3. Materials and methods

3.1 Animals used:

Male Wistar rats (*Rattus norvegicus*) were used as models of this study. They were procured from the National Institute of Nutrition (NIN), Hyderabad, India. The investigations conformed to the ‘Guidelines for the Care and Use of Laboratory Animals’ published by the US National Institute of Health (NIH Publication No. 85-25, revised 1996) and was also approved by the institutional ‘Animal Ethics Committee’, University of Calcutta (Registration No. 885/ac/05/CPCSEA), registered under the ‘Committee for the Purpose of Control and Supervision of Experiments on Laboratory Animals’, Ministry of Environment and Forests, Government of India.

All animals were maintained in a climate controlled, light regulated space with 12hr light and dark cycles in the departmental animal house of the University of Calcutta during the experimental periods. Water and standard rat chow were provided ad-libitum.

3.2 Generation of post MI model of study

3.2.1 Generation of myocardial infarction *in vivo* by left anterior descending coronary artery (LAD) ligation in adult rat

**Materials-**

1. Male Wistar rat (20 weeks of age)
2. 70% alcohol
3. Ketamine hydrochloride (Ketmin®50; Themis Medicare Ltd., India)
4. Xylazine hydrochloride (Indian Immunologicals Ltd., India)
5. Disposable syringe (1mL)
6. Silk suture
7. Operating instruments
8. Cotton
9. Betadine (Win-Medicare Pvt. Ltd., India)
10. 1X PBS (Phosphate buffered saline)

**Procedure**

20 week old male Wistar rats (*n* = 10), weighing 200± 20g were used to generate myocardial infarction, by ligating their left anterior descending coronary artery (LAD) as described by Pfeffer *et al.*, 1979, with slight modifications. Briefly, each rat was anesthetized with an i.p
(intra peritoneal) injection having a combination of 100 mg/kg body weight ketamine and 10 mg/kg body weight xylazine. Consequent to anaesthetic condition of the rat, a left thoracotomy was performed and a gentle pressure was applied on the right side of the thorax to exteriorize the heart rapidly. The left coronary artery was ligated by a silk suture between the pulmonary artery outflow tract and the left atrium. The heart was then returned to its normal position in the thoracic cavity and the thorax was immediately closed. Rats surviving 24hr post operation, were maintained on standard rat feed and water ad-libitum in the departmental animal facility. 

The experimental groups used in this study where myocardial infarction was generated were as follows- a) infarct region at 0 day MI, b) infarct region at 30 days post MI, c) infarct region at 120 days post MI, d) non-infarct region at 0 day MI, e) non-infarct region at 30 days post MI and f) non-infarct region at 120 days post MI. Due to high mortality during the post MI period and consequent non availability of the 120 days post MI group of study, myocardial infarction was alternatively generated in all the experimental rats by intra peritoneal injection of Isoproterenol hydrochloride. Thus Isoproterenol hydrochloride induced MI, was the mainstay method of model generation and use in this study.

3.2.2 Generation of myocardial infarction in vivo by intra peritoneal injection of Isoproterenol hydrochloride in adult rat

Materials-

1. Male Wistar rat (20 weeks of age)

2. 70% Alcohol

3. Ketamine hydrochloride (Ketmin®50; Themis Medicare Ltd., India)

4. Isoproterenol hydrochloride (Sigma-Aldrich, MO, USA)

5. 0.9% Sodium Chloride (NaCl) solution (Normal saline solution):
   - For 100mL- 0.9g of NaCl was dissolved in 100 mL deionised water and autoclaved for sterilization

6. 1X PBS

7. Disposable syringe (1 mL)

Procedure

Myocardial infarction was generated in 20 week old male Wistar rats (n= 10) by i.p. injection of Isoproterenol hydrochloride (dose- 100mg/ kg body weight/ day) dissolved in 0.9% saline solution for two consecutive days, as described by Korkmaz et al., 2009 and Mitra et al., 2013.
The generation of myocardial infarction by the administration of Isoproterenol hydrochloride is the non invasive method of choice since it has been well established to mimic the molecular pathophysiology of the heart that occurs during occlusion of the coronary artery (Korkmaz et al., 2009, Mitra et al., 2013). At the end of MI and post MI experimental periods, the rats were euthanized via intravenous (i.v.) Ketamine injection.

