

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

Hypothesis / aims of study

Onabotulinum neurotoxin A is a treatment for refractory overactive bladder. However, little is known about its mechanisms of action in individual bladder cell types. Our primary aim was to investigate the gene expression pattern of the SNARE proteins involved in the mechanisms of Botox [1], in three cell types from porcine bladder, and to compare expression pattern between the native tissue and the corresponding cultured cells (urothelial cells, myofibroblasts, and muscle cells). We hypothesised that Botox might reduce the release of substance P (SP) from porcine bladder, in response to by nerve stimulation. Thus, our secondary aim was to investigate SP release from mucosal and detrusor strips, prior to studying the effects of Botox in this system.

Study design, materials and methods

The genes under investigation encoded for synaptosomal-associated protein 25 (SNAP-25), vesicle associated membrane protein (VAMP) and synaptic vesicle proteins 2 (SV2: three isoforms, A, B and C). SV2 and SNAP-25 are abundant in cholinergic nerves in human bladder. SV2 is identified as the receptor for onabotulinum toxin [1], and SNAP-25 is the presynaptic target protein of the toxin. VAMP, also abundant in cholinergic nerves, is the target protein for other serotypes of the toxin including botulinum neurotoxin B [3].

The gene sequence for each protein was derived from the pig genome (published in NCBI). Primers were designed using Primer 3 software. Gene (mRNA) expression was measured by quantitative real-time polymerase chain reaction (qRT-PCR). GAPDH was used as the housekeeping gene, and the intact pig bladder tissue used as the internal calibrator. The C_t value, which is the cycle at which the fluorescent signal of the PCR reaction reaches threshold, was recorded for each gene. The authenticity of the PCR products was confirmed by the expected sizes in 2% agarose gels. The results were expressed as fold change in comparison to expression level of GAPDH. Data were analysed using the Kruskal-Wallis test followed by Dunn's multiple comparison tests. Fresh porcine bladders (female), transported on ice, were washed in Krebs, and dissected immediately. Urothelial tissue (UT) was scraped off and the remaining bladder tissue was trypsinized for 5 min at 37°C to remove the remaining urothelium, then dissected into mucosa/suburothelium (minus urothelium) (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 of urothelial (UC), suburothelial (myofibroblast) cells (MyC) and detrusor muscle cells (DC), as described [2], with cells harvested at 2 weeks. The identity of cells was confirmed by marker antibodies. The fresh tissue (-80°C) and the confluent (80%) cultured cells were homogenised in TRIZOL. Total RNA was extracted, purified (DNase treatment), and used to synthesize cDNA (Invitrogen).

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% O₂ and 5% CO₂ at 37°C). The viability and contractility of strips were examined by KCl and ACh respectively. In each group, all strips except the controls were exposed to electrical field stimulation (EFS) at 2, 10 and 20 Hz, 0.1 ms pulse width, 100 V for 20s. Aliquots of bath fluid was collected in duplicate before EFS and within 10s after onset of EFS, and snap frozen immediately at -20°C. Using a SP EIA kit (ENZO Life Science), a standard curve was prepared, and the release of peptide was estimated in each sample.

Results:

The genes for VAMP, SNAP-25, SV2B and SV2C were expressed in the three studied regions and cell types of pig bladder (Figure 1). However, no expression of SV2A was observed. In fresh tissues, the expression of SNAP-25 mainly occurred in suburothelium (MucT, fold change 3.87) and detrusor (DT, 1.18), with lower expression in the urothelium. Notably, there was a significant decrease ($p < 0.0001$) in expression of SNAP-25 in cultured myofibroblast cells and detrusor smooth muscle cells, compared with the corresponding fresh tissue. In comparison, no major differences were seen in expression pattern for VAMP, SV2B and SV2C, for both fresh tissue and cultured cells. Significant differences (Kruskal-Wallis test) were seen as follows: between UT and MucT (SNAP-25, $p < 0.05$; SV2B, $p < 0.05$, SV2C, $p < 0.001$), and between MucT and DT (SV2C, $p < 0.05$).

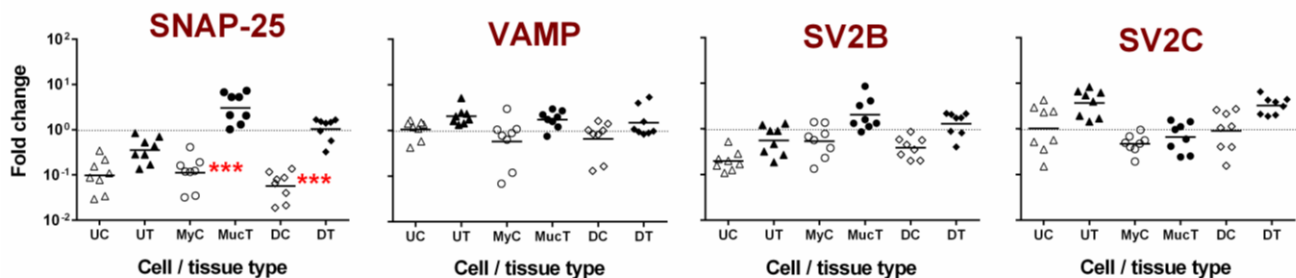


Figure 1. Expression of mRNA for SNAP-25, VAMP, SV2B and SV2C in porcine bladder ($n=8$) tissue (UT▲, MucT●, DT◆) and cultured cells (UC△, MyC○, DC◇). Data are expressed as fold change and are shown on a \log_{10} scale; the bar represents the mean. For SNAP-25, there was a significant decrease in expression in cultured cells compared with corresponding native tissue (***, $p < 0.0001$, Dunn's test).

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, Figure 2). At each frequency, EFS evoked a significant increase in SP compared with basal. EFS-induced SP release was 3 fold higher in mucosa compared with detrusor smooth muscle.

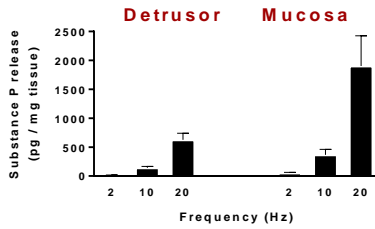


Figure 2. Frequency dependent increase in SP release in bath fluid by EFS from porcine detrusor and mucosa. Basal release has been subtracted.

Interpretation of results

Only two isoforms of SV2 (SV2B, SV2C), which are the postsynaptic target receptor for the onabotulinum neurotoxin are expressed in the bladder. The genes for SNAP-25 and VAMP are highly expressed in porcine bladder, where their corresponding proteins are the presynaptic targets for the action of onabotulinum neurotoxin type A and B respectively. To our knowledge, this is the first report of gene expression for these proteins in porcine bladder.

The continued expression of SV2 in the cell culture suggests that the neurotoxin is able to associate with and enter the cells. The down-regulation of SNAP-25 in all cultured cells, might be due to loss of expression in the culture, or that this protein was originally expressed on nerves, which did not grow in the culture. Since SNAP-25 is involved in exocytosis, this suggests that cells in culture may not be able to release any mediators. However, the uniformly high expression of VAMP in the culture may suggest that not only nerves but other bladder cell types may be involved in the action of botulinum neurotoxin type B.

In control experiments, EFS-induced SP release was higher in mucosa compared with detrusor. The cells responsible for this 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. Whether SP also might originate from the urothelium itself, as suggested in other species, is not clear.

Concluding message:

The target proteins for onabotulinum toxin A exist 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.

References

1. Coelho A et al Eur Urol 2006; 61: 1178-1184

Disclosures

Funding: University of New South Wales Departmental Funds **Clinical Trial:** No **Subjects:** NONE