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Neuropeptides and urinary bladder ischemia-reperfusion injury

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Urinary bladder decompensation following partial bladder obstruction is directly related to decreased tissue perfusion, resulting in periods of hypoxia and ischemia. Hence, it is important to look for substances which could counteract against the ischemia/reperfusion- induced neuronal damage in detrusor muscle and in such a way to ameliorate the functional disorders of urinary bladder. Many neuropeptides have been found to be synthesized, stored and released in the lower urinary tract. Some of them were reported to reduce ischemia-reperfusion injury. The purpose of this study was to examine the efficacy of vasoactive intestinal peptide (VIP), somatostatin and Sandostatin® (Novartis) to counteract the damage suffered by neurons in urinary bladder exposed in vitro to experimentally induced ischemia-reperfusion in guinea-pig. We found that VIP (0.3 μM), somatostatin (300 nM) and sandostatin (1 to 300 nM) improved significantly the response to electrical field stimulation during reperfusion as compared to the control, utreated tissues. The antioxidant activity of VIP, somatostatin and sandostatin, assessed as their capability to scavenge peroxyl radicals during linoleic acid oxidation corresponded to 6.4 ± 0.1, 6.7 ± 0.3 and 7.0 ± 0.6, respectively. The antioxidant activity of the above mentioned peptides could underlie their neuroprotective action during reperfusion, when a significant amount of free radicals has been formed.

Key words: urinary bladder, vasoactive intestinal peptide, ischemia, somatostatin, sandostatin, injury

INTRODUCTION

Many neuropeptides have been found to be synthesized, stored and released in the lower urinary tract. Some of them are released from the peripherial neural terminals of the autonomic neurvous system – vasoactive intestinal peptide (VIP), tachykinins (substance P), neuropeptide Y, calcitonin gene-related peptide, neurokinin A. Others are locally synthesized and act by para-/auto and intacrine mechanisms – for example angiotensin II. The functional role of many of these peptides has not been fully established, however they may have a sensory role, efferent function [1, 2] or serve as NANC neurotransmitters and/or neuromodulators in the bladder ganglia or at the neuromuscular junctions. Their actions have been thought to include mediation of the micturition reflex activation, smooth muscle contraction, potentiation of efferent neurotransmission and changes in vascular tone and permeability [3, 4].

Vasoactive intestinal peptide is expressed in the neural pathways regulating the lower urinary tract. VIP-immunoreactivity is present in afferent and autonomic efferent neurons innervating the bladder and urethra. In the human urinary bladder neck VIP-containing nerve fibres localized close to noradrenergic and cholinergic intramural neurons have been identified. In both types of nerve, VIP co-localizes with other transmitters, such as calcitonin gene-related peptide, neuropeptide Y, substance P, nitric oxide [5, 6]. Recently, the peptide somatostatin was found to be distributed in the sensory dorsal root ganglia neurons supplying porcine urinary bladder [7]. Sensory nerves, particularly the nociceptive nerves, may send collaterals to the smooth muscle, to the intramural ganglia and to the ganglia of the pelvic plexus [8]. This may be of great importance for the development of the unstable detrusor muscle in the urinary bladder.

Bladder outlet obstruction generally due to prostatic hyperplasia, a common problem in men over 60 years of age, is a major urologic problem that has been the subject of many clinical and experimental studies. The hyperplasia of prostate leads to obstructed micturition, during which occurs periodic bladder ischemia. The latter has been suggested to result in the partial denervation of the detrusor smooth muscle through ischemia and reperfusion injury to the post-ganglionic parasympathetic neurons within the bladder wall [9]. Previous investigations showed that *in vitro*

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ischemia-like conditions were more damaging to the nervous tissue than to the detrusor muscle [10]. Hence, it is important to look for substances which could counteract against the ischemia/reperfusion-induced neuronal damage in detrusor muscle and in such a way to ameliorate the functional disorders of urinary bladder.

It was demonstrated that the urinary bladder neuromodulator vasoactive intestinal peptide possesses the ability to scavenge oxygen free radicals and to reduce the intracellular calcium overloading in ischemic and reperfused heart [11]. Somatostatin-14 and particularly its stable analogue, the cyclic octapeptide Sandostatin®, are known to exert cytoprotective activities in peripheral tissues and in neuronal cells [12, 13]. The somatostatin peptides were also shown to afford protection against neuronal damage caused by experimentally induced cerebral ischemia in rats [10], to limit intestinal ischemia-reperfusion injury in macaques via suppression of NF-kappaB cytokine pathway [14], and to ameliorate ischemia-reperfusion injury through the early induction of heme oxygenase [15].

The purpose of this study, therefore, was to examine the efficacy of VIP, somatostatin and sandostatin, to counteract the damage suffered by neurons in urinary bladder exposed in vitro to experimentally induced ischemia-reperfusion injury.

