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Myocardial hypertrophy is associated with inflammation and activation of endocannabinoid system in patients with aortic valve stenosis

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ABSTRACT

Aims: Endocannabinoids and their receptors have been associated with cardiac adaptation to injury, inflammation and fibrosis. Experimental studies suggested a role for inflammatory reaction and active remodeling in myocardial hypertrophy, but they have not been shown in human hypertrophy. We investigated the association of the endocannabinoid system with myocardial hypertrophy in patients with aortic stenosis.
Main methods: Myocardial biopsies were collected from patients with aortic stenosis (AS) and atrial myxoma as controls during surgery. Histological and molecular analysis of endocannabinoids and their receptors, inflammatory and remodeling-related cells and mediators was performed.
Key findings: Myocardial hypertrophy was confirmed with significantly higher cardiomyocyte diameter in AS than in myxoma patients, which had normal cell size. AS patients presented compensated myocardial adaptation to pressure overload. AS patients had significantly higher: concentration of endocannabinoid anandamide, expression of its degrading enzyme FAAH, and of cannabinoid receptor CB2, being predominantly located on cardiomyocytes. Cell density of macrophages and newly recruited leukocytes were higher in AS group, which together with increased expression of chemokines CCL2, CCL4 and CXCL8, and suppression of anti-inflammatory IL-10 indicates persistent inflammatory reaction. We found higher myofibroblast density and stronger tenascin C staining along with mRNA induction of tenascin C and CTGF in AS patients showing active myocardial remodeling.
Significance: Our study shows for the first time activation of the endocannabinoid system and predominant expression of its receptor CB2 on cardiomyocytes being associated with persistent inflammation and active remodeling in hypertrophic myocardium of patients with aortic stenosis.

Introduction

Aortic valve stenosis (AS) is the most common valvular and the third most common cardiovascular disease after hypertension and coronary artery disease. AS causes a left ventricular pressure overload leading to structural, functional and molecular changes in the process of myocardial hypertrophy (Hein et al., 2003). Hypertrophic growth of cardiomyocytes and fibrosis are hallmarks of myocardial hypertrophy. Untreated hypertrophy leads over a longer period of time to ventricular dysfunction, which is irreversible and associated with advanced remodeling. At this stage most patients do not profit from valve replacement therapy anymore, but rather develop heart failure. A better understanding of the mechanisms involved in myocardial adaptation and remodeling is needed for development of novel strategies aiming at recovery and prevention of heart failure.

Myocardial remodeling of hypertrophic left ventricle is a complex process including slow loss of cardiomyocytes, differentiation of myofibroblasts, and collagen deposition in replacement fibrosis (Villari et al., 1993). Experimental studies showed association of hypertrophic remodeling with inflammation and induction of cytokines (Xia et al., 2009), while this has only been postulated for patients having compensated hypertrophy. Our previous study showed association of persistent inflammation with newly recruited leukocytes and a better recovery of human hibernating myocardium after surgical revascularization (Frangogiannis et al., 2002b). We showed in an experimental model, that timely induction of proinflammatory chemokine CCL2 and subsequent inflammatory response are necessary to prevent adverse remodeling in replacement fibrosis after myocardial infarction (Dewald et al., 2005). We also found, that CCL2 has a major relevance in development of interstitial fibrosis in a murine model of non-infarcted ischemic cardiomyopathy (Dewald et al., 2003).

Endocannabinoids and their receptors have been associated with homeostasis and pathology in many organs. An experimental study

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showed that atherosclerosis development could be attenuated using 9-Δ-tetrahydrocannabinol acting on cannabinoid receptor 2 (CB2) by suppression of the inflammatory response (Steffens et al., 2005). Also, an anti-fibrotic role has been suggested for CB2 receptor in liver fibrosis, while another study provided evidence for its cardioprotective action in an *ex vivo* rat ischemia model (Lepicier et al., 2003). Furthermore cannabinoid receptor 1 (CB1) has been associated with attenuation of heart failure development in a murine model of transverse aortic constriction (Liao et al., 2011). In a recent study activation of endocannabinoids was observed in terminal heart failure patients with dilative cardiomyopathy when compared to left ventricular myocardium samples from healthy volunteers (Weis et al., 2010). Therefore, we investigated the involvement of the endocannabinoid system in hypertrophic myocardium of patients with symptomatic aortic stenosis and preserved function, where pharmacological therapy could assist recovery after valve replacement and thereby prevent development of heart failure.

Methods

Patients and perioperative data

The ethics committee of the Medical School at the University of Bonn approved the study protocol. The investigation conforms to the principles outlined in the Declaration of Helsinki. All patients gave an informed consent. Twenty-four patients undergoing aortic valve replacement surgery (AS group) included 18 conventional surgery and six transapical aortic valve implantations. Seven patients with diagnosis of left atrial myxoma were used as a control group and were collected over a two years time period. The mean age in myxoma group was lower than in AS group and the only significantly different demographic parameter between the two groups (Table 1). There was no significant difference in non-cardiac related diagnoses and lung function. The left ventricular function was comparable between the groups. Mean aortic valve gradient was 44.46 ± 12.79 mm Hg in AS group. One patient in myxoma group had tricuspid regurgitation grades I to II, while none of them showed clinical signs of right heart dysfunction. Despite no significant difference in number of patients with renal dysfunction between the groups (Table 1) we found significantly lower serum creatinine in myxoma patients; none of them required dialysis or hemofiltration preoperatively. White blood cell count was comparable between the groups. Patients with significant concomitant diseases were excluded.

