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Elevated InsP$_3$R expression underlies enhanced calcium fluxes and spontaneous extra-systolic calcium release events in hypertrophic cardiac myocytes

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Cardiac hypertrophy is associated with profound remodeling of Ca$^{2+}$ signaling pathways. During the early, compensated stages of hypertrophy, Ca$^{2+}$ fluxes may be enhanced to facilitate greater contraction, whereas as the hypertrophic heart decompensates, Ca$^{2+}$ homeostatic mechanisms are dysregulated leading to decreased contractility, arrhythmia and death. Although ryanodine receptor Ca$^{2+}$ release channels (RyR) on the sarcoplasmic reticulum (SR) intracellular Ca$^{2+}$ store are primarily responsible for the Ca$^{2+}$ flux that induces myocyte contraction, a role for Ca$^{2+}$ release via the inositol 1,4,5-trisphosphate receptor (InsP$_3$R) in cardiac physiology has also emerged. Specifically, InsP$_3$-induced Ca$^{2+}$ signals generated following myocyte stimulation with an InsP$_3$-generating agonist (e.g., endothelin, ET-1), lead to modulation of Ca$^{2+}$ signals associated with excitation-contraction coupling (ECC) and the induction of spontaneous Ca$^{2+}$ release events that cause cellular arrhythmia. Using myocytes from spontaneously hypertensive rats (SHR), we recently reported that expression of the type 2 InsP$_3$R (InsP$_3$R2) is significantly increased during hypertrophy. Notably, this increased expression was restricted to the junctional SR in close proximity to RyRs. There, enhanced Ca$^{2+}$ release via InsP$_3$R$_2$ serves to sensitize neighboring RyRs causing an augmentation of Ca$^{2+}$ fluxes during ECC as well as an increase in non-triggered Ca$^{2+}$ release events.

Although the sensitization of RyRs may be a beneficial consequence of elevated InsP$_3$R expression during hypertrophy, the spontaneous Ca$^{2+}$ release events are potentially of pathological significance giving rise to cardiac arrhythmia. InsP$_3$R2 expression was also increased in hypertrophic hearts from patients with ischemic dilated cardiomyopathy and aortically-banded mice demonstrating that increased InsP$_3$R expression may be a general phenomenon that underlies Ca$^{2+}$ changes during hypertrophy.

Ca$^{2+}$ is key to cardiac function—stimulating myocyte contraction via...
excitation-contraction coupling (ECC)\(^5\) and controlling the transcription of genes that underlies the induction of hypertrophy.\(^6,7\) ECC is initiated by an action potential (AP) originating at the sino-atrial node. Coordinated conduction of this action potential through the heart results in a synchronized contraction of the heart chambers. The propagating action potential depolarizes the myocyte plasma membrane thereby opening voltage-sensitive L-type Ca\(^{2+}\) channels and allowing Ca\(^{2+}\) to enter the cell. This ‘trigger’ Ca\(^{2+}\), termed a sparklet,\(^3\) induces opening of ryanodine receptor (RyR) Ca\(^{2+}\) release channels on the juxtaposed sarcoplasmic reticulum (SR) intracellular Ca\(^{2+}\) store—a process called Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR).\(^9\) CICR causes a global elevation in Ca\(^{2+}\), which leads to myocyte contraction. Relaxation is then brought about by extrusion of Ca\(^{2+}\) across the sarcolemma by the Na\(^+/Ca\(^{2+}\) exchanger (NCX) and by its sequestration into the SR by the SERCA2 Ca\(^{2+}\) ATPase.

In addition to RyRs, cardiomyocytes also express inositol 1,4,5-triphosphate receptor Ca\(^{2+}\) release channels (InsP\(_3\)Rs), albeit outnumbered by RyRs 50:1.\(^10,12\) Since InsP\(_3\)Rs have been discovered in the heart, it has been questioned how the relatively small amount of Ca\(^{2+}\) that is released via InsP\(_3\) can have any physiological function in the midst of the large amount of Ca\(^{2+}\) that is cycled through RyRs during every heart beat. In mammals, there are three InsP\(_3\)R isoforms (types 1, 2 and 3),\(^13,14\) with type 2 being the main isoform in the heart.\(^10,12\) Ca\(^{2+}\) release through InsP\(_3\)Rs has been shown to mediate inotropy and arrhythmogenic Ca\(^{2+}\) release events as well as contribute to the hypertrophic effect of G\(_\text{q}\)-coupled agonists such as the vasoactive peptide endothelin-1 (ET-1).\(^15-22\)

Profound remodeling of the Ca\(^{2+}\) signaling apparatus has been reported to occur as a result of cardiac hypertrophy and failure.\(^23\) Whilst enhancement of Ca\(^{2+}\) transients facilitates greater myocyte contraction during adaptive hypertrophy, Ca\(^{2+}\) fluxes are diminished during heart failure resulting in decreased contractility and thus, cardiac output.\(^24\) Remodeling of the activity and function of the Ca\(^{2+}\) signaling proteome also underlies the increased arrhythmias associated with hypertrophy and heart failure.\(^25\) Whether altered InsP\(_3\)R signaling also contributes to remodeling of Ca\(^{2+}\) homeostasis during cardiac hypertrophy is not yet determined.

