RESULTS
4. RESULTS

4.1. Assessment of cardiac hypertrophy in different experimental groups

4.1.1. Increased heart weight to body weight (HW/BW) ratio:

Increased heart weight to body weight ratio is a hallmark of cardiac hypertrophy measurement. Heart weight (mg) to body weight (g) ratio (HW/BW) was measured in all the experimental groups. Significant increase in the heart weight to body weight ratio (HW/BW) was observed in group H (5.97±0.11, p<0.001) and group E (5.12±0.12, p<0.01) compared to group C (4.54±0.06). However, the HW/BW ratio was significantly decreased in group H X (5.07±0.16, p<0.05) after exercise training of this hypertrophic group compared to H but increased significantly after keeping the mice in rest in group E R (5.65±0.09, p<0.05) compared to E (Figure 5A).

4.1.2. Increased expression of hypertrophy marker genes:

RT-PCR analysis revealed expression of pathological hypertrophy markers (Atrial natriuretic factor [ANF] and beta myosin heavy chain [β-MHC]) and physiological hypertrophy marker (Insulin growth factor-1 [IGF-1]). ANF (6.13±0.6-fold, p<0.001) and β-MHC (4.29±0.1-fold, p<0.001) increased significantly in group H compared to either E or C. Significant decrease in expression of ANF (2.44±0.03-fold, p<0.001) and β-MHC (2.14±0.02-fold, p<0.001) was observed in group H X compared to H. Expression of both these genes increased significantly in group E R (1.97±0.12-fold, p<0.01 for ANF and 2.02±0.12-fold for β-MHC, p<0.01) compared to E (Figures 5B and 5C). Expression of IGF-1, marker of physiological hypertrophy, increased significantly in group E (2.5±0.12-fold, p<0.01) compared to H or C. IGF-1 expression was down regulated drastically in group E R (1.78±0.02-fold, p<0.01) compared to E and up regulated in group H X (1.47±0.02-fold, p<0.01) compared to H. GAPDH was used as internal control (Figures 5B and 5C).

4.1.3. Histological study:

Histological staining with Hematoxylin-Eosin revealed cross sectional area of cardiomyocyte in different experimental groups. The cross-sectional area of cardiomyocytes was significantly increased in group H (288.62±7.75 µm², p<0.001) and in group E (227.9±8.53 µm², p<0.001) compared to C (157.45±4.99 µm²). However, the myocyte cross-sectional area was significantly decreased in group H X (225±7.77 µm²,
Figure: 5 Assessment of cardiac hypertrophy

(A) Graph showing HW/BW ratio in all five experimental models: pathological (H), physiological hypertrophy (E), exercise-trained pathological hypertrophy (H*), mice kept at rest after 4 weeks of exercise training (E*), and representative control (C) (*p<0.05 for H versus E or C; #p<0.05 for H versus H*; ¶p<0.05 for E versus E*).

(B) Expression profile of pathological hypertrophy markers (ANF and β-MHC) and physiological hypertrophy marker (IGF-1) estimated by RT-PCR. GAPDH was used as loading control.

(C) Graph showing pathological hypertrophy markers (ANF and β-MHC) and physiological hypertrophy marker (IGF-1) estimated by RT-PCR. GAPDH was used as loading control. (For ANF ***p<0.001 for E versus E*; ###p<0.001 for H versus H*; For β-MHC ****p<0.001 for E versus E*; ####p<0.001 for H versus H*; For IGF-1 ***p<0.001 for E versus H; ####p<0.001 for E versus E*).
p<0.001) compared to H but increased significantly in group $E^R$ (260.1±9.38 µm$^2$, p>0.05) compared to E (Figures 6A and 6B).

4.2. Assessment of cardiac function in different experimental groups

4.2.1. Echocardiography analysis:

M-Mode echocardiography revealed cardiac function by several echocardiographic parameters. We studied left ventricular diastolic dimension (LVDD) and percent fractional shortening (%FS) for assessment of cardiac function. M-Mode echocardiography showed significantly increased LVDD and reduced %FS in group H (2.94±0.05mm for LVDD, p<0.001 and 33±0.02% for %FS, p<0.01) compared to group E (LVDD 2.24±0.12mm, p<0.001 and %FS 62.5±0.04%, p<0.05) and group C (LVDD 2.17±0.05mm, p<0.05; and 61±0.08%, p<0.01). Significantly compromised cardiac function was observed in group $E^R$ with increased LVDD and decreased %FS (LVDD 3.07±0.05mm, p<0.0 and %FS 39±0.02%, p<0.0) compared to group E. Cardiac function was improved in group $H^R$ with reduced LVDD 2.44±0.03mm and increased %FS 55±0.33% compared to group H (Figure 7A).

Assessments of left ventricular chamber dimensions revealed by echocardiographic parameter such as inter ventricular septum thickness (IVST) and posterior wall thickness (PWT). Significant increased inter ventricular septum thickness (IVST) and posterior wall thickness (PWT) in group H (0.62±0.02mm for IVST, p<0.01 and 0.51±0.08mm for PWT, p<0.05) and E (0.51±0.05mm for IVST, p>0.05 and 0.41±0.10mm for PWT, p<0.05) compared to C (IVST 0.42±0.01mm and PWT 0.26±0.06mm). Both IVST (0.47±0.01mm, p<0.05) and PWT (0.31±0.04mm, p<0.05) were significantly reduced in $H^R$ animals compared to group H indicating exercise after pathological hypertrophy ameliorates ventricular chamber thickness. Withdrawal from exercise in group $E^R$ revealed markedly increased IVST (0.59±0.02mm, p<0.05) and PWT (0.46±0.04mm, p<0.05) compared to E (Figure 7B).