Intraoperative duration of extracorporeal circulation was comparable between the groups (Table 2). We implanted biological prosthesis in 20 patients, mechanical prosthesis in three and were able to reconstruct the aortic valve by subvalvular resection in one patient. All seven myxoma patients received tumor extirpation and reconstruction of atrial septum using bovine pericardial patch. The duration of postoperative mechanical ventilation was significantly shorter in myxoma group. Postoperatively, laboratory parameters were comparable between the groups. In the AS group two patients needed hemofiltration for a short time period and two patients suffered from a stroke. None of the myxoma patients had severe complications. No mortality was observed within the 30 days postoperative period.

Tissue sampling

Briefly, myocardial tissue was excised from the septal part of the narrowed left ventricular outflow tract in AS group and divided into three samples. Three biopsies were obtained from the septum of right ventricle from myxoma patients. Two samples were designated for molecular analyses or mass spectrometry, snap frozen in liquid nitrogen immediately after excision and stored at −80 °C. The remaining sample was fixated in zinc-paraformaldehyde (Z-fix, 4%; Anatech, Battle Creek, MI, USA) for paraffin embedding and histology. In each two cases, tissue samples did not yield enough material for molecular biology or mass spectrometry, or had to be excluded because of major artifacts in histological sections in the AS; this resulted in n = 22 for each investigation in the AS group. Since separate analysis of patients with transapical valve implantation showed no difference to the conventional valve surgery we included these patients in complete analysis.

Histology

Basic histological evaluation of 5 μm sections from paraffin-embedded samples was performed using hematoxylin–eosin and picrosirius red staining. Cardiomyocyte diameter of 250 cells/slide was evaluated using planimetry software (Analysis; Olympus, Hamburg, D) on picrosirius red staining. This measurement was performed at sites with clear transverse cardiomyocyte section (90°). In order to investigate specific cell type we used following primary antibodies cross-reacting with human tissue for immunohistochemistry: MAC-2 clone 3/38 rat anti-mouse antibody for macrophages (Axxora, Lörrach, D); MAC-387 monoclonal mouse anti-human antibody for newly recruited leukocytes (NatuTec, Frankfurt, D), α-smooth muscle actin monoclonal mouse anti-mouse antibody for myofibroblasts (clone 1A4; Sigma, St. Louis, MO, USA), and tenascin C polyclonal rabbit anti-chicken antibody (Chemicon, Billerica, MA, USA). An appropriate Vectastain Elite ABC kit and diaminobenzidine were used for immunohistochemistry (Axxora). Photographic images were taken (DP70-camera, Olympus) and planimetric analysis of collagen stained area as percentage of the total myocardial area was performed

Table 1
Preoperative patient data.

	AS	Myxoma	P value
Age	72.92 ± 2.67	60.86 ± 3.07	*0.01*
Female gender (%)	14/24 (58)	4/7 (57)	0.95
BMI	26.29 ± 0.71	27.30 ± 1.89	0.63
Aortic valve stenosis (%)	24/24 (100)	0/7 (0)	*0.00*
aortic valve regurgitation (%)	13/24 (54)	0/7 (0)	*0.01*
atrial myxoma (%)	0/24 (0)	7/7 (100)	*0.00*
ejection fraction (%)	58.71 ± 2.13	64.66 ± 2.52	0.09
mild mitral valve regurgitation (%)	15/24 (63)	3/7 (43)	0.35
coronary artery disease (%)	7/24 (29)	0/7 (0)	0.10
implanted pacemaker (%)	3/24 (13)	0/7 (0)	0.32
atrial fibrillation (%)	5/24 (21)	0/7 (0)	0.19
hypertension (%)	20/24 (83)	5/7 (71)	0.48
stroke (%)	1/24 (4)	1/7 (14)	0.33
diabetes (%)	3/24 (13)	2/7 (29)	0.30
COPD (%)	3/24 (13)	0/7 (0)	0.32
renal dysfunction (%)	6/24 (25)	1/7 (14)	0.55
serum creatinine (mg/dl)	1.17 ± 0.13	0.82 ± 0.08	*0.03*
WBC count (G/l)	7.39 ± 0.45	7.93 ± 0.74	0.54

Renal dysfunction was defined with serum creatinine above the normal range of 0.6–1.3 mg/dl. Normal WBC count was 3.9–10.2 G/l. AS, aortic valve stenosis, AS—aortic stenosis, BMI—body mass index, COPD—chronic obstructive pulmonary disease, WBC—white blood cell. Significant changes between the groups are displayed in italics ($p<0.05$).

Table 2
Surgery and postoperative data.

	AS	Myxoma	P value
Bypass time (min)	64.75 ± 10.35	38.86 ± 7.66	0.06
CABG surgery (%)	3/24 (13)	0/7 (0)	
Ventilation time (h)	18.91 ± 1.54	10.86 ± 1.33	*0.001*
Postoperative arrhythmia (%)	4/24 (17)	1/7 (14)	0.88
Serum creatinine (mg/dl)	1.20 ± 0.09	1.07 ± 0.17	0.52
Troponin I (ng/ml)	1.12 ± 0.36	3.46 ± 1.35	0.14
WBC count (G/l)	9.73 ± 0.66	9.01 ± 0.70	0.46

Surgery data and laboratory data obtained 24 h postoperatively. Normal range of laboratory parameters: are: creatinine 0.6–1.3 mg/dl, troponin I <0.05 ng/ml, white blood cell count (WBC) 3.9–10.2 G/l. AS—aortic stenosis, CABG—coronary artery bypass grafting. Significant changes between the groups are displayed in italics ($p<0.05$).

using Analysis software as published before (Dewald et al., 2003). Cell density was calculated using cell count/mm^2.

