356

Bahadory F[1], Burcher E[1], Moore K H[2], Liu L[1]
1. *University of New South Wales,* ***2.*** *St George Hospital, University of New South Wales*

GENE EXPRESSION OF ONABOTULINUM TOXIN A TARGET PROTEINS IN PORCINE BLADDER: POSSIBLE EFFECT ON SUBSTANCE P RELEASE?

Hypothesis / aims of study

Onabotulinum toxin A (formerly known as Botox) has recently been approved by FDA for the treatment of neurogenic overactive bladder. It is well established that Botox inhibits cholinergic neurotransmission through the cleavage of neuronal SNARE proteins. In addition to acetylcholine, Botox may also affect the release of other neurotransmitters, including substance P (SP) [1]. However little is known about the action of Botox in individual bladder cell types. Our primary aim was to investigate the gene expression of the SNARE proteins in cultured urothelial cells, myofibroblasts, and muscle cells, in comparison with the fresh corresponding tissues. In order to investigate the effect of Botox on SP release, the method for measuring SP released from porcine mucosal and detrusor strips in response to electrical field stimulation (EFS) was established and optimised.

Study design, materials and methods

Bladders were obtained from adult female pigs freshly killed in the local abattoir, and transported to the laboratory on ice. After rinsing with Krebs solution, urothelial tissue (UT) was scraped off. The remaining bladder tissue was trypsinized for 5 min at 37˚C to remove any residual urothelium, then it was dissected into mucosa/suburothelium (MucT) and detrusor (DT). One half of these fresh dissected tissues was snap frozen and stored at -80˚C. The remainder was cultured into three cell populations: urothelial (UC), suburothelial (myofibroblast) cells (MyC) and detrusor muscle cells (DC) [2]. Cells were harvested after 2 weeks culture. The identity of the cells was confirmed by marker antibodies.

Total RNA was extracted from freshly frozen tissues and 80% confluent cultured cells using TRIZOL (Invitrogen) method, then subjected to single strand cDNA synthesis. The genes under investigation encode for SNARE proteins including synaptosomal-associated protein 25 (SNAP-25), vesicle associated membrane protein (VAMP) and synaptic vesicle proteins 2 (SV2: three isoforms, A, B and C). The gene sequences of interest derived from the pig genome (National Centre for Biotechnology Information) show high homology to their human counterparts (~90% identical). Gene expression was determined by quantitative real-time PCR using KAPA SYBR Fast. GAPDH was used as the housekeeping gene, and the intact pig bladder tissue as the internal calibrator. The results were expressed as fold change relative to GAPDH and the calibrator. Data were analysed using the Kruskal-Wallis test followed by Dunn's multiple comparison.

For functional studies, fresh bladder dome was cut into longitudinal strips of mucosa and detrusor, and mounted in organ baths in Krebs solution, bubbled with 95% O_2 / 5% CO_2 at 37˚C. After 1 h equilibrium, the bath fluid was collected for basal SP measurement. The strips were then stimulated by EFS for 20s at 2, 10 and 20 Hz, 0.1 ms pulse width, 100 V. Bath fluid was collected before (extra basal measurements) and within 10s after the onset of EFS, and snap frozen immediately at -20˚C. The amount of SP released was measured by SP EIA kit (ENZO Life Science).

Results

Figure 1 shows that the genes for SNAP-25, VAMP, SV2B and SV2C were expressed in three studied regions and cell types of pig bladder. However, no expression of SV2A was observed. In fresh tissues, the expression of SNAP-25 mainly occurred in suburothelium and detrusor, with lower expression in the urothelium. Notably, the expression of SNAP-25 was significantly decreased in cultured myofibroblast and detrusor smooth muscle cells, compared with the corresponding fresh tissues (p<0.0001). However, there was no significant reduction of VAMP, SV2B and SV2C expression in cell culture.

