DISCUSSION
5. DISCUSSION

Although, the clinical features of the two forms of cardiac hypertrophy—physiological and pathological—are well established, the molecular regulators responsible for these two forms of hypertrophy are not well documented. PKC has been identified as an important factor in different forms of hypertrophy (Bogoyevitch et al., 1994; Pass et al., 2001; Takeishi et al., 2000). However, isoform specific roles of PKC in these two distinct forms of hypertrophy have never been reported. This study for the first time, revealed specific roles of PKC isoforms in both physiological and pathological hypertrophy and dissected out precise mechanisms played by key modulators to modulate cardiac efficiency during the two forms of cardiac hypertrophy. Different animal models have been generated to study the roles of PKC isoforms. In this study cardiac hypertrophy was generated by means of renal artery ligation which is a widely used model of pathological hypertrophy (Chatterjee et al., 2011). Physiological hypertrophy was generated by swim exercise training (Galindo et al., 2009).

Mice undergoing renal artery ligation were divided into 2 groups:

1. One group designated as H or physiological hypertrophy group was generated by ligating right renal artery for 3 weeks.

2. Second group designated as $H^X$, where pathological hypertrophied animals were subjected to swim exercise training for the last 14 days out of the 3 weeks tenure of ligation.

Mice undergoing exercise training were divided into two groups:

1. One group designated as E was subjected to swim exercise training for 4 weeks which is a physiological hypertrophy group.

2. Second group designated as $E^R$, was first subjected to exercise training for 4 weeks and then were kept in rest for 2 weeks after exercise withdrawal.

A time dependent study with group $E^R$ was undertaken where animals were rested for different time frames (viz., 3, 7, 15, 20, 30 and 45 days) after exercise withdrawal (n=5).

Cardiac hypertrophy is associated with increased mRNA and rRNA levels, protein synthesis and increased protein content, increased cell size together with increased expression of several genes. Several early response genes along with some other genes which are normally expressed during fetal development are reported to express during hypertrophic
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The early genes include c-myc, c-fos and c-jun and fetal genes include β-MHC, alpha-skeletal muscle and alpha-smooth muscle actins and ANF (Wagner et al., 1999). IGF-1 is mainly produced by the liver but also within the myocardium (Bernardo et al., 2010). Increased cardiac IGF-1 expression and activation of the PI3K (p110a) pathway have been implicated in increased cardiomyocyte hypertrophy with endurance exercise (Serneri et al., 2001). Several transgenic mouse models have demonstrated that IGF-1 signalling is pivotal for physiological heart growth as IGF-1 gain-of-function results in cardiac growth with preserved/improved cardiac function (McMullen, 2008; Reiss et al., 1996). Consistent with the hypothesis that IGF-1 induces physiological cardiac hypertrophy through PI3K-Akt signalling cascade, PI3K and ensuing Akt phosphorylation were increased in the hearts of IGF-1R transgenic mice (Delauilhier et al., 1999; McMullen et al., 2004). HW/BW ratio is a common hallmark (Sen et al., 1974) for hypertrophy, markers -ANF and β-MHC exclusively induced during pathological hypertrophy (Mitra et al., 2013) and IGF-1 another specific marker exclusively induced for exercise-induced physiological hypertrophy (Ellison et al., 2012). Pathological hypertrophy in group H was evidenced by significant increase in HW/BW ratio, induced expression of markers ANF and β-MHC (Figures 5A and 5B). Physiological hypertrophy by exercise training in group E was characterized by increase in HW/BW ratio as well as induced IGF-1 expression (Figures 5A and 5B). Cardiomyocyte cross-sectional areas were increased significantly in both groups H and E compared to C (Figures 6A and 6B). Left ventricular chamber dimensions showed significant thickening in case of pathological hypertrophy (group H) as evidenced by increased IVST and PWT (Figure 7B) along with compromised cardiac function in group H with significantly increased LVDD and decreased %FS (Figure 7A). Interestingly, reversal in their condition for the two groups of hypertrophic mice was observed when group H was subjected to exercise training (group HX) and group E was rested for two weeks after the exercise regimen (group ER). Improved cardiac function, reduction in HW/BW ratio, ANF and β-MHC and cardiomyocyte cross-sectional area were recorded in HX (Figures 5, 6 and 7) while severely compromised cardiac function, significant upregulation of cardiomyocyte cross-sectional area and increase in ANF and β-MHC expression was the hallmark in group ER (Figures 5, 6 and 7). Exercise training in group HX led to the reduction in IVST and PWT (Figure 7B) whereas ER mice after exercise withdrawal showed significant increase in IVST and PWT compared to group E indicating development of pathological hypertrophy akin to group H (Figure 7B). Successful generation of hypertrophy was confirmed by significant increase in heart weight to body
weight ratio and hypertrophy marker genes. These results were also indicative of the fact that regimented exercise training may be beneficial for pathological hypertrophy patients whereas prolonged rest after exercise-induced physiological hypertrophy could be deleterious with compromised cardiac efficiency.

