Urol Res (1996) 24:229–234

ORIGINAL PAPER

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Contractile and cytoskeletal proteins in urinary bladder smooth muscle from rats treated with epidermal growth factor

Received: 3 January 1996 / Accepted: 8 March 1996

Abstract Systemic treatment with epidermal growth factor (EGF) induces growth of all wall layers in the urinary tract of pigs and rats. The present study was initiated to describe morphological and biochemical changes in the bladder smooth muscle from rats treated with EGF for 4 weeks. Eight-week-old female Wistar rats were treated with subcutaneous injections of vehicle ($n = 16$) or EGF ($n = 8$, 150 μg/kg per day) for 4 weeks. After EGF treatment the bladders were increased in weight [74.4 ± 0.4 vs 122.1 ± 0.5 mg, $P < 0.001$ (mean ± SEM)]. Sodium dedecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analyses of six bladders from each group revealed that the total amounts of actin, myosin and desmin were statistically significantly increased by 62%, 61% and 154%, respectively. The relative amounts of actin and myosin were unchanged whereas the desmin to actin ratio was significantly increased – as previously described in rat bladder smooth muscle hypertrophy. Light and electron microscopy of two bladders from each group revealed increased wall thickness involving all wall layers. The smooth muscle fibres at a midventral bladder location seemed only slightly hypertrophic – some degree of hyperplasia was therefore suspected. In conclusion, EGF treatment for 4 weeks induced a net synthesis of contractile and cytoskeletal proteins in the urinary bladder smooth muscle.

Key words Actin · Desmin · Growth factors · Myosin · Urinary bladder

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Introduction

The epidermal growth factor (EGF) family comprises several ligands and receptors [19]. The ligand EGF and five other ligands exert their action through binding to the same EGF receptor [19, 21]. The EGF family is present in almost all cells and the system plays a role in the regulation of normal, regenerative and neoplastic growth.

Many publications concern the presence of the EGF family in the kidneys (for review see Fisher [8]) but few studies have addressed the anatomical distribution and physiological significance of the family in the urinary tract. EGF is produced in the kidneys, secreted in an exocrine manner to the urine and comes into contact with the urothelium [12]. Within the urothelium, the EGF receptor and the EGF-related ligands transforming growth factor (TGFα) and amphiregulin are expressed [7]. Based on parallels in distribution and function of the ligands in the saliva and in the mucosa of the stomach [2, 17, 20], urinary EGF probably exerts a trophic action to the urothelium if damaged and the other ligands within the urothelium maintain the physiological regulation. We have recently described that systemic EGF treatment induces growth of all wall layers of the urinary tract of Goettingen minipigs and rats [25, 26]. We focused on the urothelial growth and described especially in the pigs a pronounced hyperplasia with accumulations of glycoconjugates. We found no malignant changes, but

these findings are interesting considering that members of the EGF family are often upregulated in urothelial neoplastic growth (see Neal and Mellon [16]).

It is increasingly being recognized that the EGF system might influence smooth muscle cell function, growth and differentiation. EGF exerts contractile effects on isolated smooth muscles from various organs in different species, as recently reviewed by Hollenberg [11]. In in vivo experiments EGF exerts cardiovascular effects [3, 5] and modifies smooth muscle contractility of the stomach [23]. No effects have so far been described on the smooth muscles from the urinary tract.

The aim of the present study was to further characterize the effects of EGF on the muscular coat of the urinary bladder. The rat was chosen as the experimental animal model since the contractile and structural smooth muscle proteins and the ultrastructure of the smooth muscle cells have been described in this species under other experimental growth conditions [1, 9, 10, 14].

Material and methods

Study animals

The study was conducted in 24 female Wistar rats of our own breed (Department of Pathology, Aalborg, Denmark), which were 8 weeks old. The animals were housed individually in cages on special white spanwall bedding (temperature 21 °C, humidity 55 ± 5%, dark/light cycle 12-h shift). They were fed a standard laboratory diet (Altromin 1314). The animals were throughout the study kept in a room for this specific experiment. The study complied with the Danish regulations for care and use of laboratory animals.

Study design

All procedures were carried out on a blinded basis. The 24 animals were randomly allocated into two groups. They received a subcutaneous injection of either solvent (isotonic saline) ($n = 16$) or human recombinant EGF 150 µg (25 nmol)/kg per day (Upstate Biotechnology, New York, USA) ($n = 8$) twice daily for 4 weeks.

