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Serotonin markers show altered transcription levels in an experimental pig model of mitral regurgitation

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ABSTRACT

Serotonin (5-hydroxytryptamine, 5-HT) signalling is implicated in the pathogenesis of myxomatous mitral valve disease (MMVD) through *5-HT$_{1B}$ receptor (R)*, *5-HT$_{2A}$R* and *5-HT$_{2B}$R*-induced myxomatous pathology. Based on increased *tryptophan hydroxylase-1 (TPH-1)* and decreased *serotonin re-uptake transporter (SERT)* in MMVD-affected valves, increased valvular 5-HT synthesis and decreased clearance have been suggested. It remains unknown how haemodynamic changes associated with mitral regurgitation (MR) affect 5-HT markers in the mitral valve, myocardium and circulation. Twenty-eight pigs underwent surgically induced MR or sham-operation, resulting in three MR groups: control (CON, $n = 12$), mild MR (mMR, $n = 10$) and severe MR (sMR, $n = 6$). The gene expression levels of *5-HT$_{1B}$R*, *5-HT$_{2A}$R*, *5-HT$_{2B}$R*, *SERT* and *TPH-1* were analysed using quantitative PCR (qPCR) in the mitral valve (MV), anterior papillary muscle (AP) and left ventricle (LV). MV *5-HT$_{2B}$R* was also analysed with immunohistochemistry (IHC) in relation to histological lesions and valvular myofibroblasts. All *5-HTR* mRNAs were up-regulated in MV compared to AP and LV ($P < 0.01$). In contrast, *SERT* and *TPH-1* were up-regulated in AP and LV compared to MV ($P < 0.05$). In MV, mRNA levels were increased for *5-HT$_{2B}$R* ($P = 0.02$) and decreased for *SERT* ($P = 0.03$) in sMR vs. CON. There were no group differences in *5-HT$_{2B}$R* staining (IHC) but co-localisation was found with α-SMA-positive cells in 91% of all valves and with 33% of histological lesions. In LV, *5-HT$_{1B}$R* mRNA levels were increased in sMR vs. CON ($P = 0.01$). In conclusion, these data suggest that MR may affect mRNA expression of valvular *5-HT$_{2B}$R* and *SERT*, and left ventricular *5-HT$_{1B}$R* in some pigs.

Introduction

Myxomatous mitral valve disease (MMVD) is characterised by degenerative thickening of the mitral valve and weakening of the mitral valve apparatus, resulting in valvular prolapse and mitral regurgitation (MR) (Virmani et al., 1987; Borgarelli and Haggstrom, 2010). MR is strongly associated with disease severity (Olsen et al., 1999) and leads to altered cardiac blood flow and increased shear stress (Garcia et al., 1996). With disease progression, haemodynamic changes cause volume overload and myocardial remodelling (Buchanan, 1977; Haggstrom et al., 2004).

Serotonin (5-hydroxytryptamine, 5-HT) is implicated in the pathogenesis of MMVD through activation of valvular interstitial cells (VIC) to myofibroblasts (Oyama and Levy, 2010; Hutcheson et al., 2011; Orton et al., 2012). Human and canine myxomatous mitral valves display increased abundance of tryptophan hydroxylase 1 (TPH-1), the key synthetic enzyme of 5-HT production (Disatian et al., 2010; Orton et al., 2012). In canine MMVD, additional up-regulation of the *5-HT$_{2B}$ receptor* (R) (Oyama and Chittur, 2006; Disatian and Orton, 2009) and down-regulation of the *serotonin re-uptake transporter (SERT)*, the 5-HT clearing mechanism (Disatian and Orton, 2009; Scruggs et al., 2010), support a role for increased local 5-HT signalling in MMVD.

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Serum (Ljungvall et al., 2013) and plasma 5-HT concentrations (Cremer et al., 2014a) have been correlated with the severity of MMVD. Furthermore, 5-HT culture studies with canine VIC demonstrated dose-dependent proliferation, extracellular matrix (ECM) production and collagen deposition, responses that could be antagonised with *5-HT$_{1B}$R* and *5-HT$_{2A}$R* antagonists (Connolly et al., 2009).