EXPERIMENTAL METHODS

Male guinea-pigs (300–500 g, Charles River) were anaesthetised with Ketavet and sacrificed by cervical dislocation. The animals were treated in accordance with European Commission standards concerning the care and use of laboratory animals. The urinary bladders and part of the urethra were removed and placed in oxygenated (95% O2 and 5% CO2) cold Krebs solution. The ureters were tied off using silk ligatures. The prostate gland was excised to expose the urethra and the bladder emptied. The urethra was secured to a stainless-steel tube (8 mm long, 2.5 mm diameter) sealed to a glass J-shaped tube that was connected by a three-way valve to a compensated pressure transducer. The organ was filled with 2 ml oxygenated glucose-free Krebs solution at 37°C and suspended in an insulated, 100-ml isolated organ bath containing Krebs solution maintained at 37°C and equilibrated with 95% O_2 and 5% CO_2. Silver plate electrodes on either side of the bladder connected to a Grass S48 stimulator enabled electrical field stimulation (EFS) of intrinsic nerves. Isometric contractions were evoked by EFS using 5-s trains of square pulses (20 Hz, 1 ms pulse width, 70 V) delivered at 10-min intervals. After a minimum equilibration period of 40 min the bladder was exposed to 10 μM carbachol for about 5 s to test the contractile ability of the tissue. After 30 min recovery from carbachol exposure, the intrinsic nerves were stimulated selectively every 10 min (as described above) until the response was reproducible. Ischemia-like conditions (1 h) were achieved by replacing the gas mixture with 95% N_2 and 5% CO_2 (anoxia) and the organ bath solution with glucose-free Krebs solution (glucopenia). Recovery in normal Krebs solution was then allowed for 2 h. EFS was applied every 10 min during both anoxia-glucopenia and recovery. During the recovery period the Krebs solution was changed every 30 min. Carbachol (10 μM) was applied again at the end of the experiment.

All tested compounds at different final concentrations were added to the Krebs solution during the entire period of ischemia and the first 30 min of reperfusion. The EFS responses obtained in the presence of the tested compounds were compared with those in their absence (control). Control experiments (25% of all experiments) were performed in a randomised manner during the study.

Results are given as means ± SEM. The significance of differences between means was assessed by one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons, $P < 0.05$ was considered significant. Areas under the curve (AUC) relating muscle contraction to time were calculated for ischemia and re-perfusion conditions separately, using appropriate software (Prism v. 3.03, GraphPad, San Diego, California, USA).

RESULTS AND DISCUSSION

The response to electrical field stimulation (EFS) declined rapidly in the combined absence of oxygen and substrate (ischemia-like condition), and was abolished within an hour (Fig. 1 and Fig. 2). After reintroduction of normal conditions, the recovery of the response to electrical field stimulation (neurogenic response) in control bladders was poor, reaching in 2 hours a maximum of about 25 % of the initial response (Fig. 1 and Fig. 2). At this time, however, the response of the muscle to carbachol had fully recovered (data not shown). To see if somatostatin and Sandostatin® could partially reduce the nerve damage described above, the peptides have been perfused during

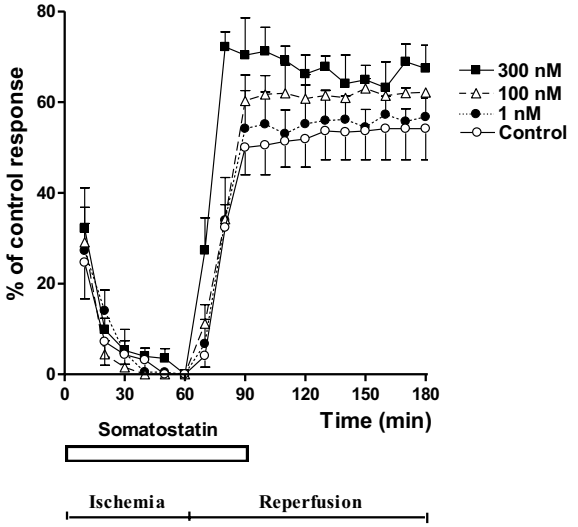

Fig. 1. Electrical field stimulation-induced contractile responses of guinea-pig whole urinary bladder subjected to 60 min of ischemia and subsequent 120 min of reperfusion. Experiments were carried out in the absence or presence of the peptide somatostatin, applied for the first 90 min. Results are expressed as mean ± SEM of six urinary bladders (n = 6).