4.3. Activation of different PKC-isoforms in all the experimental groups

Both phospho and total PKC isoforms were studied by western blot analysis using different PKC antibodies.
Figure: 6 Cardiomyocyte cross sectional area measurement

(A) Cardiomyocyte cross sectional area for different experimental groups (C=157.4±4.99, H=288.62±7.75, E=227.9±8.53, H^x=225±7.77 and E^R=260.1±9.38. Scale bar = 50 μm, Magnification = 40X. (B) Graph showing cardiomyocyte cross-sectional area (in μm^2) in groups C, H, E, H^x, and E^R (***p<0.001 for H versus E or C; ##p<0.01 for H versus H^x; ####p<0.05 for E versus E^R).
Figure: 7 Assessment of cardiac function by M-Mode Echocardiography

(A) Graph showing LVDD and %FS in all experimental groups (*p<0.05 for H versus E or C, #p<0.05 for H versus H^x, ||p<0.05 for E versus E^x). (B) Graph showing IVST and PWT in all experimental groups (*p<0.05 for H versus E or C; #p<0.05 for H versus H^x; ||p<0.05 for E versus E^x).
4.3.1. Expression status of phospho-PKC isoforms during pathological and physiological hypertrophy:

Phosphorylation status of different PKC-isoforms were studied in three experimental groups-C, H and E. Western blot analysis revealed 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) in either of these three groups (Figure 8). Apart from seven unaltered phospho-PKC, two PKC isoforms showed differential phosphorylation pattern. PKC-δ phosphorylated only in group H and PKC-α phosphorylation was found exclusively in group E (Figure 10A). RPL 32 was used as an internal loading control.

4.3.2. Expression status of total-PKC isoforms during pathological and physiological hypertrophy:

Different total PKC-isoforms were studied in these three experimental groups -C, H and E. Total PKC-ε and PKC-ζ expression levels increased significantly in group E compared to C (6.23±0.11-fold, p<0.001 for PKC-ε and 3.85±0.4-fold, p<0.001 for PKC-ζ) and H (3.61±0.28-fold, p<0.001 for PKC-ε and 1.63±0.25-fold for PKC-ζ, p<0.05), though the change was less pronounced in group H compared to E. Total PKC-θ expression level increased significantly in groups H (4.21±0.01-fold, p<0.001) and E (3.50±0.01-fold, p<0.001) compared to C. Total PKC-βII and PKD expression levels remained unaltered in all the three groups. RPL 32 was used as an internal loading control (Figures 9A and 9B).

4.3.3. Expression of PKCα and PKC-δ in different experimental groups:

Among different PKC phosphorylation, PKC-α phosphorylation was found in group E whereas PKC-δ phosphorylation was found in group H. Altered phosphorylation of PKC-α and PKC-δ was also found in rest of the groups. The phosphorylation level of PKC-δ (Thr 505) was found to be significantly high in group H (4.00±0.08-fold, p<0.001) compared to either C or E; whereas, significant increase in the phosphorylation level of PKC-α (Ser 657 and Tyr 658) was recorded in group E (6.13±0.02-fold, p<0.001) compared to either C or H (Figures 10A and 10B). Phospho-PKC-α was found to be significantly down regulated (4.73±0.07-fold, p<0.001) and phospho-PKC-δ significantly induced (2.75±0.11-fold, p<0.001) in group E compared to E. On the contrary, significant increase in the levels of phospho-PKC-α (5.10±0.10-fold, p<0.001)
**Phospho PKC isoforms**

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**Figure: 8 Expression profile of phospho-Protein Kinase-C isoforms**

Western blot analyses showing status of phospho-PKC isoforms [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)] in group C, H and E. RPL 32 was used as loading control.
Figure: 9 Expression profile of total-Protein Kinase-C isoforms
(A) Western blot analyses showing status of total PKC isoforms (PKC-ε, PKC-ζ, PKC-βII and PKD) in group C, H and E. RPL 32 was used as loading control. (B) Graph showing relative band intensity of total-PKC-ε, PKC-ζ, PKC-θ, PKC-βII and PKD in group C, H and E (For total PKC-ε: ***p<0.001 for E versus C or H; For PKC-ζ: $$$p<0.001 for E versus C or H; For total PKC-θ: ####p<0.001 for H and E versus C; Total PKC-βII and PKD expression levels remained unaltered in all the three groups).
Figure 10: Expression profile of PKC-α and PKC-δ

(A) Western blot analyses showing change in expression of PKC-α and PKC-δ (phospho and total) in groups C, H, E, H^x and E^R. PKC-δ cleavage product (41kD) is exclusive in group H, H^x and E^R. RPL 32 was used as loading control. (B) Graph showing relative band intensity of phospho-PKC-δ and phospho-PKC-α in group C, H, E, H^x and E^R as revealed by western blot analysis (For phospho-PKC-δ: ***p<0.001 for H versus C or E; ####p<0.001 for H versus H^x; |||p<0.01 for E versus E^R. For phospho-PKC-α: **p<0.01 for E versus H or C; ####p<0.001 for H versus H^x; |||p<0.01 for E versus E^R).
and decrease in phospho-PKC-δ (3.61±0.14-fold, p<0.001) was observed in group H\textsuperscript{X} compared to H (Figures 10A and 10B).

Total PKC-α expression was significantly high in both groups E (1.30±0.04-fold, p>0.01) and H (1.58±0.04-fold, p<0.001) compared to C. Total PKC-α expression increased in group H\textsuperscript{X} (1.39±0.08-fold, p<0.001) compared to H but decreased significantly in group E\textsuperscript{R} (4.30±0.12-fold, p<0.01) compared to E (Figure 10A). On the other hand, significantly increased expression of total PKC-δ was observed in group H (4.97±0.07-fold, p<0.05) compared to either C or E. Increased expression of PKC-δ was observed in group E\textsuperscript{R} (4.17±0.07-fold, p<0.05) compared to E. Total PKC-δ expression was down regulated in H\textsuperscript{X} (1.66±0.03-fold, p<0.001) compared to H (Figure 10A).

