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The potential role of myocardial serotonin receptor 2B expression in canine dilated cardiomyopathy

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ABSTRACT

Serotonin signalling in the heart is mediated by receptor subtype 2B (5-HTR2B). A contribution of serotonin to valvular disease has been reported, but myocardial expression of 5-HTR2B and its role in canine dilated cardiomyopathy (DCM) is not known. The aim of the present study was to investigate myocardial 5-HTR2B mRNA expression in dogs with DCM and to correlate results with expression of markers for inflammation and remodelling. Myocardial samples from eight healthy dogs, four dogs with DCM, five with cardiac diseases other than DCM and six with systemic non-cardiac diseases were investigated for 5-HTR2B mRNA expression using quantitative PCR (qPCR). The results were compared to mRNA expression of selected cytokines, matrix metalloproteinases (MMP) and tissue inhibitors of matrix metalloproteinase (TIMP). Laser microdissection with subsequent qPCR and immunohistochemistry were employed to identify the cells expressing 5-HTR2B.

The myocardium of control dogs showed constitutive 5-HTR2B mRNA expression. In dogs with DCM, 5-HTR2B mRNA values were significantly greater than in all other groups, with highest levels of expression in the left ventricle and right atrium. Myocytes were identified as the source of 5-HTR2B mRNA and protein. A significant positive correlation of 5-HTR2B mRNA with expression of several cytokines, MMPs and TIMPs was observed. The findings suggest that serotonin might play a role in normal cardiac structure and function and could contribute to myocardial remodelling and functional impairment in dogs with DCM.

Introduction

Serotonin (5-hydroxytryptamine) is a monoamine neurotransmitter that is produced from tryptophan by two different tryptophan hydroxylases (TPH), namely, TPH1 and TPH2, found in enterochromaffine cells of the gastrointestinal tract and in neurons of the central nervous system, respectively (Jonnakuty and Gragnoli, 2008). Serotonin is involved in platelet function, vascular and non-vascular smooth muscle contraction, as well as cardiac function (Nebigil and Maroteaux, 2003; Jonnakuty and Gragnoli, 2008). It is rapidly removed from the circulation, via cellular uptake by the serotonin transporter (SERT) and subsequently stored in platelets or metabolised in pulmonary vascular endothelial cells and hepatocytes by monoamine oxidase (Jonnakuty and Gragnoli, 2008). However, high circulating serotonin concentrations or administration of serotonergic drugs are associated with arrhythmia and valvulopathy (Sheline et al., 1997; reviewed by Kaumann and Levy, 2006; Elangbam et al., 2008; Orton et al., 2012). Furthermore, serotonin is suspected to be involved in myxomatous valvular disease (MVD) in humans and dogs (Fitzgerald et al., 2000; Arndt et al., 2009; Oyama and Levy, 2010).

Serotonin exerts its effects through seven different receptor groups (5-HTR1–7), composed of several receptor subtypes (Elangbam et al., 2005; Kaumann and Levy, 2006), all of which are members of the G-protein-coupled receptor superfamily. In the cardiovascular system, the receptor subtypes 5-HTR1B, 2A, 2B, 4 and 7 can be found, of which the receptor subtype 5-HTR2B, which is present on endothelial cells, smooth muscle cells, fibroblasts, valvular interstitial cells and cardiomyocytes, is suspected to be involved in cardiac remodelling and development of valvulopathies (Rothman et al., 2000; Kaumann and Levy, 2006; Disatian and Orton, 2009; Oyama and Levy, 2010; Hutcheson et al., 2011).

Activation of 5-HTR2B has been found to stimulate phospholipase C and A2, both of which increase the intracellular calcium concentration. In addition, downstream signalling, involving extracellular-signal regulated kinase (ERK), mediates proliferative effects by inducing transcription of transforming growth factor (TGF)-β and other effector genes, such as matrix metalloproteinases

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(MMP) and bone morphogenic protein, which potentially contribute to the pathogenesis of mitral valve disease (Disatian and Orton, 2009; Orton et al., 2012; Lacerda et al., 2012a). Furthermore, activation of 5-HTR2B has been shown to increase expression of interleukin (IL)-1, IL-6 and tumour necrosis factor (TNF)-α in murine cardiac fibroblasts (Jaffre et al., 2004). These inflammatory cytokines are known to be elevated in the blood of humans and dogs affected with congestive heart failure (CHF) and are involved in cardiac inflammation and remodelling (Anker and von Haehling, 2004; Fonfara et al., 2011). Tensile strain has been suspected to increase both TPH and 5-HTR2B and to reduce SERT in canine septal mitral valve leaflets, which might result in local serotonin synthesis and prolonged hormone activity (Elangbam et al., 2008; Scruggs et al., 2010; Lacerda et al., 2012a,b).

