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Myocardial expression of hyperpolarization-activated, cyclic nucleotide-gated proteins in healthy cats and cats with hypertrophic cardiomyopathy

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Summary

In this article the myocardial expression of different hyperpolarization-activated, cyclic nucleotide-gated (HCN) isoforms in myocardial tissue from healthy control cats and cats with hypertrophic cardiomyopathy (HCM) was evaluated. Myocardial tissue samples of the left ventricle of control cats (n = 12) and cats with HCM (n = 4) were collected. Expression of feline HCN was determined by immunoblot analysis using antibodies against HCN2 and HCN4. Optical densities of HCN bands were compared among groups by use of the Mann-Whitney Rank Sum test. HCN4 was reliably detected in myocardial tissue whereas HCN2 was not. HCN4 expression was significantly increased in left ventricular (LV) myocardial samples of cats with HCM ($P = 0.036$) compared to control cats. Results indicate that myocardial HCN4 expression can be evaluated in cats by immunoblot analysis and that HCN4 expression is upregulated in LV myocardial tissue of cats with HCM. The pathophysiological importance of HCN overexpression with regard to myocyte function and altered automaticity deserves further study.

Keywords: feline, cardiology, funny current, Western blot analysis, arrhythmia

Myokardiale Exprimierung von Hyperpolarisations-aktivierten, Zyklonukleotid-regulierten Proteinen bei gesunden Katzen und Katzen mit Hypertropher Kardiomyopathie

In diesem Artikel wird die myokardiale Exprimierung von verschiedenen Isoformen von Hyperpolarisations-aktivierten, Zyklonukleotid-regulierten Proteinen (HCN) bei gesunden Katzen und Katzen mit Hypertropher Kardiomyopathie (HCM) untersucht. Hierzu wurden Muskelgewebeproben des linken Ventrikels von 12 Kontrollkatzen und 4 Katzen mit einer Hypertrophen Kardiomyopathie gesammelt. Die Höhe der HCN Exprimierung wurde mittels Immunoblot Analyse mit Antikörpern, die gegen HCN2 und HCN4 gerichtet waren, ermittelt. Die optische Dichte der HCN Bänder wurde statistisch ausgewertet und mittels Mann-Whitney Rank Sum Test verglichen. HCN4 konnte in sämtlichen Proben gefunden werden wohingegen HCN2 nur unzuverlässig nachgewiesen werden konnte. Die Exprimierung von HCN4 im Myokard des linken Ventrikels war signifikant höher bei Katzen mit HCM verglichen mit Kontrollkatzen ($P = 0.036$). Diese Resultate zeigen, dass die Exprimierung von HCN4 bei Katzen mittels Immunoblot Analyse gemessen werden kann, und dass die HCN4 Exprimierung bei Katzen mit HCM in Proben des Myokards vom linken Ventrikel erhöht ist. Weitere Studien werden benötigt, um die pathophysiologische Bedeutung der HCN-Überexprimierung in Hinsicht auf die Funktion von Herzmuskelzellen und deren Einfluss auf die veränderte Automatie zu untersuchen.

Schlüsselwörter: Katzen, Kardiologie, Funny Kanäle, Western blot, Arrhythmie

Introduction

Cardiac pacemaking is the result of the electrical activity of pacemaker cells, determined by the interplay of several ionic currents, pumps, and exchangers (Mangoni and Nargeot, 2008). Of the cellular and molecular mechanisms involved in cardiac pacemaking, the funny current (I_f) plays an important role (Mangoni and Nargeot, 2008). The molecular correlates of native funny channels are the hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels, of which four isoforms are currently known (HCN1 to 4; Biel et al., 2002; Barbuti et al., 2007). Of these isoforms, HCN4 is the most abundant and predominantly expressed in the sinoatrial node, while HCN1 and HCN2 are less expressed (Shi et al., 1999).
The density of HCN channels in the heart does change during growth (Cerbai et al., 1999; Yasui et al., 2001), due to disease (Cerbai et al., 1997; Hoppe et al., 1998; Sridhar et al., 2006; Stillitano et al., 2008), and in response to hormonal stimuli (Renaudon et al., 2000). From a clinical point of view, mislocalized expression and/or overexpression of HCN channels may predispose myocytes from failing hearts to enhanced automaticity, a condition associated with an increased risk for ventricular and potentially fatal tachyarrhythmias and thus, sudden cardiac death (Yasui et al., 2001; Xue et al., 2007). To our knowledge, cardiac expression of HCN proteins has not been previously reported in cats. Therefore, the objective of the study reported herein was to evaluate the myocardial expression of different HCN isoforms in myocardial tissue from control cats and cats with hypertrophic cardiomyopathy (HCM). We hypothesized that expression of myocardial HCN2 and HCN4 is significantly higher in ventricular myocardium of cats with HCM.