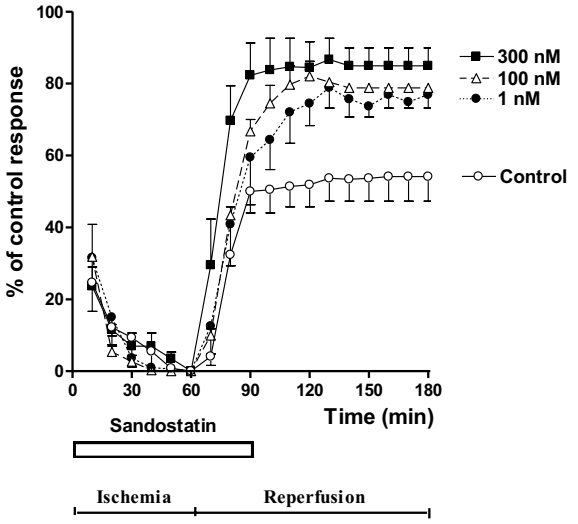

Fig. 2. Electrical field stimulation-induced contractile responses of guinea-pig whole urinary bladder subjected to 60 min of ischemia and subsequent 120 min of reperfusion. Experiments were carried out in the absence or presence of the peptide Sandostatin®, applied for the first 90 min. Results are expressed as mean ± SEM of six urinary bladders (n = 6).

ischemia and the first 30 min of reperfusion, as it is supposed that the major damage to the tissue develops not only during ischemia, but also at the beginning of reperfusion when free radicals are being formed intensively. Sandostatin® at 1, 100 and 300 nM improved significantly the EFS-induced contractile response in reperfusion phase as compared to untreated control bladders (n = 6, P < 0.01) (Fig. 3B).

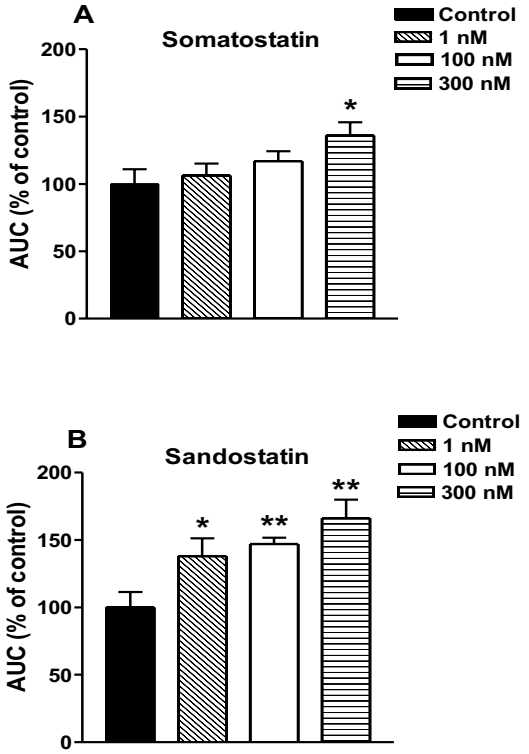

Fig. 3. Electrical field stimulation-induced contractile responses of guinea-pig whole urinary bladder subjected to 60 min of ischemia and subsequent 120 min of reperfusion. Experiments were carried out in the absence or presence of peptides somatostatin (A) or Sandostatin® (B), applied for the first 90 min. Results are expressed as mean of area under curve (AUC) ± SEM of six experiments in each group. Differences were evaluated by one-way ANOVA followed by Dunnett's *post hoc* comparison test (*P < 0.05 and **P < 0.01 versus control; n = 6).

Somatostatin at concentrations of 1 and 100 nM did not exert significant effect as compared to the control (Fig. 1, Fig. 3A) while at 300 nM it increased the recovery of contractile response during reperfusion (n = 6, P < 0.05) (Fig. 3A).

The antioxidant activity of the peptides was assessed for their capability to prevent linoleic acid peroxidation. Both vasoactive intestinal peptide, somatostatin and Sandostatin® exhibited remarkable antiperoxidant activity with a $p\mathrm{IC}_{50}$ M values of 6. 4 ± 0.1; 6.7 ± 0.3 and 7.0 ± 0.6, respectively (Table 1).

Ischemia, reperfusion, and subsequent free radical damage have been implicated in many voiding disorders. The structure and function of detrusor smooth muscle may be altered by a series of noxae including hypoxia, over distension,