Rats assigned to 0 day MI group were sacrificed 12hr after the last Isoproterenol injection while those assigned to the 30 days and 120 days post MI groups were sacrificed on the 31st and 121st days after injection respectively. In addition, age matched control rats underwent i.p. injection with 0.9% normal saline for two consecutive days without Isoproterenol hydrochloride.

3.3 Generation of cardiomyocyte (in vitro) hypoxia- reoxygenation module of study

Isolation of neonatal rat cardiomyocytes

Materials-

1. Dulbecco’s modified eagle medium [DMEM] (pH 7.4) (GIBCO™, Invitrogen™, USA)
2. Fetal bovine serum [FBS]- EU Approved Origin (GIBCO™, Invitrogen™, USA)
3. Penicillin-Streptomycin (GIBCO™, Invitrogen™, USA).
4. Collagenase Type 2 (390 U/mg, Cat# M1E4817, Lot no. 4177; Worthington Biochemical Corporation, USA).
5. Laminin (L2020, 1MG; Sigma-Aldrich, USA)

[The Laminin source is from Engelberth-Holm-Swarm murine sarcoma (basement membrane)].

6. Sterile dissection instruments

Reagents to be prepared prior to isolation

- 10% FBS medium- FBS was added (v/v) to serum free DMEM to make required quantity of FBS medium [5mL of FBS added to DMEM to make up a final volume of 50mL FBS medium]. To this FBS medium, Penicillin-Streptomycin (1%) was added before commencing the experiment [500µL Penicillin-Streptomycin for 50mL FBS medium].
- Collagenase medium- 8mg collagenase type 2 was dissolved in 40mL of 10% FBS medium and filtered before use through a syringe driven filter unit (0.45µm) (Millipore Corporation, USA).
Laminin coating of petri dish and cover glass- Laminin L2020 was mixed with serum free DMEM to make 20μg Laminin/mL of media. This solution was spread over the surface of petri dish and cover glass evenly and kept overnight in the CO₂ incubator at 37°C.

Procedure (Chatterjee et al., 2011)

Hearts from -3 day old rat (Wistar rat) pups were dissected out after etherizing the pups and placed in a petri dish having 2-3 mL of 10% FBS medium. The hearts were subsequently minced into 1mm cubed pieces by a sterile scalpel. Minced hearts in 10% FBS medium were put in a small culture flask (25cm²) and the following steps were performed sequentially-

a) 10mL of Collagenase medium (80U/mL) was added to the flask, stirred for 5min at room temperature and debris allowed to settle down for a few seconds.

b) The supernatant was carefully transferred into a fresh, sterile 15mL centrifuge tube and centrifuged at 1500 rpm for 2 min to pellet down the cells.

c) The supernatant was discarded and the pelleted cells were resuspended in 3-4mL of fresh 10% FBS medium.

d) Steps a- c were repeated four times to digest all of the heart tissues.

e) Subsequently all the cell suspensions collected were transferred to a single, fresh 15mL centrifuge tube and centrifuged at 1500 rpm for 2 min to pellet down the cells.

f) The supernatant was discarded and cells were resuspended in 12mL of fresh 10% FBS medium. This cell suspension was poured into a medium sized flask (75cm²) and incubated for 45 min at 37°C with 5% CO₂ and 18% O₂ to attach the cardiac fibroblasts to the substratum of the flask.

g) After fibroblast attachment, remaining cell suspension was collected and plated on laminin coated petri dishes and cover glasses evenly. The cells were kept in the incubator with 5% CO₂ and 18% O₂ at 37°C for attachment and further growth (Chatterjee et al., 2011).