Several studies had already indicated a prominent role of PKC during cardiac hypertrophy (Koide et al., 2003; Sil and Sen, 1997; Braz et al., 2002; Duquesnes et al., 2011; Dong et al., 2011; Liu and Molkentin, 2011) but the precise role of specific PKC-isoforms encompassing various forms of hypertrophy processes is still not understood. Our study is the first of its kind where expression of differential PKC-isoform profile was identified and compared between different hypertrophy models. For identification of the specific isoforms of PKC we have done western blot analysis with different antibodies of PKC isoforms which are fall into different subfamilies. Although, no significant difference in phosphorylation levels of PKC-ε (Ser 729), PKC-α/βII (Thr 638/641), PKC-δ/θ (Ser 643/676), PKC-θ (Thr 538), PKC-ζ/λ (Thr 410/403), PKC-μ (Ser 744/748) and PKC-µ (Ser 916) were observed (Figure 8), two isoforms- PKC-α (conventional type) and PKC-δ (novel type) showed altered expression and differential phosphorylation status among group C, H and E (Figures 10A and 10B). It was found that total PKC-ε and PKC-ζ expression level increased significantly in group E compared to both groups H and C. On the other hand total PKC-θ expression level increased significantly in group H compared to both groups E and C. But there was no alteration in expression level of total PKC-βII and PKD in group C, H and E (Figures 9A and 9B). Our study revealed altered expression and differential phosphorylation of PKC-α and -δ in group E and H respectively. Phosphorylation of PKC-δ at Thr 505 was detected exclusively in group H (Figures 10A, 10B and 11) while phosphorylated PKC-α at Ser 657 and Tyr 658 was observed in group E (Figures 10A, 10B and 12), suggesting their exclusive activation during pathological and physiological hypertrophy respectively. On the contrary, withdrawal from exercise regimen in group E resulted in reduction in phospho-PKC-α level compared to E with significant increase in total and phospho-PKC-δ whereas, significant increase in phospho-PKC-α and reduced phospho-PKC-δ level were the hallmarks of group H (Figures 10A, 10B, 11 and 12). These results reflect that prolonged rest may be responsible for the activation of PKC-δ in exercise-induced physiological hypertrophy whereas, regimented exercise training might be a potential trigger for the activation of PKC-α in pathological hypertrophy (Figures 10A, 10B, 11 and 12). This is the first report where
involvement of specific PKC-isoforms -δ and -α was recorded in pathological and physiological hypertrophy respectively with strategic reversal of the same during reversed hypertrophic conditions evidenced by PKC-δ activation in ER and PKC-α in HX groups suggesting that transition from PKC-α to -δ activation could dictate alteration from adaptive to compromised cardiac hypertrophic condition in animal model (Figures 10A, 10B, 11 and 12). Time point study with ER group of mice showed increasing trend of pathological hypertrophy markers (ANF and β-MHC) (Figures 30A and 30B) as well as increasing trend of phosphorylation of PKC-δ (Figures 29A and 29B) from 15th day of rest which increased progressively till the 45th day, with progressive deterioration in cardiac performance (Table 4). Phosphorylation of PKC-α, on the other hand, started declining progressively from the 3rd day onwards after exercise withdrawal (Figures 29A and 29B). This was an interesting observation as our data suggests that post exercise rest period could lead towards negative cardiac remodeling in hearts with efficient performances during physiological hypertrophy.

Regulation of PKC-δ activity has been reported to be mediated by different mechanisms including binding of diacyl glycerol (DAG) and its mimetics, stimulus specific modulation of phosphorylation sites in PKC-δ and proteolytic cleavage of the catalytic domain during apoptosis by activated caspase-3 and translocation of active PKC-δ to subcellular organelles (Zhao et al., 2012). According to the three-dimensional structure of PKC-δ C1 domain and phorbol ester complex, the binding of C1 domain with DAG or PMA forms a contiguous hydrophobic surface and promotes PKC-δ binding to cell membranes (Thamilselvan et al., 2009; von Burstin et al., 2010). In addition, the binding of C1 to DAG or PMA changes the conformation and exposes the catalytic domain of PKC-δ, allowing the substrates to bind (Kanthasamy et al., 2003). Several phosphorylation sites in PKC-δ are modulated in cell type and stimulus specific manner and thus have different contributions on PKC-δ activation (Johnson et al., 1996). Three conserved serine/threonine residues have been identified in the C-terminal catalytic domain of PKC-δ including Thr-505 in the activation loop, Ser-643 in the turn motif, and Ser-662 in the hydrophobic motif (Konishi et al., 2001; Steinberg, 2004). Ser-643 is an auto-phosphorylation site and the site-directed mutation of Ser-643 markedly decreased PKC-δ activity (Li et al., 1997).

