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Progressive age-associated activation of JNK associates with conduction disruption in the aged atrium

Sandra A. Jones[a,*], Matthew K. Lancaster[b]

[a] School of Biological, Biomedical and Environmental Sciences, University of Hull, Kingston-upon-Hull, HU6 7RX, UK
[b] Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, UK

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ABSTRACT

Connexin43 (Cx43) is critical for maintaining electrical conduction across atrial muscle. During progressive ageing atrial conduction slows associating with increasing susceptibility to arrhythmias. Changes in Cx43 protein expression, or its phosphorylation status, can instigate changes in the conduction of the cardiac action potential. This study investigated whether increased levels of activated c-jun *N*-terminal kinase (JNK) is responsible for the decline of Cx43 during ageing. Right atria from guinea pigs aged between 1 day and 38 months of age were examined. The area of the intercalated disc increased with age concurrent with a 75% decline in C43 protein expression. An age-dependent increase in activated-JNK correlated with a rise in phosphorylated Cx43, but also slowing of action potential conduction velocity across the atria from 0.38 ± 0.01 m/s at 1 month of age to 0.30 ± 0.01 m/s at 38 months. The JNK activator anisomycin increased activated JNK in myocytes and reduced Cx43 protein expression simulating ageing. The JNK inhibitor SP600125, was found to eradicate almost all trace of Cx43 protein. We conclude that *in vivo* activation of JNK increases with age leading to the loss of Cx43 protein resulting in impaired conduction and contributing to the increasing risk of atrial arrhythmias with advancing age.

1. Introduction

Approximately 20% of the population are over 65 years of age, and by 2050 this is predicted to increase to over a quarter of the Western population (Cracknell, 2010). Ageing is considered to be the highest risk factor for cardiac arrhythmias. Ageing associates with progressive remodelling of heart muscle and a progressive increase in the incidence of conduction abnormalities, uncoordinated contraction and diminished myocardial function. In advanced age this is particularly evident within the right atria and sinoatrial node with an age-associated increase in sick sinus syndrome and atrial fibrillation (Centurión et al., 2005; Jones et al., 2004; Roberts-Thomson et al., 2009).

Cardiac myocytes are linked at their intercalated discs. These essentially consist of 'mechanical junctions' to bond cells together, and 'gap junctions' composed from a family of proteins known as connexins (Cx) responsible for intercellular communication and propagation of the action potential (for a fuller review see Noorman et al., 2009). Cx43 is the most abundantly expressed isoform in heart muscle with additional isoforms such as Cx40 and Cx45 occurring at comparatively low levels. Thus, Cx43 predominately forms cardiac gap junctions and its expression is critical for maintaining cardiac conduction throughout the heart, with the notable exceptions being within the His-Purkinje conducting system, sinoatrial and atrioventricular nodes (Jones et al., 2004; Nikolski et al., 2003). Even moderate changes in gap junctional conductance directly correlate with changes in cardiac conductance velocity highlighting their key role in ensuring normal conduction in the heart (Dhillon et al., 2014). Depletion of Cx43 has been shown to increase the risk of arrhythmias, and a similar change during progressive ageing may contribute to the increasing risk of arrhythmia susceptibility in old age (Danik et al., 2004).

The activation of stress-associated signalling within the heart progressively increases during ageing with a number of intracellular pathways in cardiac myocytes potentially evoked such as protein kinase A, protein kinase C and the intracellular signalling family of mitogen activated protein kinase (MAPKs) including p38, p42/44 and c-jun *N*-terminal kinase (JNK) (Powers et al., 2004; Baines and Molkentin, 2005). These pathways have been implicated in the regulation of cardiac conduction during episodes of

* Corresponding author. Tel.: +44 1482 466463.
E-mail address: s.a.jones@hull.ac.uk (S.A. Jones).

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acute and chronic stress by phosphorylation of the Cx43 protein at the C-terminus (Polontchouk et al., 2002; Remo et al., 2011).

Phosphorylation of Cx43 is complex in terms of regulation and effect. Basal phosphorylation of Cx43 in the heart ensures proper gap junction assembly and targeting to the intercalated disc with de-phosphorylation in instances such as ischemia leading to gap junction disruption (Beardslee et al., 2000). Phosphorylation effects however are complex. Enhanced phosphorylation has also been shown to have the potential to alter channel gating reducing conductance, alter selectivity, and lead to down-regulation of Cx43 expression (for review see Solan and Lampe, 2009).

The stimulation of acutely cultured single cardiac myocytes and HL1 cells *in vitro* with acute stress has been demonstrated to provoke increased levels of activated-JNK (phosphorylated JNK isoform) with a reduction in both Cx43 mRNA and protein levels, (Petrich et al., 2002; Ogawa et al., 2004; Yan et al., 2013). In contrast though other studies also using acute stress simulation in culture to provoke activated-JNK have shown enhanced Cx43 expression (Shyu et al., 2004; Salameh et al., 2009). Thus, the manipulation of JNK and the ensuing effect on Cx43 protein expression within myocytes remains controversial.