After 28 days of treatment the animals were anaesthetized with ketamine (Ketalar, Parke Davis, Barcelona, Spain) 100 mg/kg and xylosin (Rompun, Bayer, Leverkusen, Germany) 15 mg/kg intramuscularly. Through a midline incision the bladder was exposed, removed, opened, rinsed, weighed and snap frozen in liquid nitrogen. Two bladders from each group were removed for morphological examination; see below.

Determination of protein composition

The bladders from six placebo and six EGF-treated animals were selected for this procedure. Five- to 15-mg tissue specimens of bladder tissue taken at a random location were homogenized in a sodium dodecyl sulphate (SDS) buffer containing 50 mM tris-(hydroxymethyl)aminomethane hydrochloride (TRIS), 25 mM dithiotreitol (DTT), 10% glycerol (vol/vol), pH 6.8, and 0.001% bromphenol blue (wt/vol). The homogenate was then boiled for 2 min. SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli [13], using a minigel system from Bio-Rad (Richmond, CA, USA). The gels were prepared with 5% or 8% total acrylamide. Each gel was loaded with four different amounts of bladder extracts and four standards of actin. The gels were stained with 1% Coomassie blue (wt/vol) in 40% methanol and 10% acetic acid and destained in the same solution without Coomassie blue in a Bio-Rad model 556 gel destainer (Bio-Rad, Richmond, CA, USA) until clarity. The gels were scanned with a GS-300 densitometer (Hoeffer Scientific, San Francisco, CA, USA). Linear relations were found between the amount of standard protein (actin) and the volume of muscle extract loaded on the gel. The actin concentration per volume extract was calculated as the relationship between the area below the actin spike of the tissue extract and that of standard actin. The total actin content of the bladder was determined by multiplying the actin concentration with the bladder weight.

The relative amounts of actin and desmin, and of actin and a protein band with an M_r of about 66 kDa, were determined on 8% gels. The relative amount of actin and myosin was determined on 5% gels, which gives a better resolution of the myosin heavy-chain bands. The total contents of myosin and desmin were thereafter determined by multiplying the actin content with the myosin/actin and desmin/actin ratios.

To confirm the position on the gels of actin (about 40 kDa) and myosin heavy chains (about 200 kDa), protein standards of actin (see above) and chicken gizzard myosin (Sigma, St. Louis, MO, USA) were run on the 8% and 5% gels, respectively. The position of the desmin (51 kDa) band was confirmed by Western blotting extracts from one control and one EGF-treated animal, as follows. Electrophoretic transfer of proteins from SDS-PAGE gels (8%) to nitrocellulose was performed using 25 mM TRIS, 192 mM glycine and 20% (vol/vol) methanol as transfer buffer. An immunoblot assay kit with alkaline phosphatase-conjugated goat anti-mouse as secondary antibody and colour development reagents BCIP and NBP was used according to the manufacturer's instructions (Bio-Rad, Richmond, CA, USA).

The band of about 66 kDa on the SDS-PAGE gels is upregulated in smooth muscle cell hypertrophia after obstruction [14]. We found in this study a similar increase of this band. Our aim therefore was to identify the protein. Its position strongly indicated that it was rat albumin. Bladder extracts and rat serum were run on SDS-PAGE gels (8%) under both reduced and non-reduced conditions.

Light and electron microscopic examination

To determine the relative amount of smooth muscle in the urinary bladder wall, two bladders from each group filled with 0.7 ml saline were immersion fixed in 5% glutaraldehyde in 100 mM cacodylate buffer, pH 7.4. At the ventral aspect of the rat bladder there is a large component of longitudinal smooth muscle running from the cranial to the caudal pole, whereas the muscles in other parts run without a preferred orientation [9]. We have previously used these longitudinal bundles to compare hypertrophic with normal smooth muscle cells [9, 14]. At the mid-ventral portion of the bladders the smooth muscles were removed, postfixed in 2% OsO_4 in cacodylate buffer, and embedded in Araldite. For light microscopy, 1- to 3-µm-thick sections were cut and stained with toluidine blue. For electron mictroscopy, 60- to 90-nm-thick sections were cut with glass knives and stained with uranyl acetate. The relative amount of smooth muscle bundles in the bladder wall was determined by point counting.