Material and Methods

This study was approved by the Animal Care and Use Committee (2008 A 0195) and the Institutional Review Board of the Department of Veterinary Clinical Sciences, College of Veterinary Medicine, The Ohio State University.

Animals

Twelve control cats and 4 cats with HCM were examined. All cats underwent a thorough echocardiographic examination prior to inclusion. Control cats were acquired from a local research laboratory undergoing observational-only studies and euthanasia. None of those cats was receiving any drugs at least 4 weeks prior to euthanasia. Cats with idiopathic left ventricular (LV) hypertrophy (end-diastolic dimension of the interventricular septum or the LV posterior wall of > 6 mm as determined by two-dimensional echocardiography) were considered affected with HCM. These client-owned cats were acquired from the Veterinary Medical Center. Hearts were examined immediately after humane euthanasia related to severe, end-stage HCM after written owner consent was obtained.

Myocardial tissue

Myocardial samples of the left ventricular mid-posterior wall (6 to 8 transmural tissue blocks of approximately 2x2x2 mm size) were harvested within 30 minutes of euthanasia from control cats and cats with HCM. Tissue from control cats and cats with HCM was acquired concurrently and thus, cell lysate proteins from LV myocardial samples of cats of both groups were analyzed simultaneously on the same nitrocellulose membranes. Optical densities of HCN bands were determined by gel analysis software, quantified, and compared side by side.

Western blot analysis

The magnitude of feline HCN expression was determined by immunoblot analysis. Myocardial samples were homogenized in ice-cold buffer containing 10mM Tris-maleate (Sigma-Aldrich, St. Louis, MO), 0.9 % NaCl (pH 6.8; Sigma-Aldrich, St. Louis, MO), and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and centrifuged (40 000 g for 30 min at 4 C). Only fresh tissue samples harvested immediately after euthanasia and processed within two hours of sampling were used. Tissue blocks were homogenized and cell lysate proteins (10 µg) were subjected to 4 % to 20 % SDS-PAGE, blotted onto nitrocellulose membranes (Bio-Rad Laboratories Headquarters, Hercules, CA) and probed with four antibodies specific for rabbit polyclonal HCN2 (Rabbit polyclonal to HCN2, Abcam Inc., Cambridge, MA and Anti-HCN2, Alomone Labs Ltd., Jerusalem, Israel) and HCN4 (Rat monoclonal to HCN4, Abcam Inc., Cambridge, MA and Anti-HCN4, Alomone Labs Ltd., Jerusalem, Israel) protein. Blots were developed with Super Signal West Pico (PIERCE Rockford, IL), scanned, and quantified in a blinded manner using gel analysis software (Image J-1.37, National Institute of Health, Bethesda, MD; Visage 2000 Blot Scanning, BioImage Systems, Jackson, M; Origin 7.0, OriginLab Corporation, Northampton, MA). For validation of the four different antibodies tested in this study, cerebral tissue (fresh and frozen) of healthy cats, where HCN2 and HCN4 is abundant (Wahl-Schott and Biel, 2009), was used as a positive control.

Statistical analysis

Statistical analysis was performed by use of commercially available software (SigmaStat, Version 3.5, SPSS Inc., Chicago, IL). Descriptive statistics were calculated from control cats and cats with HCM and data presented as median. Comparisons between groups were performed by using the unpaired Mann-Whitney Rank sum test, and significance was defined at $P < 0.05$.

Results

Of the four different antibodies evaluated, only one identifying HCN4 (anti-HCN4) revealed reliable results in both cerebral and cardiac tissue. Using this antibody, a band of ~ 150 kDa was observed for HCN4 in all samples tested (Fig. 1). The three remaining antibodies did not perform reliably in positive control (brain) samples and were therefore, considered unsuitable for inclusion. Optical densities of HCN bands revealed a 1.98-fold higher HCN4 expression in cats with HCM compared to adult control cats ($P = 0.036$; Fig. 1).

Discussion

The principal finding of this study is that HCN4 is significantly upregulated in LV myocardial tissue of cats with HCM when compared to control cats. Although it's pathophysiological importance is unknown at this time an association between HCN4 density, arrhythmogenesis, and sudden cardiac death has been speculated and thus warrants further investigation.