On culture day 2, the plated cells were incubated with fresh DMEM. On culture day 3, the petri dishes were checked for more than 80% confluency and the cells/myocytes were incubated without FBS for 6hr before experimental regimen. Ischemic condition was generated as described previously with slight modification (Mitra et al., 2013). Briefly, cardiomyocytes were subjected to 1% O₂ and 5% CO₂ for 5hr using the Galaxy 170R incubator (New Brunswick, Germany) in a glucose and serum free DMEM medium (Gibco™, USA). Cardiomyocytes were
reoxygenated for 8hr and 16hr respectively by elevating the O$_2$ level of the incubator to 18%, CO$_2$ percentage remaining the same. During reoxygenation tenure, the cardiomyocytes were maintained in DMEM (Gibco™, USA) supplemented with glucose and 10% FBS.

3.4 Determination of cardiac function by echocardiography

Materials-

1. Ketamine hydrochloride (Ketmin®50; Themis Medicare Ltd., India)
2. Xylazine hydrochloride (Indian Immunologicals Ltd., India)
3. Disposable syringe (1mL)
4. Ultrasound transmission gel (Parker Laboratories, USA)

Procedure

Two dimensional echocardiography was performed to determine cardiac function in vivo. Rats were anaesthetized with an i.p. injection of a combination of 100mg/ kg body weight Ketamine and 10mg/ kg body weight Xylazine. After removal of chest hair, the ultrasound transmission gel was evenly applied and cardiac function was assessed using the Vivis S5 ultrasound system (GE Medical Systems, India). M- mode views of the parasternal short axis were recorded with an 11MHz probe and used subsequently for cardiac function measurements. Three consecutive cardiac cycles were considered when measuring for left ventricular functional parameters. The functional state of the cardiac tissue was determined by measuring left ventricular diastolic diameter (LVDD), ejection fraction (EF), fractional shortening (FS) and end diastolic volume (EDV).

3.5 Protein extraction

Materials-

1. 1X PBS
2. In vivo lysis buffer

- Composition
  - Urea- 7M
  - Thiourea- 2M
  - 3-{(3-cholamidopropyl)dimethyl ammonio}propane sulfonic acid (CHAPS)
  - 1X EDTA free protease inhibitor cocktail tablet / 10mL of buffer volume (Roche, USA)
3. **In vitro** lysis buffer

- Composition for 1mL of buffer
  - M-PER® mammalian protein extraction reagent (Thermo Scientific): 1mL
  - EDTA : 5mM
  - EGTA (0.1M) : 10.0 μL
  - PMSF (0.1M) : 10.0 μL
  - DTT (1M) : 1.0 μL
  - Aprotinin : 10 μL (1-10 μg/mL or 80 μM)
  - Leupeptin : 10 μL (1-10 μg/mL or 2 mM)
  - Pepstatin A : 5 μL (5 μg/mL or 1mM)
  - Phosphatase Inhibitor Cocktail 2 (Sigma, MO): 10 μL

**Procedure**

For *in vivo* experiments: After the experimental period, hearts were dissected out and perfused with chilled 1X PBS. Then the heart tissues were homogenized in tissue lysis buffer using Dounce homogenizer for 30 minutes. The tissue lysates were then subjected to centrifugation at 12,500 rpm for 20 minutes at 4°C. The supernatants were collected from each sample and concentrations of protein were estimated by Bradford assay at 595nm using UV-VIS Spectrophotometer (Eppendorf, Germany).

For *in vitro* experiments: After experimental period, cells were harvested by cell scraper with 1X PBS and collected by centrifugation at 2000 rpm for 10 minutes at 4°C. Then the cells were resuspended in M-PER® Mammalian protein extraction reagent (Thermo Scientific, USA) containing protease inhibitors and kept in ice for 1hr. After that, the cell suspension was vortexed and centrifuged at 12,500 rpm for 20 minutes at 4°C. The supernatants were collected from each sample and concentrations of protein were estimated by Bradford assay at 595nm using UV-VIS Spectrophotometer (Eppendorf, Germany).