Statistical analysis

Results are expressed as means and standard error of the mean (SEM) with the number of animals indicated. Mann-Whitney's test for non-paired data was employed to test for differences from the control group. The level of significance chosen was 0.05.

Results

General observations

All animals thrived throughout the study. The animals increased in weight from 172 ± 1 to 203 ± 3 g in the controls and from 172 ± 1 to 212 ± 3 g in the EGF-treated group. The total body weight increase was significantly greater in the EGF-treated group ($P < 0.05$). This is referred to in detail elsewhere.

On macroscopic examination, the EGF bladders had an increased wall thickness with vessels of increased size (Fig. 1). The wet weight of the bladders was increased from 74.4 ± 0.4 mg in the controls to 122.1 ± 0.5 mg in the EGF-treated animals ($P < 0.001$).

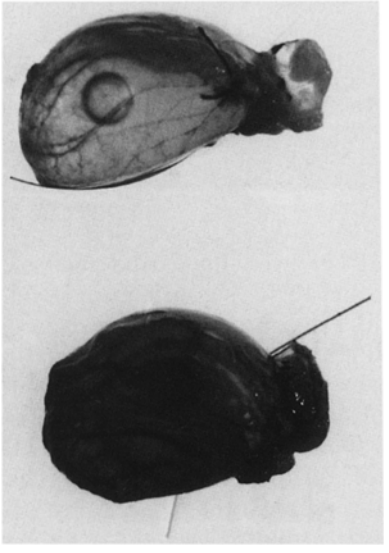

Fig. 1 Bladders from rats treated with placebo (*upper*) or EGF (150 μg/kg/day) (*lower*) for 4 weeks. The bladders were distended with 0.7 ml isotonic saline

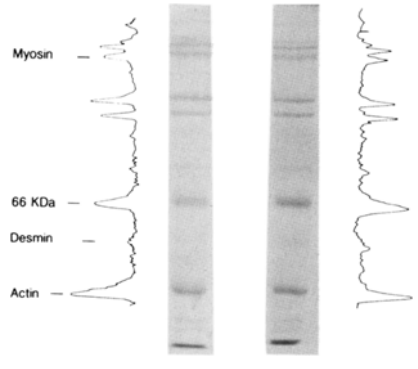

Fig. 2 Photograph of a 8% SDS-PAGE gel of bladder extracts from rats treated for 4 weeks with placebo (*left*) or EGF (150 μg/kg/day) (*right*). Positions of myosin heavy chains, actin, desmin and a protein band around 66 kDa (probably albumin) are indicated. The scans are shown beside the lanes

Table 1 Tissue amount of actin and relative amounts of contractile and structural proteins in control rats and in rats treated with EGF (150 μg/kg/day) for 4 weeks. Values are means ± SEM (*NS* not significant)

	Controls ($n = 6$)	P	EGF ($n = 6$)
Actin			
(μg/mg wet wt.)	18.2 ± 2.4	NS	17.3 ± 2.0
(μg/bladder)	1315 ± 161	$P < 0.05$	2133 ± 310
Actin/myosin	2.72 ± 0.15	NS	2.90 ± 0.29
Myosin (μg/bladder)	469 ± 63	$P < 0.05$	753 ± 100
Desmin/actin	0.18 ± 0.02	$P < 0.05$	0.27 ± 0.03
Desmin (μg/bladder)	222 ± 25	$P < 0.02$	564 ± 95.2
Band at 66 kD/actin	0.65 ± 0.11	$P < 0.01$	1.21 ± 0.13

Tissue contents of actin and myosin

A typical gel is shown in Fig. 2. Table 1 summarizes the results from the whole material. The amount of actin per milligram wet weight of the bladder was unchanged. We have previously found [15] that the staining intensities of actin and myosin are similar. We assume that this is also the case for desmin. Thus the total amount of myosin, actin and desmin per bladder can be calculated. The total amounts of actin, myosin and desmin were statistically increased by 62%, 61% and 154%, respectively. There was no change in the relative amount of actin and myosin, whereas the desmin to actin ratio was significantly increased.

The most pronounced change observed in the gels was the doubling of the relative amounts of the protein to about 66 kDa. This protein band behaved as albumin as judged from the electrophoreses. We considered the probability of this identification so high that we conducted no further analyses.