4.3.4. Immunofluorescence study:

Immunofluorescence studies that showed pronounced phosphorylation of PKC-δ (green fluorescence) in groups H and E\textsuperscript{R} compared with either group C or E (Figure 11). On the contrary, phosphorylation of PKC-α (green fluorescence) was increased in groups E and H\textsuperscript{X} compared to E, H or E\textsuperscript{R} (Figure 12). Cardiomyocyte cell specificity was confirmed by staining the cells with alpha sarcomeric actinin antibody (red fluorescence) and nuclei were stained with DAPI (blue fluorescence) (Figures 11 and 12).

4.4. PKC-δ and expression of downstream apoptotic markers in different experimental models

4.4.1. Cleavage of PKC-δ and its translocation to mitochondria and nucleus:

Subcellular fractionation followed by western blot analysis was performed in different experimental groups with ventricular extracts to determine whether cleavage of PKC-δ resulted in its translocation to mitochondria and nucleus. Significantly increased translocation of active cleaved subunit (41 kD) of PKC-δ was detected in both mitochondrial and nuclear fractions of group H compare to C or E but was significantly decreased in group H\textsuperscript{X}. Such translocation was totally absent in groups C or E but reappeared in group E\textsuperscript{R} after exercise withdrawal (Figures 13A and 13B).

4.4.2. Expression of nuclear proteins downstream to PKC-δ:

The phosphorylation status of p53, downstream nuclear target of PKC-δ was assessed by western blot analysis. Significant increase in expression level of total and
Figure: 11 Immunofluorescence micrographs (40X) showing PKC-δ expression

Immunofluorescence study showing expression of phospho-PKC-δ in different experimental groups. Tissue sections showing phospho-PKC-δ expression in panels b, f, j, n and r (green fluorescence). Sections were counter stained with alpha sarcomeric actinin antibody (panels c, g, k, o and s; red fluorescence). Nuclei were stained with DAPI (panels a, e, i, m and q; blue fluorescence) and merged images are shown in panels d, h, l, p and t. Increased expression of phospho-PKC-δ was observed in groups H and $E^R$ compared to C and E. (Scale bar = 50 μm, Magnification = 40X).
Figure: 12 Immunofluorescence micrographs (40X) showing PKC-α expression
Immunofluorescence study showing expression of phospho-PKC-α in different experimental groups. Tissue sections showing phospho-PKC-α in panels b’, f’, j’ , n’ and r’ (green fluorescence). Sections were counter stained with alpha sarcomeric actinin antibody (panels c’, g’, k’, o’ and s’; red fluorescence). Nuclei were stained with DAPI (panels a’, e’, i’, m’ and q’; blue fluorescence) and merged images are shown in panels d’, h’, l’, p’ and t’. Increased expression of phospho-PKC-α was observed in groups E and H^x compared to groups H and E^R. (Scale bar = 50 μm, Magnification = 40X).
Figure: 13 Assessment of PKC-δ associated downstream target proteins

(A) Western blot analysis with the nuclear protein revealed significantly increased translocation of cleaved PKC-δ (41kD) to nucleus in group H, H<sup>x</sup> and E<sup>R</sup> compared to either C or E. Significantly increased phospho-p53 (at Ser 46 and Ser15) and total p53 was observed in group H and E<sup>R</sup> compared to E or C whereas significantly reduced phospho-p53 (at Ser 46 and Ser15) and total p53 was observed in groups E and H<sup>x</sup> compared to H or E. RPL 32 and Lamin B were used as loading control for cytosolic proteins and nuclear proteins respectively. (B) Subcellular fractionation followed by western blot analyses with mitochondrial protein showing significantly increased translocation of cleaved PKC-δ (41kD) to mitochondria in group H, H<sup>x</sup> and E<sup>R</sup> compared to either C or E along with increased expression of PLS3, t-Bid, Bax, cytochrome-c proteins. RPL 32 and COX IV were used as loading control for cytosolic proteins and mitochondrial proteins respectively.
phosphorylated p53 (ser-46, ser-15) was observed in group H (6.48±0.21-fold, p<0.001 for total p53, 7±0.007-fold, p<0.001 at ser-46 and 5.79±0.24 fold, p<0.001 at ser-15) compared to all groups. Phosphorylated p53 level was comparatively less pronounced in \( H^X \) (1.67±0.04-fold, p<0.01 at ser-46 and 1.20±0.06-fold, p<0.05 at ser-15) compared to \( H \) with reappearance and increased phospho-p53 levels in group \( E^R \) (4.43±0.12-fold, p<0.001 for total p53, 4.66±0.20-fold, p<0.001 at ser-46 and 3.56±0.2 fold, p<0.001 at ser-15) compared to \( E \) (Figure 13A).

**4.4.3. Expression of mitochondrial proteins downstream to PKC-δ:**

The proapoptotic and downstream proteins to PKC-δ are Phospholipid scramblase-3 (PLS3), Bcl-2 associated X-protein (Bax), BH3-interacting domain death and cytochrome-c. PLS3, Bax, cleavage of Bid to truncated-Bid (t-Bid) and cytosolic/mitochondrial ratio of cytochrome-c were significantly increased in group \( H \) (3.54±0.02 fold, p<0.01 for PLS3, 4.33±0.19 fold, p<0.01 for Bax and 10.52±0.04 fold, p<0.01 for cytochrome-c) compared to either \( C \) or \( E \). Expression of these proteins were significantly reduced in group \( H^X \) (1.71±0.02-fold, p<0.001 for PLS3, 1.50±0.09-fold, p<0.05 for Bax and 2.48±0.37-fold, p<0.01 for cytochrome c) compared to \( H \) but was induced significantly in group \( E^R \) (1.91±0.02-fold, p<0.001 for PLS3, 2.83±0.08-fold, p<0.001 for Bax and 11.09±0.13-fold, p<0.01 for cytochrome c) compared to \( E \) (Figure 13B).