Study of downstream signaling pathways regulated by the specific PKC-isoforms during pathological and physiological hypertrophy revealed that induced PKC-δ phosphorylation at Thr 505 and its increased translocation to mitochondria and nucleus
during pathological hypertrophy (Figures 13A and 13B), could induce cardiomyocyte apoptosis in group H compared to E and C, as reported earlier where overexpression of PKC-δ catalytic fragment and its translocation to subcellular organelles induced apoptosis in various cell types and overexpression of PKC-δ kinase ‘dead mutants’ protected cells from apoptosis (Zhao et al., 2012). Translocated active PKC-δ interacts with PLS3 (He et al., 2007) that increases cardiolipin expression on the outer mitochondrial membrane (Liu et al., 2003b) thereby recruiting t-Bid for eventual cytochrome-c release via formation of Bax/Bak pores (Dave et al., 2011). PKC-δ has also been reported to turn on several nuclear apoptotic targets such as P53 (Zhao et al., 2012). Significant increase in activation and translocation of PLS3, cleavage of Bid to t-Bid, Bax, cytosolic cytochrome-c, phospho-P53 level along with induced cleavage of caspase-3, an upstream activator of PKC-δ (Zhao et al., 2012) and PARP, a known substrate of caspase-3 (Chatterjee et al., 2011) was observed in group H compared to other groups (Figures 13A, 13B, 14A and 14B). In tune with our earlier results all these apoptotic markers were increased significantly in E^R after exercise withdrawal in physiological hypertrophy group (Figures 13A, 13B, 14A and 14B). Thus, it can be inferred that PKC-δ activation during pathological hypertrophy induces cardiomyocyte apoptotic load resulting in compromised cardiac function (Mitra et al., 2013; Chatterjee et al., 2011) similar to what was recorded in E^R mice that has been withdrawn from a continuous exercise training regimen during physiological hypertrophy. Our results also suggest that exercise training could play a crucial role in the deactivation of proapoptotic PKC-δ and its downstream modulators resulting in improved cardiac efficiency during pathological hypertrophy in group H^X (Figures 13A, 13B, 14A and 14B). Thus, these results suggest that exercise training could play an important role in the inactivation of proapoptotic PKC-δ along with its upstream and downstream regulators whereas, withdrawal from a continuous exercise training regimen could lead to induction of cardiomyocyte apoptosis via reactivation of PKC-δ and its regulators. Proapoptotic role of PKC-δ during pathological hypertrophy was further confirmed by inhibiting both phosphorylated and total PKC-δ activity in group H mice that showed significant reduction in the levels of apoptotic regulators and caspase-3, -9 activities (Figures 20A, 20B and 21A).

Targeting directly or not, activated PKC-δ also affects other signaling cascades in apoptosis regulation including PI3-kinase/AKT pathway (Murriel et al., 2004), extracellular signal-regulated kinase (ERK) and P38 pathway (Efimova et al., 2004; Lee et al., 2002). For
example, PKC-δ formed a complex with ShcA to induce phosphorylation of ERK in MEF cells in response to H₂O₂-induced apoptosis (Hu et al., 2007). Recently serine/threonine kinase vaccinia-related kinase-1 (VRK-1) was identified as a PKC-δ target. PKC-δ activated VRK1 and P53 in nucleus, thus was involved in DNA damage-induced apoptosis. Activation of caspase-3 via the intrinsic pathway is a critical mediator of the hypertrophic responses (Chatterjee et al., 2011; Putinski et al., 2013). In addition to regulation of hypertrophic responses, caspase-3 has been shown to cleave PKC-δ for its activation during apoptosis in vascular smooth muscle cells as well as in a wide range of other cell types (Zhao et al., 2012; Kato et al., 2009). Our data has shown translocation of cleaved PKC-δ into mitochondria and nucleus (Figure 13A and 13B) which in turn induces caspase-9 mediated caspase-3 cleavage, thus forming a feedback loop that further exacerbates the hypertrophic responses (Zhao et al., 2012). These results cumulatively demonstrate that caspase mediated signaling is a key driver of the pathologic hypertrophy state and establish a clear link between caspase-3 signaling and cleavage activation of various PKC isoforms (Datta et al., 1997; Endo et al., 2000).