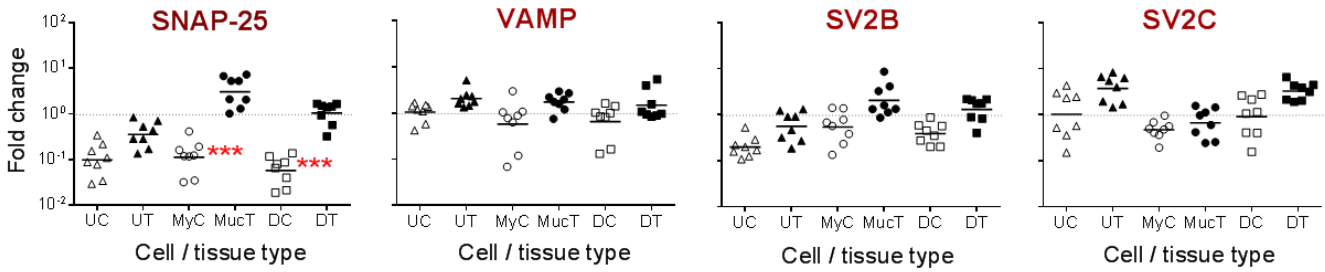

Figure 1. *SNAP-25, VAMP, SV2B and SV2C gene expression in porcine bladder (n=8) tissues (UT▲, MucT ●, DT ■) and cultured cells (UC△, MyC ○, DC □) by real-time PCR. The bar represents the mean. There was a significant decrease in SNAP-25 expression in cultured cells compared with corresponding fresh tissues (***, p<0.0001,* Kruskal-Wallis*).*

In control functional experiments, basal SP release was approximately 7 pg/mg tissue, whereas the detrusor showed a lower basal level (1.5 pg/mg tissue). EFS was effective in causing frequency-related release of SP from bladder strips (Figure 2). At each frequency, there was a significant increase in SP compared with basal (*p<0.05)*. EFS-induced SP release was 3 fold higher in mucosa compared with detrusor smooth muscle (*p<0.05)*.

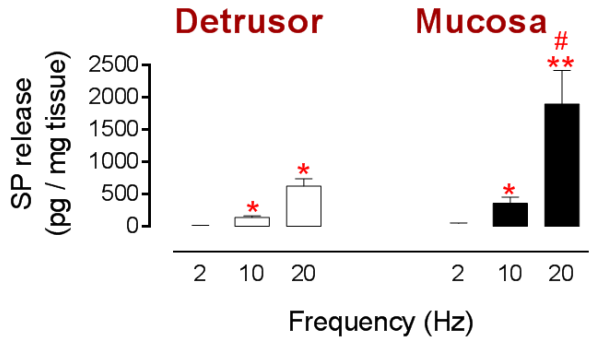

Figure 2. *Frequency-dependent increase in SP release from strips of porcine mucosa and detrusor (n=3) by EFS at 2, 10 and 20Hz (*, p<0.01). Basal release has been subtracted. Release was greater from mucosa than from detrusor (#, p<0.01,* Kruskal-Wallis).

Interpretation of results

The genes encoding SNAP-25 and VAMP are highly expressed in the porcine bladder, where their corresponding proteins are the presynaptic targets for the action of Botox A and B, respectively. The down-regulation of SNAP-25 in all cultured cells might be due to loss of expression during culture. Alternatively, in the fresh tissues SNAP-25 was originally expressed on nerve cells, which did not grow in culture. This observation is in line with the fact that SNAP-25 is presynaptically localised and involved in exocytosis. The uniformly high expression of VAMP in the cultured cells may suggest that not only nerves but other bladder cell types may participate in the action of Botox B.

SV2 proteins are the postsynaptic target receptors for Botox. Of the three SV2 isoforms, only SV2B and SV2C were present, but SV2A was undetectable. This suggests that in porcine bladder, Botox exerts its action through binding to SV2B and SV2C, a different mechanism from other species [3]. To our knowledge, this is the first report of gene expression for these proteins in porcine bladder.

In control experiments, EFS-induced SP release was higher in mucosa compared with detrusor. The cells responsible for SP release have not been identified at this time, but SP-immunoreactive fibres are present in the mucosa around blood vessels, under the urothelium as well as more sparsely in the detrusor. SP might also originate from urothelial cells.

Concluding message

The target proteins for Botox are present in porcine bladder urothelium, suburothelium and detrusor. SNAP-25 was most highly expressed in suburothelium, followed by detrusor, but was low in urothelial cells. This suggests that suburothelium may be the main effector tissue for the action of Botox in bladder. Cultured cells may be useful models for the study of botulinum toxins acting via VAMP. Bladder mucosal and detrusor strips appear suitable for the study of Botox on EFS-induced SP release.

References

1. Lucioni et al (2008) BJU Int 101: 366-70
2. Cheng Y et al (2011) Front Pharmacol 27: 1-9
3. Dong M et al (2006) Science 312: 592-96

Disclosures

Funding: NHMRC Australia **Clinical Trial:** No **Subjects:** ANIMAL **Species:** Pig **Ethics not Req'd:** No ethical approval was required for experiments performed on pig bladders obtained from abattoir.