Immunofluorescence double staining was used for localization analysis of CB2 on specific cells in the heart. Briefly, samples were permeabilized with 0.2% Tween-20 (AppliChem, Darmstadt, D) in PBS for 30 min and blocked with 5% donkey serum (Jackson Immunoresearch, West Grove, PA, USA) in PBS for another 30 min. Primary antibodies MAC-2, α-SMAC, troponin T (mouse anti-mouse antibody, clone 13/11; Thermo Scientific, Waltham, MA, USA) or CB2 (rabbit anti-mouse antibody; Cayman Chemicals, Ann Arbor, MI, USA) were incubated over night at 4 °C. For negative control primary CB2 antibody was incubated for 1 h at room temperature with its specific blocking peptide (Cayman) before using CB2 antibody on the samples. After washing the samples in PBS, secondary antibodies (Cy5 labelled donkey-anti-mouse antibody, DL649 labelled donkey-anti-rat antibody and/or DL549 labelled donkey-anti-rabbit antibody; all from Jackson Immunoresearch) were incubated for 1 h at room temperature. Nuclear staining was performed with Hoechst 33342 (Becton Dickinson, Heidelberg, D). Photographic images from immunofluorescence staining were taken on an inverted fluorescence microscope Axio Imager with ApoTome optical sectioning module (Zeiss, Jena, D).

Endocannabinoid measurements by LC–MS/MS

Myocardial samples from patients with aortic stenosis and atrial myxoma were processed for endocannabinoid extraction and quantification using the protocol described in detail by Schulte et al. (Schulte et al., 2012). Analyses were performed on a LC–MS/MS system (Agilent 1200 LC system; 5500 QTrap; AB SCIEX, Darmstadt, D). Concentration of endocannabinoids anandamide and 2-arachidonoyl glycerol, as well as arachidonic acid were measured and normalized to protein weight of the tissue sample.

Molecular analysis

Myocardial mRNA was isolated using standard phenole/chloroform extraction (Trizol, Invitrogen, Karlsruhe, D). First-strand cDNA was synthesized with high capacity cDNA transcription kit (Applied Biosystems, Foster City, CA, USA) using random hexameric primers as described by the manufacturer protocol. Expression of mRNA was analyzed using Taqman® real time quantitative PCR system (RT-qPCR; Applied Biosystems). RT-qPCR was performed and analyzed on an ABI Prism 7900HT Sequence Detection System and SDS2.2 Software (Applied Biosystems) with 1/10 diluted cDNA following manufacturer's instructions. Target gene-expression was normalized to an internal control and housekeeping gene GAPDH. All primers were measured using FAM TAMRA® chemistry and relative standard curve method. Dissociation curve analysis was performed in order to ascertain the amplification of a single PCR product.

Statistical analysis

Data are reported as mean ± SEM. Statistical analysis was performed using unpaired chi-square or t-test in GraphPad Prism 5.0 software. Differences with $p < 0.05$ were considered significant.

Results

Endocannabinoid system is active in human hypertrophic myocardium

Basic histological evaluation in atrial myxoma samples showed normal myocardial structure with narrow interstitial space (Fig. 1A). In contrast, patients with aortic stenosis showed prominent characteristics of myocardial hypertrophy with wide interstitial space and enlarged cardiomyocytes (Fig. 1B). This difference was confirmed in planimetric evaluation of cardiomyocyte size with significantly larger diameter in AS group than in myxoma group (Fig. 1C). Next we investigated hypertrophy related mediators and found a strongly induced level of both atrial and brain natriuretic proteins in AS when compared to the myxoma samples, as expected (Fig. 1D and E). The mRNA expression of α-myosin heavy chain (MHC) isoform was comparable between the groups (Fig. 1F). However, we found a significantly stronger induction of the β-MHC isoform in hypertrophic myocardium of AS patients (Fig. 1G) indicating changes in myocardial contractile elements towards better economy in force generation under pressure overload.

Since experimental data suggested that CB2 is associated with cardiac adaptation to injury we investigated its localization on specific cells in myocardium using immunofluorescence double staining. We found a weak expression of CB2 on cardiomyocytes in myxoma hearts (Fig. 2A) in contrast to very strong CB2 signals on cardiomyocytes in AS hearts (Fig. 2B and Supplementary material online Fig. A–D). We confirmed the specificity of this staining using negative controls and CB2-specific blocking peptide (Supplementary material online Fig. E and F). The double staining for other cells showed CB2-positive macrophages and smooth muscle cells in blood vessel wall, but no CB2-positive myofibroblasts (Supplementary material online Fig. G-I). We measured the mRNA expression of CB2 in myocardium and found a significantly higher level in AS than in myxoma group (Fig. 2C). The expression of cannabinoid receptor CB1 showed only a tendency to higher expression in AS (Fig. 2D). The major degradation enzyme for endocannabinoids, fatty acid amide hydrolase (FAAH), showed also a tendency to higher mRNA expression in the AS group suggesting a higher endocannabinoid production (Fig. 2E). Indeed, mass spectrometry measurements revealed a significantly higher concentration of endocannabinoid anandamide in myocardium of AS patients (Fig. 2F). The concentration of endocannabinoid 2-arachidonoyl glycerol (myxoma 1.81 ± 0.35 nmol/g vs. AS 1.25 ± 0.13 nmol/g, n.s.) and endocannabinoid degradation product arachidonic acid (myxoma 39.35 ± 4.69 nmol/g vs. AS 43.85 ± 2.81 nmol/g, n.s.) were comparable between the groups. These data show that the endocannabinoid system is strongly activated in hypertrophic myocardium, and its CB2 receptor is expressed on cardiomyocytes of patients with aortic stenosis.