During conditions such as heart failure, atrial and ventricular myocytes can re-express I_f during the adult life as a consequence of electrophysiological remodeling (Hoppe et al., 1998; Cerbai et al., 1997; Sridhar et al., 2006; Stillitano et al., 2008). Previous studies in humans (Cerbai et al., 1997; Stillitano et al., 2008) and rats (Cerbai et al., 1996; Sridhar et al., 2006; Stillitano et al., 2008) revealed an increase in both expression and density of HCN in hypertrophied ventricular cardiomyocytes. The degree of hypertrophy was positively correlated with I_f density (Cerbai et al., 1996), and the magnitude of HCN and I_f upregulation was most pronounced in cardiac regions with the highest pressure load (Fernandez-Velasco et al., 2003). This indicates that the development of hypertrophy and ion channel expression are interrelated.

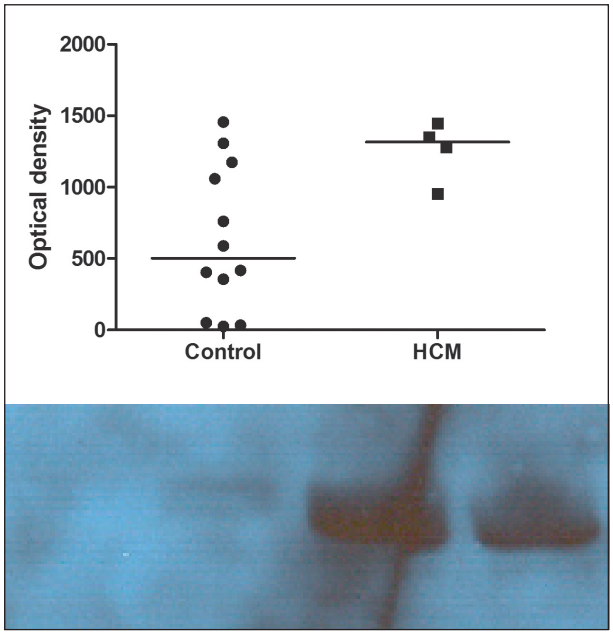

Figure 1: Myocardial expression of HCN4 in left ventricular tissue from 12 control cats and 4 cats with hypertrophic cardiomyopathy (HCM). A: Representative immunoblots of HCN4 in left ventricular myocytes from control cats (n = 2) and cats with HCM (n = 2). B: Optical densities of HCN4 of left ventricular tissue obtained from control cats (n = 12) and cats with HCM (n = 4). Results are expressed as mean and SEM. *, $P < 0.05$.

To our knowledge, expression of HCN proteins has not been reported in healthy cats or cats with HCM. Overexpression of HCN4 in LV tissue was confirmed in cats with HCM in this study. However, due to the small number of cats included no attempt was made to evaluate the effect of disease severity and sample location within the left ventricle on the magnitude of HCN expression.

We evaluated the potential use of four different antibodies specific for HCN2 or HCN4, which all have been utilized in prior studies of various species (Stevens et al., 2001; Han et al., 2002; Much et al., 2003; Fyk-Kolodziej and Pourcho, 2007). Only one antibody detecting HCN4 (anti-HCN4, Alomone Labs Ltd., Jerusalem, Israel) revealed reliable results in our study. Using this antibody, a strong ~ 150-kDa signal was detected in all myocardial and cerebral samples studied which is in agreement with previous findings (Han et al., 2002). The three other antibodies (two different HCN2 and one other HCN4 antibody) used in our study failed to consistently detect their corresponding HCN bands in positive control (brain) samples. Therefore, no conclusion with regard to HCN2 expression in feline myocardial tissue can be drawn. A possible explanation for the inability of these three antibodies to detect HCN may be related to structural differences of these proteins between species.

Overexpression of HCN channels in ventricular myocardium may predispose myocytes of failing hearts to enhanced automaticity (Xue et al., 2007), an arrhythmogenic mechanism that may be important in the pathogenesis of sudden cardiac death (Yasui et al., 2001; Xue et al., 2007). Along with reduced expression of the inwardly rectifying K + current as observed in failing hearts of rats (Sridhar et al., 2006) and humans (Hoppe et al., 1998) leading to increased diastolic excitability, the higher myocardial expression of functional HCN channels may further contribute to electrical inhomogeneity and thus, electrical instability (Xue et al., 2007). This may result in the development of fatal arrhythmias especially in presence of high sympathetic tone (Xue et al., 2007). Thus, in addition to anti-ischemic properties related to heart rate reduction, selective I_f inhibition may reduce the risk of ventricular tachyarrhythmias and/or sudden death, although the latter has not been substantiated by clinical evidence. Studies in cats with HCM are needed to validate the potential benefits of selective I_f blockade on arrhythmogenicity and outcome.