In a recent study,\(^26\) we established that augmented expression and Ca\(^{2+}\) release through InsP\(_3\)Rs contribute to enhanced Ca\(^{2+}\) fluxes and increased spontaneous arrhythmogenic Ca\(^{2+}\) events associated with hypertrophy. As a model to study hypertrophy-dependent changes in Ca\(^{2+}\) signaling, we used hypertrophic ventricular cardiomyocytes from spontaneously hypertensive rats (SHR) and non-hypertrophic ventricular cardiomyocytes from Wistar Kyoto rats (WKY) as controls.\(^26\) We found that in hypertrophic myocytes InsP\(_3\)R2 expression was specifically increased in the junctional SR membrane, thereby increasing the numbers of InsP\(_3\)Rs in close proximity with type 2 RyRs (RyR2), the expression of which was unchanged as a result of hypertrophy. Enhanced Ca\(^{2+}\) release through these InsP\(_3\)Rs sensitized their neighboring RyRs, thereby enhancing CICR leading to bigger Ca\(^{2+}\) transients and greater myocyte contraction. Moreover, the sensitization of RyR opening through increased InsP\(_3\)-induced Ca\(^{2+}\) release (IICR) resulted in a higher propensity for the generation of elementary Ca\(^{2+}\) release events and extracellular Ca\(^{2+}\) transients, which could act as a substrate for cardiac arrhythmia in vivo. Interestingly, we could not only show that InsP\(_3\)R2 expression was increased in hypertrophic rat cardiomyocytes but also in cardiomyocytes isolated from aortically-banded mice and in human hearts displaying ischemic dilated cardiomyopathy. We showed by immunoblotting and, as presented here, by immunohistochemistry that InsP\(_3\)R2 expression is increased in diseased human hearts compared to control hearts (Fig. 1). These micrographs also illustrate that InsP\(_3\)R2 expression is restricted to myocytes and is not present in non-myocyte cells, for example surrounding the vessels.

An increase in InsP\(_3\)R2 expression therefore seems to be a general phenomenon occurring during cardiac disease. This is surprising considering the lack of consensus regarding the different effects of hypertrophy on Ca\(^{2+}\) signaling.\(^23\) Although amplification of Ca\(^{2+}\) cycling is considered as a general mechanism for increasing cardiac output during hypertrophy, myocytes isolated from certain hypertrophy models exhibit either no change, or even suppression, in their ECC-mediated Ca\(^{2+}\) fluxes.\(^27,28\) As such, the mechanisms underlying remodeling of Ca\(^{2+}\) cycling during hypertrophy are not fully understood. In hypertrophic failing myocytes, Ca\(^{2+}\) cycling is suppressed causing decreased contractility. In this condition, defects in coupling between L-type channels and RyRs due to a breakdown in the t-tubular network,\(^27\) decreases in SERCA-mediated Ca\(^{2+}\) sequestration into the SR lumen,\(^29\) and increased Ca\(^{2+}\) leak from the SR via hyper-phosphorylated RyRs\(^30\) have all been shown to be involved in pathology.

The reason for this array of Ca\(^{2+}\) signaling phenotypes during hypertrophy may lie in the panoply of hypertrophy models employed. For example, hypertrophy is induced by haemodynamic loading (such as aortic banding), interruption of myocardial blood supply (infarction) and interfering of heart rate (pacing or AV block).\(^23,31\) More recently, genetic models of hypertrophy have also been employed.\(^32\) Even more variability between studies is introduced by the hypertrophic stage at which Ca\(^{2+}\) signaling measurements are made and the location of the myocardium from which myocytes are isolated.\(^33\) The SHR model offers considerable advantages. The temporal development of hypertrophy in SHRs has been well characterized and develops slowly between the age of 2 and 6 months subsequent to the onset of systemic hypertension.\(^34\) After the development of hypertrophy, there is also a long latency before the development of heart failure at 20–24 months.\(^35\) This contrasts with surgically-induced models where hypertrophy develops 1–2 weeks post surgery.\(^31\) However, our results show that the increase in InsP\(_3\)R expression seems to be independent of how hypertrophy has been induced.\(^36\)

Considering the pro-arrhythmic effect of increased InsP\(_3\)R expression, which is clearly evident in our study, this raises the question of why InsP\(_3\)R expression is enhanced in hypertrophic and failing cardiomyocytes? During hypertrophy a decrease in the coupling efficiency
between plasma membrane L-type channels and RyR channels due to an increase in the width of the dyadic cleft has been observed. This deficit in ECC did not significantly affect contractile function and was overcome by beta-adrenergic stimulation. Here, sensitization of RyR opening due to increased Ca\(^{2+}\) release through InsP\(_3\)Rs could compensate for the lack of coupling efficiency, even under basal conditions.