Persistent inflammatory reaction in hypertrophic myocardium

Animal studies showed association of inflammation in myocardial hypertrophy (Kuwahara et al., 2004; Xia et al., 2009) and with activation of endocannabinoid system (Steffens et al., 2005). Therefore, we investigated the cellular and molecular involvement of inflammation and found a low macrophage infiltration of myocardium in myxoma patients (Fig. 3A). In contrast, AS patients showed a strong macrophage infiltration concentrated to the interstitial, fibrotic areas (Fig. 3B). Quantitative evaluation showed significantly higher macrophage density in AS than in the myxoma group (Fig. 3C). Macrophages have different roles during tissue remodeling after injury and may not necessarily reflect a continuous replenishment of inflammatory cells. In order to investigate the postulated persistent infiltration we stained the slides with a marker for newly recruited leucocytes (Fig. 3D and E) (Frangogiannis et al., 2002b) and found a significantly higher MAC-387 + cell density in the AS group (Fig. 3F). Next we measured expression and found a significantly higher expression of chemokines CCL2, CCL4 and CXCL8 (IL-8), indicating that chemokines maintain this persistent low-level inflammation in myocardial hypertrophy (Fig. 3G–I). The mRNA expression of chemokine CCL3 and chemokine receptors CCR2, CCR4, CCR5, CXCR1 und CXCR2 showed no difference between the groups (data not shown). We also measured expression of inflammatory mediators and found no difference in mRNA expression of proinflammatory cytokine TNF-α (data not shown) or interleukin (IL)-1β (Fig. 3J). In contrast, The expression of anti-inflammatory cytokine IL-10 was significantly down regulated in AS group (Fig. 3H), thus

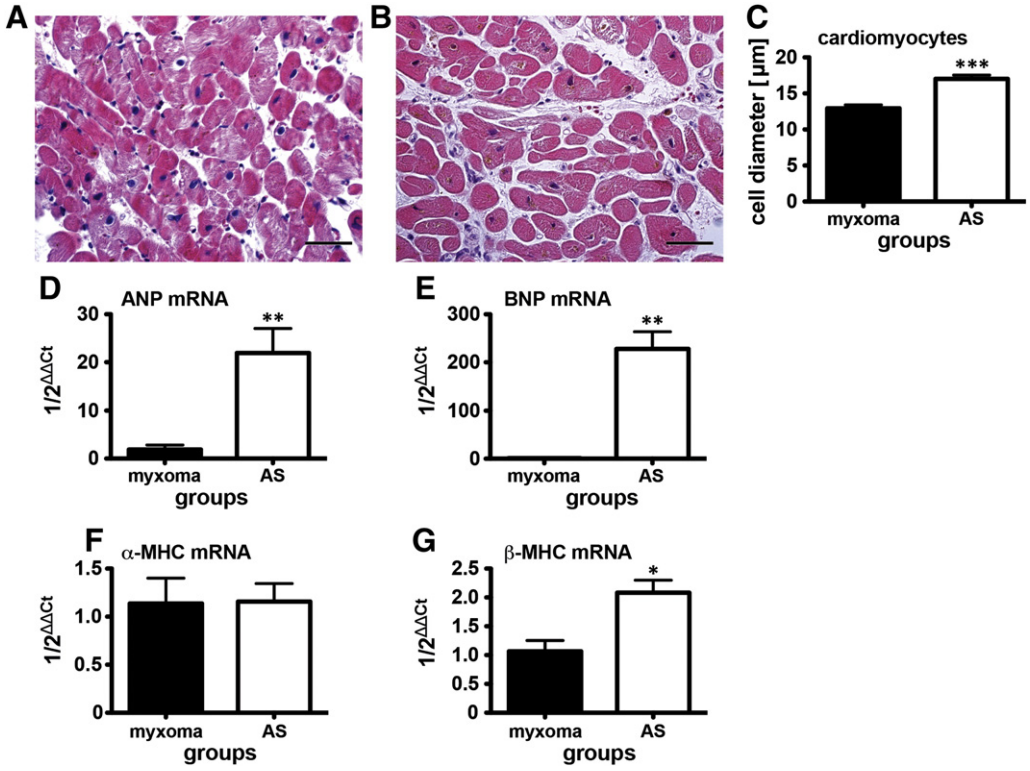

Fig. 1. Cardiomyocyte adaptation in hypertrophic myocardium. HE-staining of a representative myocardial biopsy from (*A*) a patient with left atrial myxoma shows normal myocardial architecture, while (*B*) a patient with aortic stenosis (AS) presents with myocardial hypertrophy. (*C*) Planimetric evaluation of cardiomyocyte diameter between the groups confirms a significantly higher diameter of patients with AS. mRNA expression of stress related (*D*) atrial natriuretic peptide (ANP) and (*E*) brain natriuretic peptide (BNP) in myocardium of patients with aortic stenosis (AS) and atrial myxoma was measured in RT-qPCR. Expression of myosin heavy chain (MHC) isoforms α (*F*) and β (*G*) indicated compensated myocardial adaptation to pressure overload. AS: n = 22/group; myxoma: n = 7/group. mRNA expression in RT-qPCR is related to controls and GAPDH using comparative ΔΔCt-method. *, $p < 0.05$; **, $p < 0.01$.

showing suppression of the resolution of inflammatory response and a shift in balance towards proinflammatory milieu. These findings show a persistent and predominantly chemokine-driven inflammatory reaction involving macrophages and newly recruited leukocytes in the human hypertrophic myocardium.