**4.4.4. Expression of end point apoptotic markers:**

Western blot analysis revealed active catalytic fragments of two end point markers caspase-3 and Poly ADP ribose polymerase (PARP) during pathological hypertrophy. Caspase-3 was found to be cleaved into two prominent active catalytic fragments (19 kD and 17 kD) in groups \( H \) and \( E^R \) that were absent in \( C \) or \( E \) (Figure 14A). However, significantly reduced cleavage of fragments (19 kD and 17 kD) of caspase-3 was observed in group \( H^X \) compared to \( H \) (Figure 14A). Cleaved 89 kD active fragment of PARP was also detected in groups \( H \) and \( E^R \) that was absent in groups \( E \) or \( C \). Interestingly, PARP active fragment was significantly reduced in \( H^X \) compared to \( H \) (Figure 14A).
Figure: 14 Assessment of apoptosis by end point apoptotic markers

(A) Western blot analysis showing cleavage of caspase-3 and PARP in group H, H^x and E^R compared to C or E. RPL 32 was used as internal loading control. (B) Caspase-3 activity assay showing similar changes in all the experimental groups. No significant difference in caspase-3 activity was detected between groups E and C (*p<0.05 for H versus C; ###p<0.001 for H versus H^x; †††p<0.01 for E versus E^R).
4.4.5. Caspase-3 activity assay:

Caspase-3 activity level was checked by activity assay kit as described earlier. Caspase-3 activity significantly increased in group H (7.89±0.23 fold, p<0.01) compared to C or E. However, caspase-3 activity was significantly increased in group E\textsuperscript{R} (3.28±0.69-fold, p<0.01) compared to E and reduced activity of caspase-3 was observed in group H\textsuperscript{X} (3.36±0.24-fold, p<0.001) compared with H (Figure 14B).

4.5. Assessment of fibrosis and profibrotic modulators in different experimental models

4.5.1. Increase in collagen transcript:

RT-PCR analysis revealed a significant increased collagen expression (type-1 and type-3) in group H (3.34±0.06-fold, p<0.05 for Col 1; 3.89±0.06-fold, p<0.05 for Col 3) compared to E or C. However both of the collagen genes was highly upregulated in group E\textsuperscript{R} (3.07±0.06-fold, p<0.01 for Col 1; 3.12±0.09-fold, p<0.01 for Col 3) compared to E; whereas collagen expression (type-1 and type-3) was down regulated in group H\textsuperscript{X} (1.38±0.07-fold, p<0.001 for Col 1; 1.29±0.02-fold, p<0.001 for Col 3) compared to H (Figures 15A and 15B).

4.5.2. Increase in total ventricular collagen content:

Hydroxyproline assay revealed the total collagen content for all the experimental groups. Ventricular collagen concentration was significantly increased in group H (0.613±0.02 µg/mg wet tissue, p<0.05) compared to E (0.463±0.01 µg/mg wet tissue, p<0.01) and C (0.418±0.008 µg/mg wet tissue). Interestingly, ventricular collagen concentration was significantly increased in mice after exercise withdrawal (group E\textsuperscript{R}: 0.693±0.01 µg/mg wet tissue, p<0.01) compared to E and was significantly decreased in pathological hypertrophy group undergoing exercise training (group H\textsuperscript{X}) (0.517±0.04 µg/mg wet tissue, p<0.05) compared to H (Figure 16A).

Masson’s Trichrome (MT) staining revealed changes of collagen profile among control and experimental mice hearts. Collagen infiltration was quite prominent as observed by Masson’s Trichrome staining and was indicated by appearance of blue collagen fibers in the tissue sections. Blue collagen fibers were prominent in both the tissues of group H and E\textsuperscript{R} mice. Group E or C showed no blue deposition of collagen and the intra-myofibrillar spaces remained empty. Collagen fibers were less in group H\textsuperscript{X} mice compared to H (Figure 16B).
Figure: 15 Expression profile of collagen genes
(A) Expression of *collagens* (1 and 3) in all experimental groups estimated by RT-PCR analysis. *GAPDH* was used as loading control. (B) Graph showing relative band intensity of *col 1* and *col 3* in groups C, H, E, H<sup>+</sup> and E<sup>R</sup>. (For *Col 1*: *p*<0.05 for H versus C; ###*p*<0.001 for H versus H<sup>+</sup>; ||*p*<0.01 for E versus E<sup>R</sup>; For *Col 3*: *p*<0.05 for H versus C; ###*p*<0.001 for H versus H<sup>+</sup>; ||*p*<0.01 for E versus E<sup>R</sup>).
Figure: 16 Estimation of collagen by Hydroxyproline assay and Masson trichrome staining

(A) Graph showing ventricular collagen concentration in groups C, H, E, Hx, and Er estimated by hydroxyproline assay [*p<0.05 for H versus Hx; **p<0.01 for E versus Er]. (B) Masson trichrome staining revealed collagen accumulation in ventricle of all experimental groups (C, H, E, Hx, and Er) [Scale Bar=50 μm, Magnification = 40X].
4.5.3. Activation of STAT3 protein:

Phosphorylation of STAT3 proteins was studied in all the experimental groups by western blot analysis. Result showed significant phosphorylation of STAT3 at position Tyr-705 and Ser-727 in group H (4.01±0.01-fold, p<0.05 for Tyr-705; 3.87±0.01-fold, p<0.05 for Ser-727) mice compared to E or C. Phosphorylation of both position of this protein was totally absent in group E mice. However, phosphorylation of this protein was upregulated (4.8±0.02-fold, p<0.01 for Tyr-705; 4.11±0.01-fold, p<0.01 for Ser-727) when mice were kept at rest condition after exercise regimen (E^R group) and was down regulated (4.05±0.01-fold, p<0.001 for Tyr-705; 3.90±0.01-fold, p<0.001 for Ser-727) in H^X group. Total STAT3 level also remained unchanged in all the experimental groups. RPL 32 expression was used as an internal loading control (Figures 17A and 17B).