Major pathological changes that occur during cardiac hypertrophy include apoptosis, necrosis and fibroblast proliferation, dynamic changes in ECM components and excess synthesis and deposition of collagen proteins leading to cardiac fibrosis. Collagen1 and collagen3 are the major ECM proteins in the heart which maintain structural integrity of the myocardium (Bishop et al., 1994). Cardiac fibrosis is considered to be a major event which is associated with ventricular dysfunction and arrhythmia that potentially contributes to the development and progression of heart failure and sudden death (Gonzalez et al., 2002; Sugimoto et al., 2009). Studies had reported that LVH is the combined result of increased collagen synthesis and decreased degradation (Eghbali and Weber, 1990; Weber et al., 1995; Varo et al., 2000). Aortic banding in animal models of heart failure showed increase in both collagen1 as well as collagen3 leading to myocardial fibrosis (Barth et al., 2000). During pressure overload induced hypertrophy increased collagen synthesis is also reported (Diez et al., 2002; Ahmed et al., 2006; Martos et al., 2007; Solomon et al., 2007; Bradshaw et al., 2009). A number of studies have shown that PKC-δ modulates the expression of collagen genes and increased PKC-δ has been associated with the development of pathologic tissue fibrosis (Wermuth et al., 2011). Different studies report that IL-6, Ang II, ET-1 and TGFβ-1 induced PKC-δ is an important axis that regulate collagen gene expression both at RNA and protein levels (Jimenez et al., 2001; Chintalgattu and Katwa, 2009). During pressure
overload-induced hypertrophy, activity of P38MAPK is increased (Takeishi et al., 2001; Frey and Olson, 2003). P38 MAPK inhibition reduces hypertrophy and end organ damage during hypertensive heart disease (Behr et al., 2001; Frantz et al., 2007). Activation of P38MAPK has been described in animal models as well as in human heart failure (Cook et al., 1999; Behr et al., 2001; Frantz et al., 2007) and also development of maladaptive eccentric hypertrophy has been ascribed to activation of P38 MAPK pathways (Miyamoto et al., 2004; Fischer and Hilfiker Kleiner, 2008). Different activators of P38 MAPK (e.g. MKK3, MKK6) also induce myocyte hypertrophy, suggesting an important role of P38 MAPK in this process (Wang et al., 1998; Frey and Olson, 2003). Not only that, in vivo hypertrophy after aortic banding resulted in activation of TAK-1 (TGF-β-activated kinase-1), upstream of MKK3/6, which further suggests the role of P38 MAPK in hypertrophic process (Zhang et al., 2000; Frey and Olson, 2003). Activation of STAT3 along with P38 modulates collagen synthesis (Mir et al., 2012).

MMPs are zinc-dependent extracellular proteolytic enzymes and main contributors of extracellular collagen degradation associated with LV remodeling in diseased heart (Zhang et al., 2009). Although, there are reports saying no or only a little involvement of MMPs in fibrosis (Zhou et al., 2004; Heymans et al., 2005; Nishida et al., 2007; Reif et al., 2005), several studies have established that both MMP-2 and MMP-9 are involved in this process (Cheng and DH Lovett, 2003; Dwivedi et al., 2006; Lim et al., 2006; Bergman et al., 2007; Matsumoto et al., 2009). Since, MMPs are responsible for degradation of several components of the ECM, their activity is very tightly regulated which is important for maintaining normal structure and function of the heart. A group of four endogenous, naturally occurring inhibitors termed TIMPs (TIMP-1, 2, 3 and 4) regulate the activity of these MMPs (Vanhoutte et al., 2010). TIMPs non-covalently bind to zinc-binding site in the catalytic domain of active MMPs in 1:1 molar ratio and block their binding to their substrate molecules and thereby prevent extracellular matrix degradation (Cawston et al., 1990; Brew et al., 2000; Chirco et al., 2006; Nagase et al., 2006; Phatharajaree et al., 2007; Vanhoutte et al., 2010). Out of four known TIMPs, TIMP-1 and TIMP-2 are the most studied ones (Spinale, 2007). The activities of almost all MMPs except MMP-2 and MT1-MMP are inhibited by TIMP-1 whereas; TIMP-2 inhibits the activity of all MMPs except MMP-9 (Phatharajaree et al., 2007).
All these reports corroborate with our finding of increased collagen synthesis and deposition in the myocardium (Figures 15 and 16), increased activation of STAT3 (Figure 17A and 17B), P38 MAPK, activators of P38 MAPK (e.g. MKK3, MKK6) and TAK-1 (upstream of MKK-3/6) (Figures 18A and 18B) were found in hypertrophied mice heart (group H) compared to C or E. In tune with earlier results, all these proteins expression and collagen synthesis and deposition in the myocardium were increased significantly in E$^R$ after exercise withdrawal in physiological hypertrophy group (Figures 15, 16, 17 and 18). Exercise training might be a potential trigger for the deactivation of STAT3, P38 MAPK and associate proteins and decreased synthesis and deposition of collagen in the myocardium in group H$^X$ (Figures 15, 16, 17 and 18). In our study, increased expression of MMP-2 and TIMP-1 gene was found in group H mice compared to E or C. However, MMP-2 and TIMP-1 expression was down regulated in group H$^X$ mice compared to H and up regulated when mice were kept at rest condition (E$^R$ group) compared to E (Figures 19A and 19B). Fibrotic role of PKC-δ during pathological hypertrophy was further confirmed by inhibiting phosphorylated PKC-δ activity in group H mice that showed significant reduction in the levels of fibrotic regulators (Figure 22B). PKC-δ inhibition resulted in significant decrease in ventricular collagen concentration in both in vivo and in vitro models of pathological hypertrophy along with significant reduction of phospho-P38 and phospho-STAT3 (at Tyr-705 and Ser-727; Figures 22A and 22B), an important regulator of ventricular collagen synthesis as described earlier by our group (Mir et al., 2012).

