker et al, 1988). Together these data reinforce the principle that innervation plays an important role in the maintenance of a normal tissue protein milieu.

The present study has profound clinical implications.

It is well established that fertility is severely impaired in many men following spinal cord injury (SCI) (for review see Linsenmeyer and Perkash, 1991). The two major causes of infertility in SCI men are poor semen quality and anejaculation. When electroejaculatory procedures are utilized, semen quality, in general, remains poor and fertilization rates are dismal. The present data suggest that the disruption of neuronal input following SCI may affect the protein milieu within the epididymis and consequently the fertility of spermatozoa stored therein. Semen obtained from SCI patients following electroejaculation contains reduced levels of fructose, albumin, glutamic oxaloacetic transaminase, and alkaline phosphatase; these alterations may also contribute to seminal dysfunction (Hirsch et al, 1991).

In conclusion, the present study demonstrates that sympathetic innervation emanating from the IMG is important for the preservation of the normal protein composition within the luminal fluid of the rat cauda epididymidis. Although the mechanism is unknown, it is apparent that the epididymis requires not only hormonal stimulation but also neuronal input for the maintenance of the luminal fluid microenvironment that is essential for the functional preservation of spermatozoa.

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