4.5.4. Phosphorylation status of TAK-1, MKK 3/6 and P38 MAPK:

Similar to STAT3, phosphorylation of TAK-1, MKK-3/6 and P38 MAPK were also significantly higher in group H (3.54±0.02-fold, p<0.05 for TAK-1; 3.33±0.08-fold, p<0.05 for MKK-3/6; 3.93±0.09-fold, p<0.05 for P38 MAPK) mice compared to E or C, as shown by western blot analysis. Phosphorylation of these proteins was minimal in group E. However phosphorylation of these proteins was upregulated when mice that were kept at rest after exercise training (E^R group) (2.35±0.02-fold, p<0.001 for TAK-1; 2.274±0.04-fold, p<0.01 for MKK-3/6; 2.87±0.04-fold, p<0.01 for P38 MAPK) compared to E and was significantly down regulated in hypertrophied mice undergoing exercise training (H^X group) (1.71±0.03-fold, p<0.05 for TAK-1; 1.8±0.04-fold, p<0.05 for MKK-3/6; 1.20±0.04-fold, p<0.05 for P38 MAPK) compared to H (Figures 18A and 18B). Expression of total TAK, MKK 3/6 and p38 MAPK proteins were also remained unchanged in all groups. RPL 32 was used as loading control for these experiments.

4.5.5. Expression of collagen modulators MMP-2 and TIMP-1:

Western blot analysis also showed significant increase in MMP-2 (2±0.02-fold, p<0.05) and TIMP-1 (2.22±0.01-fold, p<0.05) expression in group H mice compared to E or C. However expression of MMP-2 (1.45±0.05-fold, p<0.001) and TIMP-1 (2.22±0.06-fold, p<0.001) was down regulated in group H^X mice compared to H. MMP-2 (2.38±0.03-fold, p<0.01) and TIMP-1 expression was upregulated (2.87±0.01-fold,
Figure: 17 Phosphorylation status of STATs in different groups
A) Western blot analysis showing expression status of phospho STAT3 (Tyr 705 and Ser 727) in groups C, H, E, H^{x} and E^{R}. RPL 32 was used as an internal loading control. (B) Graph showing relative band intensity of phospho STAT3 (Tyr 705 and Ser 727) in groups C, H, E, H^{x} and E^{R}. (For STAT3 Tyr 705- *p<0.05 for H versus C; ###p<0.001 for H versus H^{x}; p<0.01 for E versus E^{R}; For STAT3 Ser 727- *p<0.05 for H versus C; ###p<0.001 for H versus H^{x}; p<0.01 for E versus E^{R}).
Figure: 18 Phosphorylation status of TAK-1, MKK-3/6 and P38 MAPK

(A) Western blot analysis showing expression status of phospho-TAK-1, MKK-3/6 and P38 MAPK in groups C, H, E, Hx and Er. RPL 32 was used as an internal loading control. (B) Graph showing relative band intensity of phospho-TAK-1, MKK-3/6 and P38 MAPK in groups C, H, E, Hx and Er. (For phospho-TAK-1:*p<0.05 for H versus Hx; ###p<0.001 for E versus Er; For phospho-MKK-3/6:*p<0.05 for H versus Hx; #p<0.01 for E versus Er; For phospho-P38 MAPK:*p<0.05 for H versus Hx; ##p<0.01 for E versus Er).
RESULTS

p<0.01) when mice were kept at rest condition (E R group). RPL 32 was used as internal control for these experiments (Figures 19A and 19B).

4.6. PKC-δ activation results in induction of apoptosis and fibrosis during pathological hypertrophy

4.6.1. Inhibition of PKC-δ resulted in down regulation of associate mediators of apoptosis:

Both phosphorylated and total PKC-δ expression was significantly reduced in PKC-δ siRNA treated pathological hypertrophy group (H) along with significant down regulation of Bax (2.91±0.06 fold, p<0.01), cytosolic/mitochondrial ratio of cytochrome-c (6.29±0.08 fold, p<0.01) and cleavage of PARP compared to non specific siRNA treated group H (Figures 20A and 20B). The PKC-δ siRNA treatment also resulted in significant reduction in activities of caspase-3 and -9 in group H (7.83±0.53 fold, p<0.05 and 3.56±0.28 fold, p<0.05 respectively) compared to non specific siRNA treated group H mice (Figure 21A). Similar results were observed when group H mice were treated with chemical inhibitor Rottlerin compared to vehicle treated ones (data not shown). PKC-δ siRNA or Rottlerin treatment did not alter the expression of these proteins during physiological hypertrophy (data not shown).