Changes in shear stress due to MR may result in endothelial dysfunction (Pedersen et al., 2003; Moesgaard et al., 2012), which could influence 5-HT signalling. The endothelium has the potential to affect valvular, myocardial and circulating 5-HT (Chen and Lopez, 2005; Villalon and Centurion, 2007) via local 5-HT receptors and interaction with circulating platelets, the storage site of circulating 5-HT (Jonnakuty and Gragnoli, 2008). The degree to which MR might contribute to altered valvular 5-HT signalling has not been investigated, and 5-HT signalling in the myocardium is largely unexplored in MMVD.

In the current study, the influence of MR on valvular and myocardial gene expression of *5-HT$_{1B}$R*, *5-HT$_{2A}$R*, *5-HT$_{2B}$R*, *SERT*, *TPH-1* and serum 5-HT concentration were investigated in a surgical porcine model of induced MR (Ravn et al., 2014). The spatial location of *5-HT$_{2B}$R* was assessed by immunohistochemistry (IHC) to explore its relationship with previously described markers of MMVD severity (Cremer et al., 2014b), including valvular histopathology and myofibroblast transformation of VIC, characterised by the expression of α-smooth muscle actin (α-SMA).

Materials and methods

Experimental animals

The study was approved by the Danish Inspectorate for Animal Experimentation (reference number 2006/561–1174; date of approval 19 July 2006). The study followed the Guide for Care and Use of Laboratory Animals and the directive 2010/60/EU of the European Parliament. The animals received humane care and were euthanased if they failed to thrive or showed signs of refractory pain. Blood sampling, echocardiography and euthanasia were performed under general anaesthesia. The pigs in the current study have been part of three previous publications (Cirera et al., 2013; Cremer et al., 2014b; Ravn et al., 2014).

Forty-three female Danish Landrace/Yorkshire pigs, aged 10–12 weeks and weighing ~20 kg, were included at the start of the study. All animals were considered to be healthy on the basis of clinical examination, complete blood cell count (CBC), biochemical profile and echocardiography. The pigs were randomly divided into two groups: (1) an intervention group ($n = 30$) that underwent surgically induced MR; and (2) a sham-operated control group (n = 13) (Ravn et al., 2014). A thoracotomy was performed, the pericardium was opened and the P2 segment of the posterior leaflet of the mitral valve (MV) was ligated under ultrasound guidance. Sham-operated pigs underwent thoracotomy and opening of the pericardium. The timespan from surgery to euthanasia was 8 weeks. Based on the MR area (evaluated by echocardiography in relation to the left atrial area rounded to the nearest 5%; Pedersen et al., 1999), the pigs of the intervention group were divided into two groups: a mild MR group (mMR), with MR >10% and ≤50%; and a moderate to severe MR group (sMR), with MR >50%. A maximum of 10% MR was accepted in the control group (CON) based on the natural occurrence of MR in all pigs at the start of the study.

Table 1
Oligonucleotide primers for quantitative PCR.

Gene	Oligonucleotide sequences (5′→3′)	Amplicon length (base pairs)	Efficiency (%)
5-HT$_{1B}$R[a]	F: ACTACATTTACCAGGACTCCAT R: CAGTGACCGTGTACATGGTGC	228	85.6
5-HT$_{2A}$R	F: GTTTCCTTGTCATGCCTGTG R: GTTGAATCGCCTGTGATGG	191	95.9
5-HT$_{2B}$R	F: CTCACGAGCTACAGCATTCATC R: CCAGTGAGCCAAAGAGCATG	170	94.8
SERT	F: AGTACCACCGAAATGGATGC R: AGGAGTTCTTGCAGCTGGTC	189	96.8
TPH-1	F: TGGATCTGAACTGGATGCTG R: CGGTTCCCCAGGTCTTAATC	155	88.1
TBP[b]	F: AACAGTTCAGTAGTTATGAGCCAGA R: AGATGTTCTCAAACGCTTCG	153	93.0
ACTB[b]	F: CACGCCATCCTGCGTCTGGA R: AGCACCGTGTTGGCGTAGAG	100	97.0
RPL4[b]	F: CAAGAGTAACTACAACCTTC R: GAACTCTACGATGAATCTTC	122	97.0

5-HTR, serotonin receptor; *ACTB*, β actin; *RPL4*, ribosomal protein L4; *SERT*, serotonin re-uptake transporter; *TBP*, TATA box binding protein; *TPH-1*, tryptophan hydroxylase 1.

[a] Ullmer et al. (1995).

[b] Nygard et al. (2007).