What remains to be determined is how InsP\(_3\)R expression during hypertrophy is regulated and how the striking junctional pattern of InsP\(_3\)R2 expression is achieved. Our data would suggest that InsP\(_3\)R expression increases concomitant with or is a product of hypertrophy and is not causal. In support of this conclusion, non-hypertrophic hearts from four month old SHRs do not exhibit increased InsP\(_3\)R expression compared to WKY controls. Moreover, hypertrophy is induced in vitro in an InsP\(_3\)-dependent manner within 24 hours, a time period that would not allow for elevated InsP\(_3\)R expression to have effect. Thus, it is likely that the role of increased InsP\(_3\)Rs detected during hypertrophy is to modify calcium signals, and possibly also act to sustain the hypertrophic phenotype. The subcellular localization of InsP\(_3\)R2 expression could be mediated via interactions with accessory proteins such as Ankyrin B, which has been shown to localize InsP3Rs. Whether the mechanisms that mediate a preferential increase in InsP\(_3\)Rs in the junctional zones and not around the nucleus during hypertrophy play any role in restricting the recently described pro-hypertrophic action of InsP\(_3\)-induced calcium release (IICR) in the nucleus also remains to be determined.

A dramatic consequence of hypertrophy for Ca\(^{2+}\) signaling observed in our

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**Figure 1.** Expression of InsP\(_3\)R2 is increased in cardiomyocytes from human patients suffering from ischemic dilated cardiomyopathy. Immunohistochemical detection of InsP\(_3\)R2 (brown) in cardiomyocytes from control patients (A) and in cardiomyocytes from patients showing ischemic dilated cardiomyopathy (B). Higher magnification images for (A and B) are presented in (C and D) respectively. Control staining of hypertrophic myocytes without using InsP\(_3\)R2 primary antibody are presented at low and high magnification in (E and F) respectively. These data are representative of images taken from slides prepared from two control and two diseased hearts. Non-myocyte cells surrounding a blood vessel (indicated by the black arrow) do not stain for InsP\(_3\)R2. The size of the bar is indicated in each image. The nuclei are indicated in blue.
study was the increased occurrence of extra-systolic Ca\(^{2+}\) transients following ET-1 or InsP\(_3\) ester (cell permeant InsP\(_3\)) application. The relatively low incidence of InsP\(_3\)-stimulated Ca\(^{2+}\) release events in WKY myocytes is consistent with our previous work in Wistar rat ventricular myocytes.\(^{10,20-22}\) The enhanced incidence of extra-systolic Ca\(^{2+}\) transients in hypertrophic ventricular myocytes is reminiscent of previous findings in atrial myocytes, which express ~6-fold more InsP\(_3\)Rs under normal conditions.\(^{17,20,22,36}\) Due to their higher basal expression of InsP\(_3\)Rs, stimulation of atrial myocytes with InsP\(_3\) or InsP\(_3\)-generating agonists, such as ET-1, potently induce extra-systolic Ca\(^{2+}\) release events.\(^{20}\)

Although insignificant by themselves, these miniature Ca\(^{2+}\) release events can provide sufficient Ca\(^{2+}\) to catalyze further Ca\(^{2+}\) release through CICR and cause AP-independent increases in [Ca\(^{2+}\)]\(_i\) that could potentially result in arrhythmia. The role of Ca\(^{2+}\) release through InsP\(_3\)Rs has been substantiated in InsP\(_3\)R2-deficient mice, in which ET-1- and InsP\(_3\)-stimulated arrhythmias in atrial myocytes were completely abolished.\(^{17}\) Interestingly, InsP\(_3\)Rs in atrial myocytes are located on the SR underlying the sarcolemma—the functional equivalent of the junctional zone created by the juxtaposition of the SR terminal cisternae and the t-tubular membrane in ventricular myocytes. Thus, in hypertrophic ventricular myocytes, ECC may mirror that observed in atrial myocytes.