Structural changes associated with EGF treatment

The wall thickness of the two control bladders was 0.169 and 0.178 mm compared with 0.200 and 0.214 mm for the two EGF bladder walls. The relative amounts of muscle tissue were 44.8 and 49.4% in the controls bladders and 53.3% and 63.3% in the EGF-treated animals. The general appearance of the smooth muscle cell profiles in the EGF bladders was completely normal. As demonstrated in Fig. 3, the number of smooth muscle cell profiles per cross-sectional area was slightly lower in the EGF-treated animals. Some degree of smooth muscle cell hypertrophia was therefore suspected. In our previous experiments, two

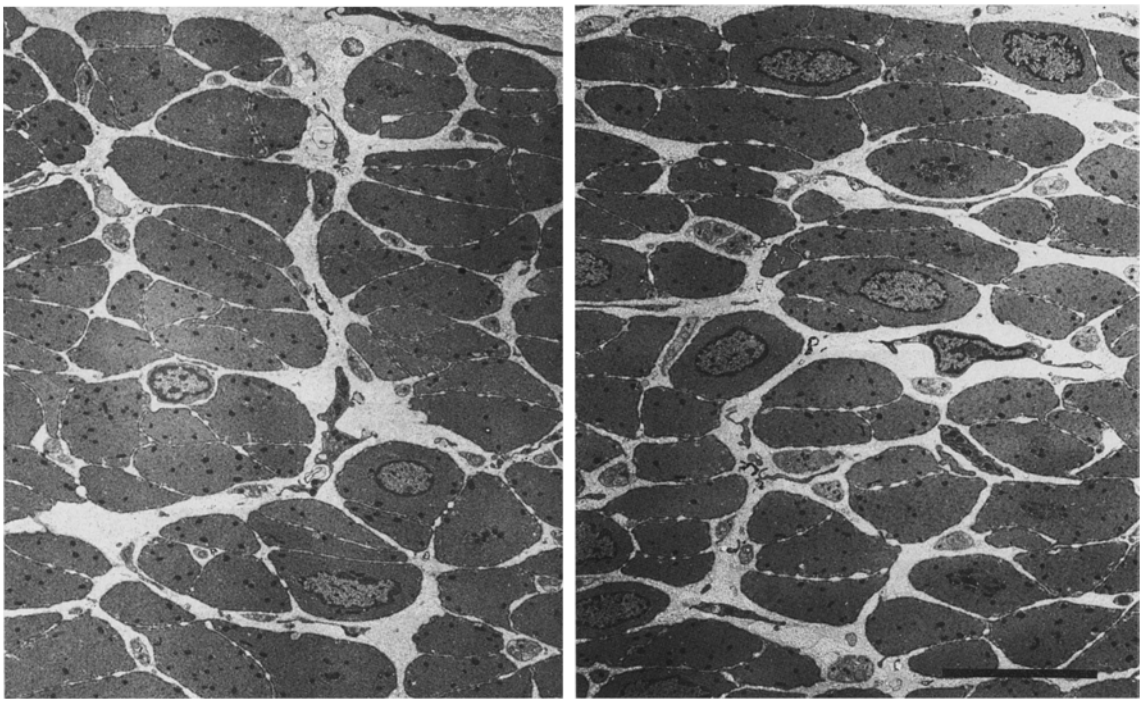

Fig. 3 Electron micrograph of muscular coat of the bladder of a control rat (*left*) and a rat treated with EGF (150 μg/kg/day) (*right*) for 4 weeks (×4110, *bar* indicates 5 μm)

animals from each group were sufficient to demonstrate obvious hypertrophic changes. This was not the case in the present study. We therefore believe that some degree of smooth muscle hyperplasia was aslo involved.

Discussion

Systemic treatment with EGF is a new approach to growth induction of the urinary tract. The main difference between this approach and the growth seen in relation to hyperdiuresis or urinary overload is the more pronounced urothelial proliferation.