Studies of transgenic mice *in vivo* using the cre-LoxP-mediated system to express MKK7D (a specific activator of JNK under the control of the murine α-MHC promoter) demonstrated that cardiac specific over-expression of activated-JNK resulted in a 90% reduction of Cx43 protein expression compared with their control littermates (Petrich et al., 2004). This was accompanied by the functional affect of a 40% reduction in action potential conduction velocity and sudden death of the transgenic mice at 6–8 weeks highlighting the potential arrhythmogenic consequences. Furthermore, using cardiac myocytes from MKK7D over-expressing mice the activity of JNK was inhibited *in vitro* and shown to attenuate Cx43 protein expression (Petrich et al., 2002). Thus, the transgenic over-expression mouse model illustrates JNK acts as an important mediator in the regulation of Cx43 expression. This is further supported by the recent finding that in rabbits activation of JNK with anisomycin lead to a 34% decline in Cx43 associating with a 50% increase in activated JNK within the left atria. This was accompanied by an increased susceptibility to atrial arrhythmias (Yan et al., 2013).

Cx43 protein has previously been identified as a molecular correlate inversely linked to susceptibility of atrial arrhythmias (Kostin et al., 2002). Atrial arrhythmias, in particular atrial fibrillation, show increasing prevalence with age and are present at a high incidence within the elderly population (Kostin et al., 2002; Brembilla-Perrot, 2003). Previous studies illustrate the relationship of the protein levels of activated-JNK to Cx43, along with Cx43 phosphorylation status, indicating their potential significance in the mechanistic underpinnings of a higher risk for atrial arrhythmias in old age. Therefore our hypothesis was that progressive activation of JNK signalling and subsequent modification of Cx43 contributes to the "aged-heart" phenotype; a phenotype predisposed to atrial arrhythmias.

2. Materials and methods

2.1. Animal model

Healthy female tricolor guinea pigs were obtained at 1 day (0.03 month), 1 month, 18 months, 26 months and 38 months of age ($n = 5$ per age group). This age range covers the previously determined life expectancy of this in-bred guinea pig strain (Jones et al., 2004). All animal procedures were performed in accordance with the United Kingdom Animals (scientific procedures) Act 1986 and reviewed by the University of Hull and Leeds Biomedical Sciences ethics committees. Animals were humanely sacrificed by intravenous administration of an overdose of pentobarbital followed by removal of the heart and isolation of the right atrium.

2.2. Extracellular electrode recording

The intact right atria was pinned endocardial surface uppermost in bicarbonate buffered Tyrode's solution, maintained at 37 °C, and continued to generate spontaneous impulses (Jones et al., 2004). Using two extracellular modified bipolar electrodes, one as a stationary reference electrode and the second as a moving electrode, the local action potential activation time across the muscle was measured as previously described (Yamamoto et al., 1998). For each animal examined, action potential conduction was determined in the main direction of propagation in the atrial muscle – perpendicular to the crista terminalis.

2.3. Immunofluorescence

Single cardiac myocytes were isolated from right atrial tissue by enzymatic digestion (Lancaster et al., 2004). Cells or 10 μm frozen sections were subjected to immunolabelling as previously described (Jones et al., 2002). Details of the primary antibodies utilised are listed in Table 1. FITC-conjugated anti-rabbit or anti-mouse antibodies, as appropriate for the primary antibody (Dako, Denmark) were used to fluorescently tag the primary antibody. Following immunolabelling, myocytes were incubated for 2 h with wheat germ agglutinin (WGA) conjugated to rhodamine, a lectin that binds to *N*-acetylglucosamine within membranes (Vector, Burlingame, USA) to permit visualisation of membrane morphology.

To prevent photo-bleaching, myocytes were mounted in vectorshield (Vector, USA) and all slides were stored in the dark at 4 °C prior to examination by laser scanning confocal microscopy (Zeiss, Hertfordshire, UK). All fluorescent images were individually collected for each optical slice, using the same microscope and settings for the lasers and detector, from superimposing 6–10 optical slices taken at ≤1 μm intervals at each wavelength. Images of each intercalated disc were analysed by measuring their axes and area using the confocal software (LSM, Zeiss). Analysis of images such as density of label were performed using ImageJ v2.11x (NIH, USA).

Table 1
The antibodies used in this study with concentrations used and their source companies.

Antibody	Source company	Host	Immunofluorescence μg/ml	W-blot μg/ml
Cx40	Chemicon, Hampshire, UK	Rabbit	20	10
Cx45	Chemicon, Hampshire, UK	Mouse	20	10
Total endogenous Cx43	Cell signalling Tech., Hertfordshire, UK	Rabbit	1	0.1
Phosphorylated Cx43	Cell signalling Tech., Hertfordshire, UK	Rabbit	–	1
Non-phosphorylated JNK	Cell signalling Tech., Hertfordshire, UK	Rabbit	–	1
Phosphorylated JNK	Cell signalling Tech., Hertfordshire, UK	Rabbit	–	1
Desmin	Dako, Denmark	Mouse	–	6