As well as being manifest as spontaneous Ca\(^{2+}\) release events and extra systolic Ca\(^{2+}\) transients, increased InsP\(_3\)R expression resulted in a greater rise in diastolic [Ca\(^{2+}\)]\(_i\) in hypertrophic myocytes following ET-1 or IICR stimulation.\(^{15}\) As a result, diastolic [Ca\(^{2+}\)]\(_i\) was brought closer to the threshold for activation of RyRs, thereby increasing the incidence of stochastic Ca\(^{2+}\) release events and accelerating the generation of pacing-evoked Ca\(^{2+}\) transients. These findings are consistent with data from InsP\(_3\)R2-deficient atrial myocytes which exhibited a decrease in the ET-1-evoked elevation in diastolic [Ca\(^{2+}\)]\(_i\).\(^{17}\)

The arrhythmogenic effect of InsP\(_3\)-stimulated Ca\(^{2+}\) release events in the ventricles is potentially more catastrophic than in the atria. Whereas atrial fibrillation only has potentially serious consequences over the long term, ventricular fibrillation is potentially immediately life-threatening. This situation is further exacerbated by increases in the expression of additional components of the pathway that leads to InsP\(_3\) generation. Specifically, levels of cardiac ET-1 and also that of its receptor (ET-R), are elevated during development of hypertension and hypertrophy (Fig. 2).\(^{38,39}\)

Moreover, phospholipase C beta1 levels have also been recently reported to be increased during atrial hypertrophy. Together, enhanced activity of this signaling pathway as a result of hypertrophy would promote greater Ca\(^{2+}\) release through InsP\(_3\)Rs, and heighten the susceptibility for arrhythmias, even under non-stressed conditions.

Our recent study\(^{15}\) presents a plausible mechanism why hypertrophic hearts are more prone to cardiac arrhythmia and proposes InsP\(_3\)R2 as a possible target for the development of new drugs against hypertrophy-related arrhythmia.

**Materials and Methods**

**Animal models.** Male SHRs and normotensive Wistar-Kyoto (WKY) rats were obtained from Harlan, UK, and were housed under control conditions with ad libitum food and water. All experiments were performed in accordance with the guidelines from the code of practice for humane killing under Schedule 1 of the Animals (Scientific Procedures) Act 1986.

**Patients.** Left ventricular tissue samples of human failing hearts were from individuals undergoing heart transplantation due to end-stage heart failure. All samples were obtained from male caucasians, aged 41-62. Samples from non-failing donor hearts were provided by the “UK Human Tissue Bank.” After cardiectomy, left ventricular samples were frozen in liquid nitrogen and stored at -80°C. Detailed information about the patients is included in Harzheim et al. 2009. All experiments involving human tissue samples have been approved by the Cambridgeshire Research Ethics Committee.

**Immunohistochemistry.** Immunohistochemistry was performed as previously described.\(^{41}\) Paraffin tissue sections were prepared from non-diseased human hearts and from hearts with ischemic dilated cardiomyopathy (tissue source described above). Sections were deparaffinized and antigen retrieved using basic citrate buffer, after which slides were washed with PBS and blocked with 0.3% H\(_2\)O\(_2\)/PBS for 10 mins. After a further wash in PBS, slides were incubated with 5% BSA/PBS for 1 hour at room temp. Slides were then incubated overnight at 4°C with polyclonal rat anti InsP\(_3\)R2 antibody\(^{15}\) diluted at 1:50. As a result, diastolic [Ca\(^{2+}\)]\(_i\) was brought closer to the threshold for activation of RyRs, thereby increasing the incidence of stochastic Ca\(^{2+}\) release events and accelerating the generation of pacing-evoked Ca\(^{2+}\) transients. These findings are consistent with data from InsP\(_3\)R2-deficient atrial myocytes which exhibited a decrease in the ET-1-evoked elevation in diastolic [Ca\(^{2+}\)]\(_i\).\(^{17}\)
room temp after which they were washed, visualized with DAB, counterstained with Harris' haematoxylin and mounted.

Immunoblot detection of ET₄R. Protein expression was detected as previously described. In brief, proteins were extracted from ventricular myocytes from WKY rats and SHR in lysis buffer (10 mM Tris-HCl, pH 7.6, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, protease and phosphatase inhibitor cocktail (Sigma)). Protein extracts (25 μg) were then subjected to SDS-PAGE (10% Bis-Tris NuPage, Invitrogen) and subsequent immunoblot analysis with antibodies directed against the ET₄R (Alomone labs) and alpha-actinin (Sigma). Immunocomplexes were detected by enhanced chemiluminescence with anti-mouse or anti-rabbit IgG coupled to horseradish peroxidase as the secondary antibody (Jackson Immuno Research Laboratories).

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