The role of serotonin and its receptors in canine DCM is not fully understood. Dilated cardiomyopathy is associated with left ventricular dilatation and ventricular wall thinning, therefore increasing wall stress. This is likely to stimulate mechanoreceptors, which could cause local serotonin production and increased 5-HTR2B expression, as reported for valve leaflets exposed to tensile strain (Disatian and Orton, 2009; Lacerda et al., 2012b). This appears likely since in rats, chronic serotonin administration has been shown to increase valvular 5-HTR2B expression (Elangbam et al., 2008). In humans with heart failure, plasma serotonin concentrations are also elevated, which has led to the proposal that heart failure is mediated by 5-HTR2B (Jaffre et al., 2009; Shyu, 2009).

Through induction of IL-1, IL-6, TNF-α and TGF-β1, it is suspected that serotonin contributes to cardiomyocyte hypertrophy, increased fibrosis and ventricular stiffness, leading to reduced cardiac contractility and heart disease (Anker and von Haehling, 2004; Jaffre et al., 2004: Disatian and Orton, 2009; Jaffre et al., 2009). Increased concentrations of TGF-β1, a potent profibrotic cytokine, have been reported in canine MVD (Aupperle et al., 2008; Disatian and Orton, 2009) and CHF (Fonfara et al., 2011). We have recently shown that dogs with end-stage cardiac disease exhibit increased myocardial mRNA expression of inflammatory cytokines, TGF-β1, MMP-2 as well as tissue inhibitor of matrix metalloproteinase (TIMP)-1 and TIMP-2 (Fonfara et al., 2013a,b). Considering these findings and the characteristic pathological changes seen in canine DCM, we hypothesized that the serotonin/5-HTR2B system plays a role in the pathogenesis of canine DCM. We therefore designed a study to investigate cardiac expression of 5-HTR2B and its association with expression of IL-1, IL-6, TNF-α, TGF-β1, MMP-1, -2, -3, -13, TIMP-1 and -2, comparing healthy control dogs with other groups of dogs affected with DCM, other cardiac diseases or systemic non-cardiac disease.

Materials and methods

Animals and tissues

Eight control Beagles (group 1) were used for the quantitative PCR assay (four entire males and females; median age 2.75 years, sourced from a pharmaceutical company). Dogs were euthanased, autopsied and samples taken from the myocardium (left and right atrium [LA, RA], left and right ventricle [LV, RV]) and immersed in RNAlater (Ambion). Two Doberman Pinscher dogs (one female neutered 2 years old, one male entire 6 years old), with no gross or histological evidence of cardiac disease, served as controls for the immunohistological examination.

Details of clinical cases recruited into the study are shown in Table 1. These consist of dogs affected with DCM (group 2; $n = 4$), cardiac diseases other than DCM (group 3; $n = 5$) and dogs with systemic, non-cardiac disease (group 4; $n = 6$) (Fonfara et al., 2013a,b). All dogs with cardiac and systemic diseases were clinical cases that had undergone diagnostic investigations, according to their underlying disease and presenting clinical signs. Investigations were performed by clinical specialists in their respective fields or Board-registered residents under supervision. For cardiac cases, investigations included a cardiac work-up, comprising blood pressure measurement, electrocardiography, echocardiography and thoracic radiography, at different time points prior to death (Fonfara et al., 2013b). Diagnoses were made, based on applying standardised criteria for clinical assessment of cardiac cases. For classification of heart failure, the ABCD scheme was used (Strickland, 2008). Dogs had been euthanased at the owners' request, due to poor prognosis and/or impaired quality of life, with one exception, where a dog developed ventricular fibrillation and died (Fonfara et al., 2013b). Informed consent was obtained from owners prior to inclusion into the study and samples were anonymised by assigning identification numbers. The study was approved by the University of Bristol Committee on Research Ethics.