Table1 Inhibition of lipid peroxidation

Antioxidant	IC_{50} (mM) ± SEM	pIC_{50} (mM) ± SEM
DTBHA	0.088 ± 0.006	7.1 ± 0.5
Sandostatin®	0.097 ± 0.011	7.0 ± 0.6
Somatostatin	0.164 ± 0.019	6.7 ± 0.3
Vasoactive intestinal peptide	0.380 ± 0.008	6.4 ± 0.1
BHA	0.428 ± 0.005	6.3 ± 0.4
β-TAG	1.040 ± 0.330	6.0 ± 0.4
Propofol	3.100 ± 0.380	5.5 ± 0.3
β-GLU	9.910 ± 2.480	5.0 ± 0.4

diabetes, and ischemia. It was shown that both experimental ischemia and partial outlet obstruction of the urinary bladder induce similar dysfunction with regard to the contractile responses to electrical field stimulation [16]. Previous investigations demonstrated that the response to nerve-mediated stimulation declined rapidly as O_2 and glucose were withdrawn, and recovered only to a limited extent when perfusion with O_2 and glucose started again [17, 16]. We reported that VIP improved this recovery by protecting nervous tissues against anoxia/glucopenia damage [18]. To elicit neuroprotection, however, we incubated the guinea-pig detrusor strips with VIP during the anoxia/glucopenia (i.e. experimental ischemia) phase and the first 30 min of reperfusion. In the last years, increasing evidence has been accumulating that reactive oxygen species (ROS), i.e. hydrogen peroxide (H_2O_2) as well as superoxide anion ($O_2^{\cdot}$) and hydroxyl radical (·OH) generated in greater amounts after reperfusion of the ischemic tissue [19, 20] are important mediators of tissue injury during reperfusion following an ischemic insult. Reactive oxygen species, including the singlet molecular oxygen ($^1O_2$), by promoting lipid peroxidation can damage cell membranes, causing severe dysfunction with impairment of intracellular Ca^{2+} homeostasis. In turn, increases of intracellular Ca^{2+} concentration during reperfusion following ischemia, may activate a cascade of events, which leads to neuronal cell death [11]. Indeed, it was confirmed that reperfusion results in greater injury than 2 h of ischemia in the rabbit bladder, and that the mechanisms of this injury involve mitochondrial and neuronal damage [21]. The reactive oxygen species burst at the beginning of reperfusion and cytosolic Ca^{2+} overload are two proposed mechanisms to explain cell injury. It is difficult to separate cause-and-effect relationship between these variables, since ROS by activating kinases upstream may induce Ca^{2+} overload, and both ROS and Ca^{2+} are known to be mediators in the regulation of mitochondrial permeability transition pore formation [22], a key step in the process of reperfusion injury.

In the present study, a remarkable antioxidant activity of Sandostatin®, somatostatin and vasoactive intestinal peptide has been found, which could underlie their neuroprotective action during reperfusion, when a significant amount of free radicals has been formed. The exact mechanism by which the above mentioned peptides protect the detrusor muscle nerves from reperfusion injury is only a matter of speculation, though a loss of VIP and somatostatin among other sensory neuropeptides in the obstructed human bladder has been previously described [23]. Probably activation of some peptide receptor/receptors is involved in their protective action. Indeed, it was recently reported that activation of somatostatin receptor (sst 5) protects rat retina from neurotoxicity [24], and that changes in VIP and associated receptor transcripts and protein expression in micturition pathways resemble some, but not all, changes observed after induction of urinary bladder inflammation [25]. In summary, the pharmacological action of drug Sandostatin® (Novartis), outlined in the present study, may represent a new therapeutic option for the control of functional disorders of the urinary bladder.

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НЕВРОПЕПТИДИ И УВРЕЖДАНЕ НА ПИКОЧНИЯ МЕХУР ВСЛЕДСТВИЕ ИСХЕМИЯ И РЕПЕРФУЗИЯ

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Декомпенсацията на пикочния мехур, следваща частичната обструкция, е директно свързана с намаляване на тъканната перфузия, което води до поява на хипоксия и исхемия. Следователно е важно да се търсят субстанции, които намаляват предизвиканото от исхемията и реперфузията увреждане на невроните в детрусорния мускул и по такъв начин да подобрят функцията на пикочния мехур. Установено е, че много невропептиди се синтезират, съхраняват и освобождават в долната част на пикочните пътища. Показано е, че някои от тях намаляват увреждането, предизвиканото от исхемия и реперфузия. Целта на настоящата работа беше да се изследва способността на вазоактивния интестинален пептид (ВИП), соматостатина и Сандостатин® (Novartis) да намалят увреждането на невроните при експериментална исхемия и реперфузия в изолиран пикочен мехур от морско свинче при условия ин витро. Установихме, че ВИП (0.3 □M), соматостатин (300 nM) и сандосттин (1 до 300 nM) подобряват значително електрически-предизвикания съкратителен отговор на пикочния мехур по време на реперфузията в сравнение с този в контролните пикочни мехури. Антиоксидантната активност на ВИП, соматостатин и сандостатин, определяна съобразно тяхната способност да предотвратят пероксидацията на линолеовата киселина, беше съответно 6.4 ± 0.1, 6.7 ± 0.3 и 7.0 ± 0.6. Антиоксидантната активност на изследваните пептиди вероятно е в основата на невропротективното им действие по време на реперфузията, когато се образува значително количество свободни радикали.