Active interstitial remodeling in patients with aortic stenosis

Our previous study showed that persistent macrophage infiltration is associated with an active interstitial remodeling in hibernating human myocardium (Frangogiannis et al., 2002a). Therefore, we investigated

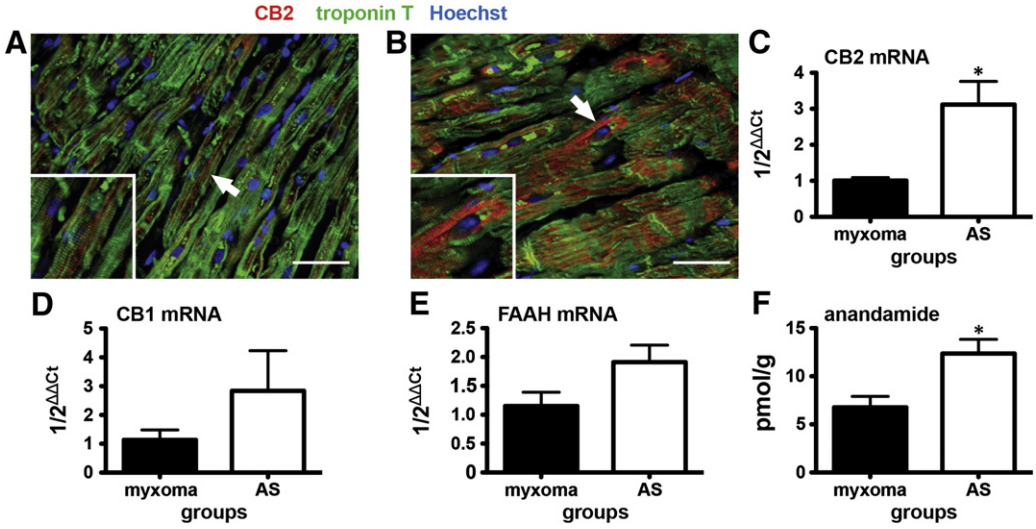

Fig. 2. Activation of endocannabinoid system in patients with aortic stenosis. Double staining with CB2 antibody (red) and troponin T (green) for cardiomyocytes (nuclei blue) reveals a very weak CB2 positive signal on cardiomyocytes in myxoma patients (*A*), whereas a strong CB2 expression was found on cardiomyocytes in AS (*B*). mRNA-expression of (*C*) CB2, (*D*) CB1 and (*E*) endocannabinoid degrading enzyme fatty acid amide hydroxylase (FAAH) in myocardium was measured with RT-qPCR. (*F*) Tissue concentration of endocannabinoid anandamide was measured using liquid chromatography mass spectometry and confirmed the activation of endocannabinoid system. Scale bar in A, B, D & E: 50 µm; for insets in D & E: 25 µm. White arrows in D & E indicate origin of insets. AS: n = 22/group; myxoma: n = 7/group. mRNA expression in RT-qPCR is related to controls and GAPDH using comparative ΔΔCt-method. *, $p < 0.05$; ***, $p < 0.001$.