4.6.2. Inhibition of PKC-δ modulates collagen concentration and down regulates fibrosis both in vivo and in vitro:

In vivo

Inhibition of PKC-δ resulted in significant regression of ventricular collagen concentration in siRNA treated pathological hypertrophy (H) (0.377±0.02 µg/mg, p<0.01) samples compared to non specific siRNA treated group H (0.597±0.01 µg/mg, p<0.01) (Figure 22A). PKC-δ siRNA treated pathological hypertrophy mice group showed significantly reduced phosphorylation status of PKC-δ along with P38 MAPK. The PKC-δ siRNA treatment also resulted in reduction of phosphorylation of STAT3 (Tyr-705 and Ser-727) in group H mice compared to non specific siRNA treated group H (Figure 22B). Similar results were observed when group H mice were treated with chemical inhibitor Rottlerin compared to vehicle treated ones (data not shown).
Figure: 19 Expression pattern of collagen modulators (MMP and TIMP)

(A) Western blot analysis showing expression status of MMP-2 and TIMP-1 in groups C, H, E, H^X and E^R. RPL 32 was used as an internal loading control. (B) Graph showing relative band intensity of MMP-2 and TIMP-1 in groups C, H, E, H^X and E^R. (For MMP-2, *p<0.05 for H versus C; ###p<0.001 for H versus H^X; \( \dddot{\dddot{p}} < 0.01 \) for E versus E^R; For TIMP-1, *p<0.05 for H versus C; ###p<0.001 for H versus H^X; \( \dddot{\dddot{p}} < 0.01 \) for E versus E^R).
Figure: 20 Inhibition of PKC-δ reduces expression of downstream target proteins

(A) Western blot analysis showing successful down regulation in expression of both phospho and total PKC-δ along with significant decrease in the level of bax, cytosolic cytochrome-c and PARP cleavage in mice treated with PKC-δ siRNA in group H. RPL 32 was used as loading control. (B) Graph showing significantly decreased phospho-PKC-δ, Bax and cytochrome-c (cytosolic/mitochondrial ratio) in mice treated with PKC-δ siRNA compared to non specific siRNA treated mice [*p<0.05 for H (non specific siRNA) versus H (PKC-δ siRNA)].
Figure: 21 Inhibition of PKC-δ leads to decreased caspase-3 and -9 activities
Graph showing caspase-3 and caspase-9 activities in the two experimental groups (C and H) treated with siRNA and non-specific siRNA. Significant decrease in caspase-3 and caspase-9 activities occurred in mice treated with PKC-δ siRNA compared to mice treated with non-specific siRNA [*p<0.05 for H (non specific siRNA) versus H (PKC-δ siRNA)].
In vitro

Inhibition of PKC-δ by specific siRNA treatment resulted in significant regression of ventricular collagen concentration in cardiac fibroblast treated with Ang-II (2.90±0.01 ng/ml, p<0.05) compared to cardiac fibroblast treated with Ang-II (4.06±0.21 ng/ml, p<0.05) (Figure 22A). PKC-δ siRNA treated hypertrophied fibroblasts showed significantly reduced phosphorylation of PKC-δ along with P38 MAPK. The inhibitor treatment also resulted in reduction of phosphorylation of STAT3 (Tyr-705 and Ser-727) in Ang-II treated cardiac fibroblast compared to cardiac fibroblast (Figure 22B). In vivo and in vitro treatment with PKC-δ siRNA had no significant effect on total P38 and STAT3 (data not shown). Similar reduction in expression of these proteins was observed following Rottlerin treatment in group H mice and Ang-II treated cardiac fibroblast (data not shown).

4.7. Activation of PKC-α and expression of its downstream mediators in different experimental groups

4.7.1. Activation status of Akt and ERK-1/2 proteins downstream to PKC-α:

The phosphorylation status of Protein kinase B (Akt) and ERK-1/2, downstream pro-survival targets of PKC-α, was studied in all the experimental groups. Akt phosphorylation at serine 473 was observed only in group E along with its reappearance in group H'. Phospho-Akt to Akt ratio was 2.66±0.03-fold (p<0.001) higher in group E compared to groups C or H. On the other hand, phospho-Akt to Akt ratio was significantly increased in group H' (1.90±0.03-fold, p<0.01) compared to H and decreased in group E'' (1.52±0.02-fold, p<0.01) compared to E. Similarly, phospho-ERK-1/2 to ERK-1/2 ratio was increased significantly in group E (5.3±0.02-fold, p<0.001) and in group H (1.76±0.07-fold, p<0.05) compared to C. However, phospho-ERK-1/2 to ERK-1/2 ratio was significantly increased in group H' (1.43±0.006-fold, p<0.01) compared to H and decreased in group E'' (1.44±0.15-fold, p<0.01) compared to E (Figures 23A and 23B).

4.7.2. Caspase-9 activity assay:

Proapoptotic caspase-9 activity was found to be significantly decreased in group E (5.95±0.67-fold, p<0.05) compared to group H. It was also revealed that caspase-9 activity was significantly increased in group E'' (5.17±0.50-fold, p<0.05) compared to E;
**Figure: 22 Effect of PKC-δ on downregulation of collagen synthesis**

**(A)** Graph showing ventricular collagen concentration in groups C, H and H + PKC-δ siRNA (*in vivo*) and in groups C, Ang-II and Ang-II + PKC-δ siRNA (*in vitro*) estimated by hydroxyproline assay. [***p<0.01 for H (non specific siRNA) versus H (PKC-δ siRNA) and **p<0.01 for Ang-II (non specific siRNA) versus Ang-II (PKC-δ siRNA)]. **(B)** Western blot analysis showing expression status of phospho PKC-δ, phospho P38, phospho STAT3 (Tyr 705 and Ser 727) following PKC-δ siRNA treatment along with pathological hypertrophy (H) in comparison to hypertrophy alone *in vivo* as well as *in vitro*. RPL 32 was used as loading control.
Figure: 23 Expression of PKC-α and downstream survival kinase proteins