Table 2
Group summary of mitral regurgitation (MR), immunohistochemistry of valvular *serotonin receptor 2B (5-HT$_{2B}$R)* and valvular myofibroblasts (α-SMA), and valvular histopathology, from 28 pigs subjected to surgically induced mitral regurgitation (MR) or sham operation (CON).

Group	CON	mMR	sMR
n	12	10	6
MR pre (%)[d]	5 (0–5)	5 (1.25–10)	5 (7.5–10)
MR post (%)[d]	5 (3.75–10)[b,c]	22.5 (20–30)[a,c]	95 (82.5–100)[a,b]
5-HT$_{2B}$R (%) (none/mild/moderate/heavy)	10/70/10/10	0/45/22/33	0/17/66/17
VIC activation/α-SMA (%)[d] (none/mild/moderate/heavy)	0/89/11/0[b,c]	0/12.5/75/12.5[a]	0/17/66/17[a]
Histopathology (%)[d] (none/focal/moderate/extensive)	45/55/0/0[c]	50/12.5/12.5/25	0/20/40/40[a]

The groups were: control (CON), mild MR (mMR) and moderate-severe MR (sMR). α-SMA, α-smooth muscle actin; LV, left ventricle; post, study end; pre, study start (before surgery); VIC, valvular interstitial cells.
Within each row, superscript numerals indicate that the group is statistically significantly different from CON[a], mMR[b] and sMR[c]. Non-italicised superscripts indicate $P < 0.05$.

[d] MR, α-SMA and valvular histopathology were reported in two parallel studies (Cremer et al., 2014b; Ravn et al., 2014).

Sample collection

Left-sided thoracotomy was performed and venous blood was collected from the pulmonary artery. Blood samples were handled and stored within 30 min of collection. Ethylene diamine tetraacetic acid(EDTA)-treated blood was used for CBC and serum was used for biochemical analysis and stored at –80 °C for later 5-HT enzyme immunoassay (EIA). The heart was excised surgically and samples from the myocardium and valves were harvested immediately. The anterior MV was graded macroscopically according to Whitney (1967). Samples of the posterior MV leaflet, anterior papillary muscle (AP) and left ventricle (LV) were collected and placed in RNA-later for 24 h at 4 °C, then stored at –20 °C. Samples of anterior MV leaflet, AP and LV were placed in 10% neutral-buffered formalin (pH 7.4) for up to 48 h and embedded in paraffin for histopathological and immunohistochemical analyses.

RNA isolation, cDNA synthesis, primer design and qPCR

Total RNA was isolated from individual cardiac locations (MV; anterior papillary muscle, AP; left ventricle, LV) using the RNeasy Fibrous Tissue Mini Kit (Qiagen) using approximately 50 mg of tissue for the extraction. The quantity and quality of the RNA samples were assessed with the NanoDrop 1000 (Thermo Scientific) with a 260/280 'ratio' of 1.8–2.2 considered to be acceptable. The integrity was assessed with the Experion system (Bio-Rad) using the Eukaryote Total RNA StdSens kit (Bio-Rad). RNA quality index (RQI) > 6.8 was accepted, with the exception of one sample which had an RQI of 4.7. The RNA samples were stored at –80 °C.

Total RNA was transcribed to cDNA; 1 μg total RNA was added to 1.0 μL 5× Moloney murine leukaemia virus reverse transcriptase (RT) buffer (5 μL), 10 mM (1.3 μL) deoxynucleotide triphosphates (dNTP), 2 μg/μL (0.2 μL) random hexamer primer, 0.5 μg/μL (0.4 μL) Oligo(dt) and 0.8 μL RNase inhibitor (Promega). The RT reaction was performed on a Biometra T Gradient machine at 25 °C for 10 min, 42 °C for 60 min and 95 °C for 5 min, then cooled to 4 °C. cDNA samples were diluted 1:10 and stored at –80 °C until amplification by real-time quantitative PCR (qPCR).

Primers were designed for *5-HT$_{2A}$R*, *5-HT$_{2B}$R*, *SERT* and *TPH-1* using Primer 3 software[1] and taken from Ullmer et al. (1995) for *5-HT$_{1B}$R* (Table 1). All primers were designed to span over an intron. Reference genes were *TATA box binding protein* (*TBP*), β *actin* (*ACTB*) and *ribosomal protein L4* (*RPL4*). Selection of the reference genes was based on high expression stability in porcine heart (Nygard et al., 2007). Cq, melting curve and PCR efficiency were assessed, and efficiencies of 85–110% were

[1] See: http://primer3.ut.ee/.