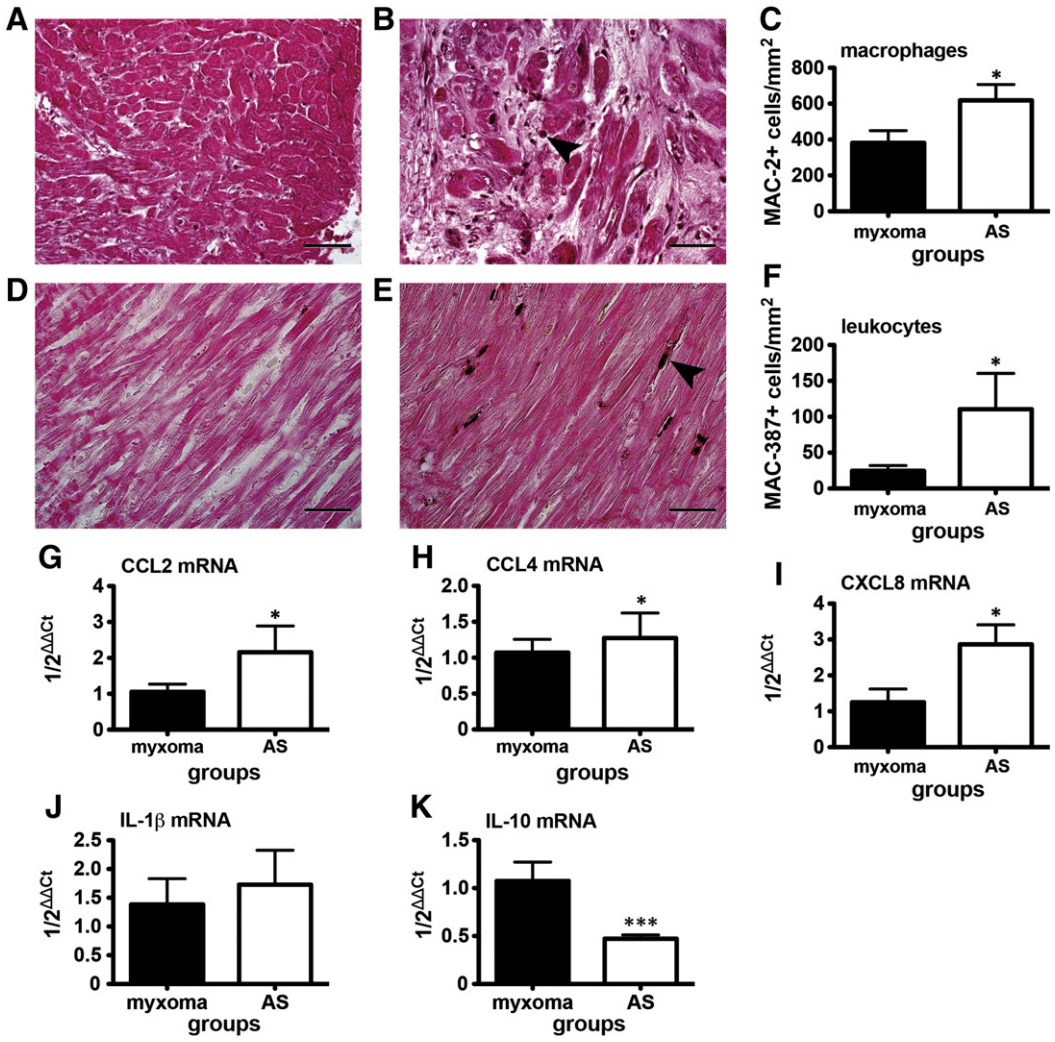

Fig. 3. Persistent inflammatory reaction in myocardial hypertrophy. Representative macrophage staining using MAC-2 antibody of myocardium from patient with (*A*) myxoma and (*B*) aortic stenosis (AS) showed increased cellularity in wide interstitial space of hypertrophic myocardium (arrow). (*C*) Quantitative analysis confirmed higher macrophage density in AS group than in myxoma patients. Representative staining of newly recruited leukocytes into myocardium using MAC-387 antibody in (*D*) myxoma and (*E*) AS myocardium revealed persistent influx of fresh inflammatory cells (arrow) in myocardial hypertrophy. (*F*) Cell count of newly recruited leukocytes showed significantly higher cell density in AS group. The mRNA expression of chemokines (G) CCL2, (H) CCL4 and (I) CXCL8, as well as (*J*) proinflammatory cytokine Il-1β and (*K*) anti-inflammatory cytokine IL-10 were measured in RT-qPCR and demonstrated proinflammatory milieu in hypertrophic myocardium. Scale bar in A, B, D & E: 50 μm. AS: n = 22/group; myxoma: n = 7/group. mRNA expression in RT-qPCR is related to controls and GAPDH using comparative ΔΔCt-method. *, $p < 0.05$; ***, $p < 0.001$.

cells and mediators involved in active myocardial remodeling. We observed only rare interstitial myofibroblasts along with expected arteriolar vessel staining in myxoma group (Fig. 4A). In contrast the AS group slides presented more interstitial myofibroblasts (Fig. 4B). Quantitative evaluation revealed a significantly higher myofibroblast density in the AS group (Fig. 4C). In parallel to these findings we observed only thin interstitial collagen fibers in myxoma patients (Fig. 4D) when compared with dense interstitial collagen deposition in AS patients, which also may be attributable to a loss of cardiomyocytes (Fig. 4E). The planimetric evaluation showed a significant difference in collagen stained area between the myxoma and the AS group (Fig. 4F). In order to investigate the associated mediators we evaluated tenascin C, an early marker of tissue remodeling, which is also expressed during embryonic development (Fluck et al., 2008). Immunohistochemistry showed only a weak tenascin C signal in myxoma patients (Fig. 4G) when compared with the strong staining in AS patients (Fig. 4H). Consistently we measured a significantly higher mRNA expression of tenascin C in AS group (Fig. 4I). Furthermore, we measured a no induction of TGF-β1 in AS group (Fig. 4J), but a strongly and significantly induced mRNA expression of collagen deposition related connective tissue growth factor (CTGF) (Fig. 4K). Taken together, these data strongly support the concept of active interstitial remodeling in the myocardium of patients with aortic stenosis.

Discussion

This study investigated activation of endocannabinoid system in hypertrophic myocardium of patients with aortic stenosis. Histology revealed that myxoma patients have normal cardiomyocyte diameter when compared with previously reported data (Mundhenke et al., 1997) and are therefore a valid control for the aortic stenosis patient group. We found a strong mRNA induction of stress related myocardial markers atrial and brain natriuretic peptide in hypertrophic hearts, which is comparable to previous studies using protein levels in the blood (Opie et al., 2006). Their low expression in patients with atrial myxoma also supports our choice of these patients as controls because they do not show morphological or functional signs of cardiac stress or dysfunction when compared to coronary artery bypass patients, which have been used as controls in another study (Heymans et al., 2005). In order to assess the compensatory adaptation to hypertrophy we further measured the mRNA expression of myosin heavy chain isoforms and found comparable levels in α-isoform between the groups, as well as