(A) Western blot analysis showing expression of phospho-Akt to total-Akt and phospho-ERK-1/2 to total-ERK-1/2 ratio were significantly increased in group E compared to either H or C. Phospho-Akt/total-Akt and phospho-ERK-1/2 to total-ERK-1/2 ratio were significantly increased in group H° compared to H and decreased in group E° compared to E. RP L32 was used as loading control. (B) Graph showing relative band intensity of phospho-Akt/total-Akt and phospho-ERK-1/2/toal-ERK-1/2 in group C, H, E, H° and E°. (For phospho-Akt/total-Akt: ***p<0.001 for E versus C; ##p<0.01 for H versus H°; ¶¶p<0.01 for E versus E°. For phospho-ERK-1/2/total-ERK-1/2: ***p<0.001 for E versus C; ##p<0.01 for H versus H°; ¶¶p<0.01 for E versus E°).
RESULTS

whereas significant reduction in caspase-9 activity was observed in group HX (2.5±0.3-fold, p<0.05) compared to H (Figure 24).

4.8. PKC-α dependent cell survival pathway during physiological hypertrophy

4.8.1. Inhibition of PKC-α down regulates associated survival mediators:

Pro-survival role of PKC-α during physiological hypertrophy was further confirmed by treating exercised mice group (E) with PKC-α siRNA. Significant down regulation in the levels of phospho and total PKC-α along with phospho-Akt and phospho-ERK-1/2 levels was observed in group E mice treated with PKC-α siRNA compared to non specific siRNA treatment (Figures 25A and 25B). Gö 6976 (chemical inhibitor of PKC-α) treatment showed similar results for phosphorylation of Akt and ERK levels (data not shown). Inhibition of PKC-α activity did not alter phosphorylation status of either Akt or ERK-1/2 during pathological hypertrophy (H) (data not shown).

4.9. Inhibition of PKC isoforms reverses cardiac adaptation during pathological and physiological hypertrophy

4.9.1 PKC-α inhibition activates PKC-δ during physiological hypertrophy:

PKC-α siRNA treatment during physiological hypertrophy, led to significant increase in the phospho-PKC-δ level in group E mice along with increase in ratio of cytosolic/mitochondrial cytochrome-c (8.74±0.2 fold, p<0.01) and cleavage of caspase-3 compared to non specific siRNA treated group E mice (Figure 26A). Significantly induced caspase-3 (10.54±0.06 fold, p<0.05) and caspase-9 (14.42±2.16 fold, p<0.05) activities were also recorded in PKC-α siRNA treated group E samples (Figure 26B). However, inhibition of PKC-α during pathological hypertrophy did not show alteration of phospho-PKC-δ expression and apoptotic markers in group H (data not shown).

Apart from induced apoptotic signaling, altered activation from PKC-α to PKC-δ resulted in compromised cardiac function with significantly increased LVDD and decrease in %FS in PKC-α siRNA treated group E mice, compared to non specific siRNA treated mice of the same group (LVDD:2.87±0.02mm vs 2.24±0.12mm; %FS: 49.8±0.02% vs 62.5±0.04%; p<0.05) (Figures 27A and 27B).
Figure: 24 Caspase-9 as a downstream survival protein

Caspase-9 activity assay showing changes in all the experimental groups (*p<0.05 for H versus E; †p<0.05 for E versus E^R).
Figure: 25 inhibition of PKC-α reduces expression of downstream target proteins

(A) Western blot analyses reveal successful knockdown of phospho and total PKC-α along with significant decrease in the phosphorylation level of Akt and ERK-1/2 in mice treated with PKC-α siRNA. RPL 32 was used as loading control. (B) Graph showing significantly decreased phospho-PKC-α along with significant decrease in the phosphorylation level of Akt and ERK-1/2 in mice treated with PKC-α siRNA compared with non specific siRNA treated mice [*p<0.05 for E (non specific siRNA) versus E (PKC-α siRNA)].
Figure: 26 PKC-α inhibition activates PKC-δ during physiological hypertrophy
(A) Western blot analysis showing inhibition of PKC-α in physiological hypertrophy mice with PKC-α siRNA resulting in significant phosphorylation of PKC-δ and translocation of active PKC-δ (41kD), cytosolic cytochrome-c and caspase-3 cleavage compared to non-specific siRNA treated physiological hypertrophy mice. RPL 32 was used as loading control. (B) Graph showing PKC-α siRNA treatment during physiological hypertrophy generation showed significant increase in caspase-3 and caspase-9 activities compared to non-specific siRNA treatment during physiological hypertrophy [*p<0.05 for E (non specific siRNA) versus E (PKC-α siRNA)].
4.9.2. Inhibition of PKC-δ activates PKC-α isoform and subsequent Akt-ERK 1/2 activation during pathological hypertrophy:

PKC-δ siRNA treated group H mice on the other hand, showed significant increase in the level of both phosho-PKC-α (5.73±0.33 fold, p<0.01) and total PKC-α (1.54±0.04 fold, p<0.01) level compared to non specific siRNA treated pathological hypertrophy group (Figure 28A). Inhibition of PKC-δ also significantly increased the expression of phosho-Akt (2.57±0.04 fold, p<0.01) and phosho-ERK-1/2 (2.61±0.02 fold, p<0.01) in this mice group (Figure 28A). However, PKC-δ inhibition did not alter expression of phosho-Akt and phosho-ERK-1/2 in group E (data not shown).

Inhibition of PKC-δ and subsequent activation of phosho-PKC-α level in group H resulted in notably improved cardiac function with significant decrease in LVDD (1.75±0.05mm, p<0.05) and increase in %FS (72.6±0.03%, p<0.05) compared to otherwise severely compromised functional parameters in non specific siRNA treated mice with pathological hypertrophy (Figure 28B).