In our first paper describing this growth in pigs, we focused on the urothelial changes, and showed that the effect of EGF was most likely an effect of the systemically administered EGF directly on the basal cells of the urothelium. In the present experiment, it was not possible to ascertain whether the growth of the muscular coat was a direct effect of the EGF on the smooth muscles or an effect secondary to growing urothelial and submucosal tissues. It is, however, well established in other organ systems in various species that EGF is able to exert direct effects on smooth muscles in vitro as well as in vivo [3, 5, 11, 23]. The possible involvement of the members of the EGF family in muscle growth is increasingly being recognized with the demonstration of upregulation of the gene encoding for heparin-binding EGF in growing cardiac muscle cells [18] and in growing aortic smooth muscle cells [24]. We have, in addition, recently demonstrated that EGF exerts a direct contractile effect on rat bladder smooth muscles in vivo and in vitro. (Vinter-Jensen et al., unpublished data). It is therefore possible that at least part of the smooth muscle cell growth is a direct action of EGF on the smooth muscle cells.

The involvement of other growth factors in bladder smooth muscle growth is a field of growing interest. For example, in rats insulin-like growth factor I (IGF-I) [6] and nerve growth factor (NGF) [22] are both upregulated in the hypertrophying bladder after urethral obstruction. In the latter study, it was also demonstrated that administration of calcium channel antagonists partly inhibited the obstructive growth, a finding implicating cytosolic calcium in bladder smooth muscle growth. This may also be relevant for the EGF-induced bladder smooth muscle growth, as an EGF-induced increase in cytosolic calcium has previously been associated with contractility and growth of smooth muscle cells from the rat aorta [4].

The total weight increase of the EGF-treated bladders was approximately 80% in 4 weeks. This growth response is less pronounced than in experimental infravesical bladder obstruction in rats, where the bladder grows threefold in 10 days and 10-fold in 7 weeks [14]. In the infravesical obstruction, the muscular coat increases relatively more in weight than the other layers, which is also the case after EGF treatment as judged from a single estimate from the midventral location of the bladder. The total amount of bladder

smooth muscle was evidently increased as the unbiased estimates of the muscular proteins actin, myosin and desmin increased with an increase in the desmin to actin ratio. In smooth muscles, the intermediate filaments are composed of vimentin and desmin in varying amounts in different tissues. In the rat bladder, desmin accounts for approximately 95% of the intermediate filaments in ordinary smooth muscle and even more in hypertrophic smooth muscle [14]. We found, despite the modest (compared to obstruction) weight gain after EGF treatment, an increased desmin to actin ratio. The increased desmin to actin ratio reflects the relatively increased content of intermediate filaments. Therefore, these biochemical alterations in response to EGF treatment resemble, to a large extent, those described in experimental obstruction. It has been suggested that the increased amount of desmin reflects an increased cytoskeleton in the hypertrophic muscle cells. In the present study there was, however, a discrepancy between the limited (if any) increase in smooth muscle cell size and the substantial increase in desmin to actin ratio. It could be that desmin is synthesized in growing smooth muscle irrespective of whether the growth is through hypertrophy or hyperplasia.

In bladder obstruction an increased actin to myosin ratio has been demonstrated [14]. In the present study, we only observed a minor insignificant increase. Another much more pronounced change was an increase in the band around 66 kDa – most likely albumin. This band also increased in experimental obstructive growth [14]. We suggest that the increased contents of albumin in the growing rat bladder is caused by a leak of intravascular albumin to the extravascular space from newly formed capillaries. It is outside the scope of this paper to examine this issue further.

In conclusion: We have described that systemic EGF treatment for 4 weeks causes bladder growth with an increase in the total amount of smooth muscles. The amount of actin, myosin and desmin increased with an increase in the desmin to actin ratio. This distribution resembles the distribution seen in infravesical bladder obstruction even though only slight (if any) smooth muscle hypertrophia was present.

Acknowledgements This study was supported by Professor J.C. Christoffersens Mindefond, The Danish Medical Research Council (12-1377-1) and The Swedish Medical Research Council (14x-9479, 04x-8268). The recombinant EGF was a generous gift from Professor Esam Z. Dajani, International Drug Development Consultants, a Division of Midgulf, USA, Inc., Long Grove, Illinois, USA. Technicians Ole Sørensen and Torben Madsen, Department of Pathology, Aalborg Hospital, Denmark, and laboratory assistant Chistina Svensson, Department of Physiology and Neuroscience, Lund University, are thanked for their handling of the animals and conducting the biochemical procedures, respectively.

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