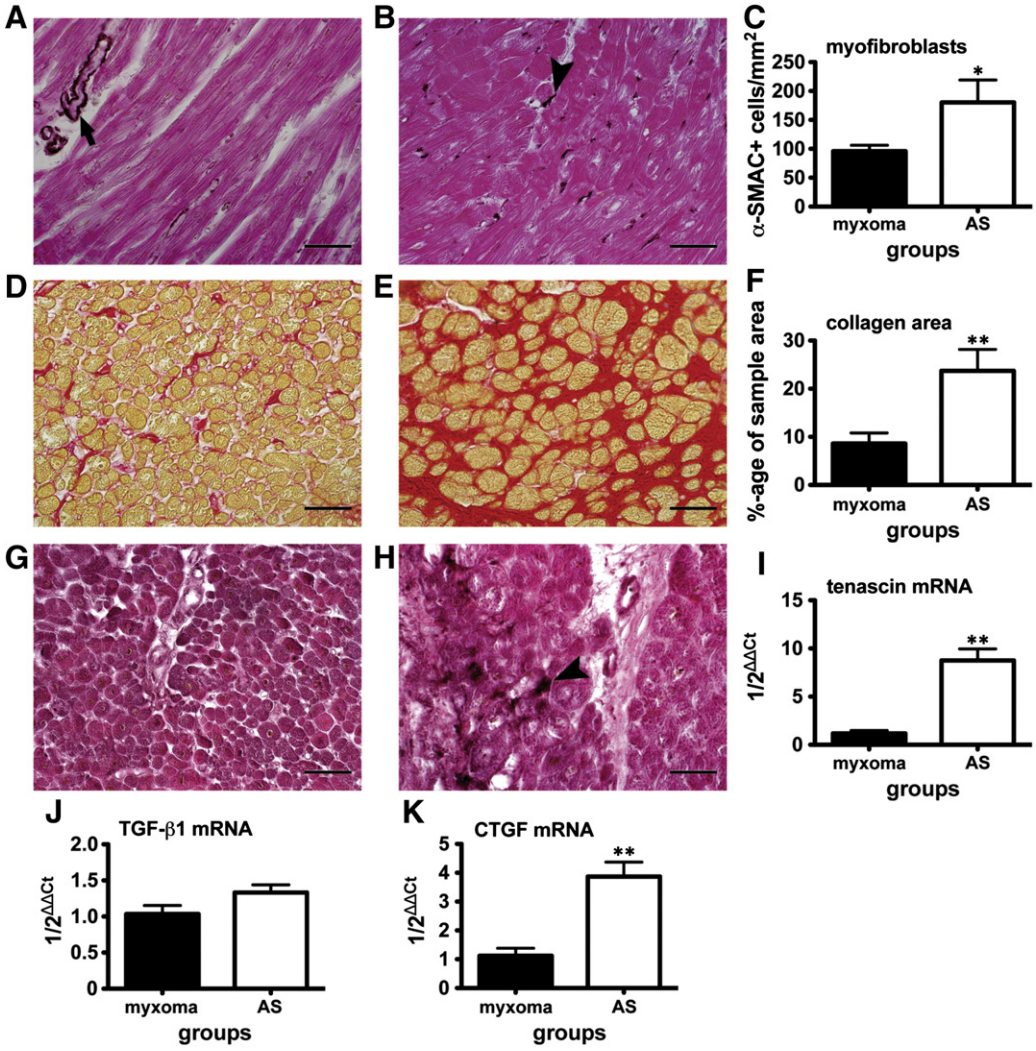

Fig. 4. Myocardial hypertrophy in patients with aortic stenosis is associated with active interstitial remodeling. Myofibroblast staining using α-smooth muscle antibody of representative myocardial biopsy from (*A*) myxoma patient shows only the positive arteriolar staining (arrow), in contrast to (*B*) numerous positive interstitial cells (arrowheads) in addition to arterioles in myocardium of aortic stenosis (AS) patient indicating active remodeling. (*C*) Myofibroblast cell count confirmed significantly higher cell density in myocardial hypertrophy of AS patient group. Collagen staining using picrosirius red in myocardium of (*D*) myxoma and (*E*) AS patient delineated higher collagen deposition and fibrosis in myocardial hypertrophy, which is also confirmed by (*F*) planimetric evaluation of this staining. Tenascin C, an early remodeling marker, is barely detectable in (*G*) myxoma patients, in contrast to (*H*) strong interstitial signals in myocardium of AS patient (arrow), which supports the hypothesized active remodeling in hypertrophy. The mRNA expression of (*I*) tenascin C, (*J*) transforming growth factor (TGF) β1 and (*K*) collagen deposition-associated connective tissue growth factor (CTGF) was measured in RT-qPCR and further supports our concept. Scale bar in A, B, D, E, G & H: 50 μm. All experiments, AS: n = 22/group; myxoma: n = 7/group. mRNA expression in RT-qPCR is related to controls and GAPDH using comparative ΔΔCt-method. *, $p < 0.05$; **, $p < 0.01$.

increased β-isoform in AS group. This pattern of myocardial contractile element expression is associated with more economical force generation (Razeghi et al., 2003), because β-MHC isoform consumes less ATP due to reduced contractile velocity. Thereby it reflects a situation of compensated hypertrophy in our patients in contrast to heart failure patients, which have decreased expression of both isoforms (Vanderheyden et al., 2008).

A recent study described involvement of endocannabinoid system in terminal heart failure patients when compared with commercially available left ventricular myocardium from healthy volunteers (Weis et al., 2010). Our study clearly shows for the first time the activation of endocannabinoid system in compensated human myocardial hypertrophy. CB2 receptor expression was much stronger in AS when compared to myxoma group. Furthermore its strongest expression was found on cardiomyocytes than on other cell types in AS group suggesting that the major part of its action and RNA expression in the heart could be on cardiomyocytes. We assume that CB2 receptor is helping the hypertrophic heart to survive pressure overload, similarly to its previously suggested cardioprotective role in experimental studies in ischemia models. Analogous to previous animal or *in vitro* studies (Steffens et al., 2005) CB2 receptor was also expressed on MAC-2 positive cells in AS hearts, predominantly representing macrophages. The induction of CB2 receptor in AS group was associated with increased concentration of anandamide and expression of its degradation enzyme FAAH. Together with increased endocannabinoid degradation product arachidonic acid the data clearly show increased activity of endocannabinoid system, when compared to myxoma controls. We found a non-significant induction of CB1 receptor, which was recently reported to be up regulated in degenerated human aortic valve cusps (Naito et al., 2010). CB1 receptor has been associated with attenuation of heart failure development in a murine pressure overload model (Liao et al., 2011), while another experimental study suggested its contribution to development of ventricular dysfunction in cirrhotic rats (Batkai et al., 2007). Nevertheless, the CB1 receptor antagonist rimonabant, which was recently withdrawn from the market, showed controversial effects on blood pressure in experimental and clinical studies, which only proved the difficulties when translating experimental data into clinical setup. The currently available data demonstrate a need for more specific CB agonists or antagonists in order to reach clinically relevant effects.