Various studies have reported PKC-α to be a pro-survival isoform that suppresses apoptotic signaling and promotes cell survival via direct phosphorylation of Akt at serine 473 (Partovian and Simons, 2004) and also as a negative regulator of cardiac functional efficiency shown by its increased expression in various models of cardiac injury or failure (Steinberg, 2012; Liu and Molkentin, 2011). PKC-α dependent regulation of cardiomyocyte hypertrophy requires ERK-1/2 activation (Braz et al., 2002) for cardio-protection during ischemia reperfusion injury through activation of the several anti-apoptotic and pro-survival signaling cascades (Cuadrado et al., 2011). Our study has revealed significantly increased phosphorylation of Akt and ERK-1/2 along with exclusive activation of PKC-α in group E during physiological hypertrophy (Figures 23A and 23B). Their pro-survival role in these animals was also ascertained by significantly decreased caspase-9 activity (Figure 24), corroborating earlier reports that claimed responsibility of these kinases for the inactivation...
of proapoptotic caspase-9 (Lee et al., 2011). Inhibition of both phosphorylated and total PKC-α during physiological hypertrophy resulting in significant deactivation of phospho-ERK-1/2 and -Akt level further confirms the pro-survival role of PKC-α (Figures 25A and 25B) and indicates that exercise training could play a crucial role in the activation of PKC-α and its downstream pro-survival proteins. In contrast, deactivation of PKC-α along with decreased phosphorylation of Akt and ERK-1/2 leading to the activation of pro-apoptotic markers and caspase-3, -9 activities which were recorded in group E₀ (Figures 23A and 23B), again confirms that withdrawal from exercise training during physiological hypertrophy leads to onset of pathological hypertrophic characters marked by induction of myocyte apoptosis and compromised cardiac function in group E₀. Moreover PKC-δ inhibition in group H interestingly, led to the reactivation of PKC-α along with induced downstream pro-survival markers viz. phospho-Akt and phospho-ERK-1/2 with significant improvement of cardiac function during pathological hypertrophy (Figures 28A and 28B), similar to the condition achieved through exercise training during pathological hypertrophy in group Hₓ. This result was corroborated by an earlier report where PKC-δ inhibition led to activation of PKC-α isoform in prostate cancer cells (Tanaka et al., 2003). On the other hand, PKC-α inhibition during physiological hypertrophy resulted in activation of PKC-δ in group E along with induction of apoptotic regulator proteins and severely compromised cardiac function (Figures 26A, 26B and 27) similar to the phenotypes observed in group E₀. This result corroborates with an earlier report that showed induced PKC-δ dependent apoptotic program upon inhibition of PKC-α in salivary epithelial cells (Matassa et al., 2003). Thus, our study clearly demonstrated that PKC-α and -δ isoforms are the prime modulators of cardiac adaptation during physiological and pathological hypertrophy respectively. Further, this study also established that cardiac adaptive processes during transition of an efficiently functioning heart to a functionally compromised heart could be modulated by reversal of activation of PKC-α to-δ isoform.