4.10. Switching of PKC isoform through model dictates cardiac adaptation during physiological hypertrophy

4.10.1. Increased expression of PKC-δ in exercise withdrawal animals (E<sup>R</sup> group):

Expression of PKC-δ and-α was studied in exercised withdrawn animals with increasing period of rest (3, 7, 15, 20, 30 and 45 days post exercise). Phosphorylation of PKC-δ was observed in exercise withdrawn animals from the 15th day of rest and was maximally induced on the 45th day of rest period, as observed by western blot analysis. Phosphorylation of PKC-δ was increased by 2.73±0.3-fold, p<0.01 on the 15th day, 2.79±0.06-fold, p<0.01 on the 20th day, 2.88±0.15-fold, p<0.001 on the 30th day and 3.24±0.08-fold, p<0.01 on the 45th day of rest compared to exercised animals (E) (Figures 29A and 29B). However, phosphorylation of PKC-α was observed till the 3rd day after exercise withdrawal but could not be detected with increasing time of rest (Figure 29A).
Figure: 27 PKC-α inhibition improves cardiac function during physiological hypertrophy
(A) Graph showing significant increase in LVDD in mice treated with PKC-α siRNA compared to non specific siRNA treated mice [*p<0.05 for E (non specific siRNA) versus E (PKC-α siRNA)].
(B) Graph showing significant decrease in %FS in mice treated with PKC-α siRNA compared to non specific siRNA treated mice [*p<0.05 for E (non specific siRNA) versus E (PKC-α siRNA)].
Figure: 28 Inhibition of PKC-δ activates PKC-α isoform and modulates cardiac function during pathological hypertrophy

(A) Western blot analyses showing significantly increased phosphorylation of PKC-α, Akt and ERK-1/2 and PKC-α expression in pathological hypertrophy mice treated with PKC-δ siRNA. RP L32 was used as loading control. (B) Graph showing significant decrease in LVDD and increase in %FS in mice treated with PKC-δ siRNA compared to non specific siRNA treated mice [*p<0.05 for H (non specific siRNA) versus H (PKC-δ siRNA)].
4.10.2. Increased expression of hypertrophy marker genes in exercise withdrawal animals (E\textsuperscript{R} group) with time:

Expression of ANF and β-MHC was studied in exercised withdrawn animals with increasing period of rest (3, 7, 15, 20, 30 and 45 days post exercise). Expression of ANF and β-MHC was observed in exercise withdrawn animals from the 15th day of rest and was maximally expressed on the 45th day of rest period. Expression of ANF and β-MHC was increased by 1.22±0.23, p<0.01 fold for ANF and 1.10 ±0.03 fold, p<0.05 for β-MHC on the 15th day, 1.59±0.06-fold, p<0.001 for ANF and 1.99±0.06 fold, p<0.01 for β-MHC on the 20th day, 1.68±0.15 fold, p<0.001 for ANF and 2.10 ±0.03 fold, p<0.001 for β-MHC on the 30th day and 2.04±0.08-fold, p<0.01 for ANF and 2.23 ±0.03 fold, p<0.01 for β-MHC on the 45th day of rest compared to exercised animals (E). GAPDH was used as an internal loading control (Figures 30A and 30B).

4.10.3. Deterioration of cardiac function with time increment in exercise withdrawal animals (E\textsuperscript{R} group):

Cardiac function was also found to deteriorate from 15th day of rest and was almost equally compromised till 45th day of rest as evidenced by significantly increased LVDD and reduced %FS, compared to 0 or 3 days of post exercise rest group (Table-4).

Table-4: M-mode echocardiography data

<table>
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<th>Experimental Groups</th>
<th>LVDD in mm</th>
<th>FS</th>
<th>PW thickness in mm</th>
<th>IV septum thickness in mm</th>
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<tr>
<td>C</td>
<td>2.17±0.05</td>
<td>61±0.08%</td>
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<td>0.51±0.08</td>
<td>0.62±0.02</td>
</tr>
<tr>
<td>H\textsuperscript{*}</td>
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<td>55±0.02%</td>
<td>0.31±0.04</td>
<td>0.47±0.03</td>
</tr>
<tr>
<td>E</td>
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<td>62.5±0.04%</td>
<td>0.37±0.01</td>
<td>0.5±0.05</td>
</tr>
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<td>37.5±2.33%</td>
<td>0.49±0.02</td>
<td>0.67±0.03</td>
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Figure: 29 Increased expression of PKC-δ in exercise withdrawal animals with increasing period of rest

(A) Western blot analyses showing changes in expression of phosphorylated and total PKC-δ and PKC-α in exercise withdrawn animals (E^R) rested for different time periods. RPL 32 was used as loading control. (B) Graph showing relative band intensity of phospho-PKC-δ at different period of rest (3, 7, 15, 20, 30 and 45 days). **p<0.01 for E versus 15D; ##p<0.01 for E versus 20D; $$$p<0.001 for E versus 30D; ###p<0.01 for E versus 45D.
Figure: Increased expression of hypertrophy marker genes in exercise withdrawal animals with increasing period of rest.

(A) Expression profile of pathological hypertrophy markers (ANF and β-MHC) in exercise withdrawn animals (E<sup>α</sup>) rested for different time periods estimated by RT-PCR. GAPDH was used as loading control.

(B) Graph showing relative band intensity of ANF and β-MHC at different period of rest (3, 7, 15, 20, 30 and 45 days). (For ANF: **p<0.01 for E versus 15D; ###p<0.001 for E versus 20D; $$$p<0.001 for E versus 30D; ▲▲▲p<0.01 for E versus 45D. For β-MHC: *p<0.05 for E versus 15D; ##p<0.01 for E versus 20D; $$$p<0.001 for E versus 30D; ▲▲▲p<0.01 for E versus 45D).