Hearts were removed within 1 h of death and examined for any gross pathological abnormalities. Myocardial samples (interventricular septum [IVS], RA, RV, LA, LV) were collected and stored in RNAlater at −20 °C until use. Hearts were subsequently fixed in 10% formalin for a minimum of 48 h and tissue samples from the same sites as those for RNA extraction were prepared and paraffin wax embedded, according to routine procedures for histological and immunohistological examinations. The hearts from the immunohistology control dogs were formalin-fixed and subsequently processed as for the other hearts.

Laser microdissection

A LV sample from a dog with DCM, stored in RNAlater, was embedded in OCT compound (Tissue-Tek) and frozen at −40 °C. Cryosections (8–10 μm) were prepared and placed onto specific membrane slides (Carl Zeiss), which had been incubated in dry heat at 180 °C for 4 h prior to use, in order to inactivate RNase enzymes. The slides with cryosections were air dried, stained with haematoxylin as recommended by the manufacturer (Carl Zeiss) and immediately used.

Cardiomyocytes were isolated with a Zeiss Palm microbeam laser microdissection microscope, using a 40× lens. Cutting was performed with 52–55% energy, 60–62% focus and 10% speed and collection with 65% energy and 47% focus. Three samples of approximately 100–150 cardiomyocytes were collected into a 500 μL adhesive cap tube (Carl Zeiss). After collection, tissue was lysed into 50 μL RLT buffer containing 10 μL/mL 2-mercaptoethanol (Qiagen). Samples were subsequently frozen at −20 °C for 18–22 h and RNA isolation was performed using the RNeasy Mini Kit (Qiagen).

Quantitative assessment of 5-HTR2B mRNA expression

Total RNA was extracted from myocardial samples and cDNA synthesized as reported previously (Fonfara et al., 2011). For the samples obtained by laser microdissection, 14 μL of RNase-free water were used to elute RNA from the column prior to cDNA synthesis. The forward (sense) and reverse (antisense) primer sequences for the canine housekeeping gene, GAPDH were 5′-CTGGGGCTCACTTGAAAGG-3′ and 5′-CAAACATGGGGGCATCAG-3′, respectively, and for 5-HTR2B were 5′-CCCAATGAGGCTCTGCAGTT-3′ and 5′-CTGTGATGAGAAGTGTATCTAGTAGAATGATT-3′, respectively. The reaction efficiency for the GAPDH primer pair was 100% and for 5-HTR2B was 97%. PCR was performed and analyzed according to standard protocols, with relative expression of 5-HTR2B mRNA normalized to GAPDH expression, calculated using the $2^{-\Delta\Delta Ct}$ method, as previously described (Fonfara et al., 2013a,b).

Statistical analysis

For statistical analysis of the quantitative PCR results, Minitab 16 was used. Following performance of basic descriptive statistics, 5-HTR2B mRNA values were log transformed to improve normality and the model assumptions necessary for parametric analysis. For comparison of different groups and/or cardiac regions, one-way ANOVA tests were employed. Results are displayed as means and standard deviation.

To test for a potential correlation between 5-HTR2B and cytokine, MMP or TIMP expression in the hearts of dogs with DCM, the qPCR results for IL-1, IL-6, TNF-α, TGF-β1, MMP-1, 2, -3, -13, TIMP-1 and TIMP-2, obtained from the same myocardial samples, were used (Fonfara et al., 2013a,b). Expression was examined with scatterplots and then tested with the Pearson correlation test. Statistical significance was defined as $P < 0.05$.

Histology and immunohistochemistry

For the histological examination, 3–5 μm thick sections were prepared from the paraffin wax-embedded tissue blocks and stained with haematoxylin and eosin. Immunohistochemistry for 5-HTR2B was performed on sections from the RA and the RV and/or LV using a murine anti-human 5-HTR2B antibody (clone A72-1; Becton Dickinson) employing the horseradish peroxidase method (HRP mouse kit, Dako), following antigen retrieval by incubation in citrate buffer (pH 6.0) and microwaving (Disatian and Orton, 2009). Consecutive sections were incubated with an isotype-control mouse monoclonal antibody and sections from canine stomach, tissue from a case of suppurative myocarditis and smooth muscle cells present in myocardial arteries served as positive controls.