Our previous experimental study demonstrated that adaptive interstitial fibrosis is also dependent on presence of free radicals and low-level inflammatory response in a murine model of repetitive, short ischemia episodes without infarction (Dewald et al., 2003). Therefore we investigated the cellular infiltration and found significantly higher macrophage density in the AS group, suggesting a persistent proinflammatory response. This assumption was confirmed with increased density of newly recruited leukocytes in AS group, thus showing continuous replenishment of myocardium with fresh inflammatory cells. The significant upregulation of CCL2, CCL4 and CXCL8 shows a predominantly chemokine-driven, persistent inflammation assisted by suppression of anti-inflammatory IL-10 found in our patients with myocardial hypertrophy. Macrophage infiltration and chemokine induction were also shown in a murine model of transverse aortic constriction leading to myocardial hypertrophy (Xia et al., 2009). Using the same model in rats it has been shown that chemokine CCL2 is associated with fibrotic response in hypertrophy (Kuwahara et al., 2004). In contrast, that experimental data also showed adverse remodeling after myocardial infarction in CCL2-deficient mice (Dewald et al., 2005). So, it remains to be further elucidated whether CCL2 could be a potential therapeutic target in myocardial hypertrophy.

The concept of myocardial remodeling has been rather associated with infarction including an important role for macrophages and inflammatory reaction in scar formation. Recent studies showed aspects of active remodeling in myocardial hypertrophy. A study on aortic stenosis patients showed, that the progression of compensated hypertrophy towards heart failure is based on cardiomyocyte loss due to autophagy and oncosis (Hein et al., 2003). At the same time they correlated the expression of TGF-β with the degree of fibrosis in the failing human heart. Another recent study proposed serum procollagen type I propeptide as an indicator of increased myocardial collagen preceding development of hypertrophy (Ho et al., 2010). An earlier study correlated expression of tissue inhibitors of matrix metalloproteinases with fibrosis development in aortic stenosis patients while comparing them with biopsies from coronary artery bypass surgery patients (Heymans et al., 2005). The patients in our study showed compensated myocardial hypertrophy with normal ventricular function and presented with different aspects of active myocardial remodeling. Beside increased collagen we found significantly more myofibroblasts in AS group, which have been clearly associated with production of collagen and other components of extracellular matrix in active remodeling. Still, we found CB2 receptor only on α-actin positive smooth muscle cells in the vessel wall and not on myofibroblasts. It seems that CB2 does not directly modulate myofibroblasts in remodeling, but acts indirectly through regulation of inflammatory response. Active remodeling was further underlined by the higher expression of early remodeling marker tenascin C in AS group. Tenascin C has been associated with replacement fibrosis in heart failure patients (Tamura et al., 1996) and its serum level seems to reflect the severity of heart failure (Terasaki et al., 2007). Further support to our concept gave the increased expression of collagen deposition-related CTGF in AS patients. An experimental study suggested CTGF as determinant of myocardial fibrosis in a rat model of pressure overload, while showing its staining in biopsies from heart failure patients (Koitabashi et al., 2007). Another experimental study associated CTGF with development of myocardial hypertrophy after stimulation with angiotensin II (Panek et al., 2009). Interestingly, our AS patients showed no mRNA induction of TGF-β isoforms suggesting that the fibrotic turnover in our patients may not be very high. Still, this does not necessary reflect the active protein which we could not measure due to the very limited tissue sample size. TGF-β has been associated with development of myocardial hypertrophy in a murine model (Teekakirikul et al., 2010). Also TGF-β plasma levels have been reported to be elevated in aortic stenosis patients, but they reported only levels of MHC-isoforms in relation to the TGF-β due to the lack of control group (Villar et al., 2009). We made an attempt to illustrate the complex interactions of endocannabinoids and their receptors with different cells in human hypertrophic myocardium (Fig. 5).

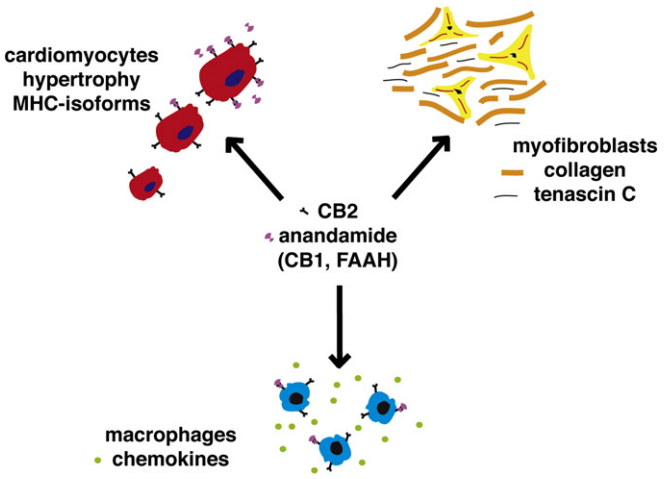

Fig. 5. Interaction of endocannabinoids and their receptors in myocardium.

One limitation of our descriptive study is the low patient number in myxoma group, which is attributable to the very low patient number per center per year. Myxoma patients may have a certain degree of myocardial stress due to the size of tumor causing dyspnea and bringing the patients to the doctor, but the lack of right ventricular dysfunction, tricuspid regurgitation or pulmonary hypertension makes this point rather speculative. Another limitation is the unknown duration of aortic stenosis-induced pressure overload before valve replacement surgery, since patients present only when symptomatic. Also, follow-up biopsies could not be retrieved because of ethical considerations.

Conclusion

Our study describes the involvement of ligands, receptors and degrading enzymes of the endocannabinoid system in human hypertrophic myocardium for the first time. We also present novel evidence for a chemokine-driven persistent inflammatory response being associated with active myocardial remodeling and tenascin C expression in patients with aortic stenosis. Since myocardial hypertrophy is associated with progressive replacement fibrosis and ventricular dysfunction leading into heart failure, the presented study and further investigations may open a window for novel therapeutic targets.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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