Certain limitations of the study should be emphasized. First, the number of cats included was small and thus, no attempt was made to evaluate the HCN expression in various regions of the LV (e.g., papillary muscles or LV posterior wall). We only evaluated HCN protein expression but not mRNA expression or I_f current. Therefore, no conclusions can be drawn regarding ionic conductance of the upregulated HCN4 channels in cats. The stage of cardiac disease and the severity of LV hypertrophy in cats with HCM were variable and no attempts were made to specifically relate HCN4 optical densities to disease stage or LV mass. Histological exam was not available in all cats of the present study. Therefore, the diagnosis was solely based on echocardiography. Finally, HCN proteins degrade rapidly in feline myocardial tissue. Therefore, detection is limited to fresh, un-frozen myocardium, and only cell lysates from freshly harvested samples can be used. As HCN degradation was not specifically studied, and the time between animal death and preparation of cell lysate varied between 30 minutes and two hours, mild protein degradation may have been occurred in some samples possibly affecting quantitative HCN4 analysis.

In summary, detection and quantification of myocardial HCN4 is feasible in cats. Results indicate that HCN4 expression is upregulated in ventricular myocardium in cats with HCM. Although still hypothetical, myocardial HCN4 upregulation may predispose myocytes from failing hearts to enhanced automaticity and thus may potentially increase the risk of sudden cardiac death. Further studies are needed to evaluate the association between HCN4 density and outcome in cats with HCM.

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Expression myocardique des protéines activées par hyperpolarisation et régulées par des cyclonucléotides chez des chats sains et des chats souffrant de cardiomyopathie hypertrophique

Dans cet article, on étudie l'expression myocardique de diverses iso-formes de protéines activées par hyperpolarisation et régulées par des cyclonucléotides chez des chats sains et des chats souffrant de cardiomyopathie hypertrophique (HCM). On a collecté pour cela des échantillons de muscle provenant du ventricule gauche chez 12 chats de contrôle et chez 4 chats souffrant d'une cardiomyopathie hypertrophique. Le niveau de l'expression des HCN a été mesuré par une analyse sur immunblot avec des anticorps dirigés contre HCN2 et HCN4. La densité optique des lignes HCN a été analysée statistiquement et comparée au moyen du Mann-Whitney Rank Sum Test. Des HCN4 ont été trouvés dans tous les échantillons, alors les HCN2 n'étaient mises en évidence que de façon peu fiable. L'expression des HCN4 dans le myocarde du ventricule gauche était significativement plus élevée chez les chats souffrant de HCM par rapport aux chats de contrôle ($P = 0.036$). Ces résultats montrent que l'expression de HCN 4 chez les chats peut être mesurée par analyse immunoblot et que cette expression est plus élevée dans le myocarde du ventricule gauche chez les chats souffrant de HCM. D'autres études seront nécessaires pour examiner la signification pathophysiologique de la surexpression des HCN par rapport à la fonction des cellules du myocarde et son influence sur leur automatie modifiée.

Espressione miocardica di proteine attivate in iperpolarizzazione modulata da nucleotidi ciclici in gatti sani e gatti con cardiomiopatia ipertrofica

In questo articolo, l'espressione del miocardio di diverse isoforme di proteine attivate in iperpolarizzazione modulata da nucleotidi ciclici (HCN) è stata studiata in gatti sani e gatti con cardiomiopatia ipertrofica (HCM). A questo scopo sono stati raccolti campioni di tessuto muscolare del ventricolo sinistro in 12 gatti di controllo e in 4 gatti con cardiomiopatia ipertrofica. La quantità dell'espressione di HCN è stata determinata mediante l'analisi immunoblot utilizzando anticorpi diretti contro HCN2 e HCN4. La densità ottica delle bande HCN è stata valutata statisticamente e confrontata con il test Mann-Whitney Rank Sum. L'HCN4 è stata trovata in tutti i campioni, mentre l'HCN2 solo raramente. L'espressione di HCN4 nel miocardio del ventricolo sinistro era significativamente maggiore nei gatti con HCM rispetto ai gatti di controllo ($P = 0.036$). Questi risultati dimostrano che l'espressione di HCN4 nei gatti può essere misurata mediante analisi immunoblot, e che l'espressione di HCN4 nei gatti affetti da HCM è aumentata in campioni di miocardio provenienti dal ventricolo sinistro. Ulteriori studi sono necessari per capire la fisiopatologia della sovraespressione di HCN rispetto alla funzione del muscolo cardiaco e l'influenza sulle alterazioni dell'automatismo.

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