**3.6 Trypsin digestion and iTRAQ labelling**

**Materials**-

1. 8-plex iTRAQ reagent (AB Sciex, USA; iTRAQ™)
2. Dithiothreitol [DTT] - 25mM
3. Iodoacetamide [IAA] - 55mM
4. Modified Trypsin V511 (Promega, USA)
5. Ethanol

**Procedure (Maity et al., 2014)**

Proteomic analysis was performed with biological triplicate of all the six experimental conditions and two technical replicates of control per biological replicate. For this purpose, 8-plex iTRAQ labelling (AB Sciex, USA) was used after tryptic digestion of protein samples as per manufacturer’s instructions. Briefly, for each biological replicate, 70µg of protein from each sample was first reduced with 25 mM DTT for 30 min at 56°C and then treated with 55 mM IAA at room temperature for 15- 20 min for blocking the cysteine residues. The samples were then incubated with modified trypsin (Promega, V511) in a 1: 10 ratio for 18hr at 37°C. After digestion, the different samples were labelled with the eight different iTRAQ tags (113, 114, 115, 116, 117, 118, 119 and 121). The supplied tags were kept at room temperature for 15- 20 min before being resuspended in 70µL ethanol and mixed properly. To each of the digested sample a different reconstituted tag was mixed and kept at room temperature for 2hr to allow the tagging to occur. The sample corresponding to the tag used was noted for future reference. Each 8 plex experiment corresponds to each biological replicate. All the eight individual tagged samples were pooled together and mixed in a single centrifuge tube and subjected to drying under vacuum at 30°C using the vacuum concentrator (Eppendorf, USA).

3.7 Two dimensional separation of iTRAQ labelled peptides and MS/MS analysis

**Materials**-

1. SCX cartridge (AB Sciex, USA)- 5µm, 300Å bead and cartridge holder (AB Sciex, USA)

2. Buffer A (pH 3.0)
   - Composition
     - Ammonium formate- 8mM
     - Acetonitrile- 30% (v/v)
     - Formic acid- 1% (v/v)

3. Elution buffer with gradients (pH 3.0)
   - Composition
     - Ammonium formate- 35mM, 70mM, 100mM, 125mM, 150mM and 250mM
     - Acetonitrile- 30% (v/v)
     - Formic acid- 1% (v/v)
4. Buffer B

- Composition
  - Double distilled water
  - Formic acid- 1% (v/v)

5. Chromo LC Trap column- 200µm x 0.5mm

6. C-18 column (Eksigent)- 75µm x 15cm

7. Liner step gradient elution buffer

- Composition
  - Acetonitrile- 100%
  - Ammonium acetate- 10mM
  - Formic acid- 0.1% (v/v)

Procedure (Maity et al., 2014)

The first dimension separation of the pooled, iTRAQ labelled peptides was achieved by cation exchange chromatography (SCX) using a SCX cartridge (5µm, 300Å bead from AB Sciex, USA) and a cartridge holder (AB Sciex, USA). The pooled and dried peptides (each 8plex experiment) were reconstituted in 1mL buffer A (8mM ammonium formate, 30% v/v acetonitrile and 0.1% formic acid; pH- 3.0) and then applied to the SCX cartridge using a hand syringe system. The sample was fractionated using a step gradient of increasing concentration of ammonium formate based elution buffer (35 mM, 70 mM, 100 mM, 125 mM, 150 mM and 250 mM ammonium formate along with 30% v/v acetonitrile and 0.1% formic acid; pH- 3.0). 500 µL of each eluted fraction was collected and vacuum dried. Each dried fraction was reconstituted in 70 µL of loading buffer/ buffer A (100% H₂O, 0.1% formic acid).

The reconstituted fractions were then analysed on a TripleTOF 5600 (AB Sciex, USA) mass spectrometer coupled to an Eksigent NanoLC-Ultra 2D plus system. 10 µL of each fraction obtained previously was loaded onto a reverse phase peptide ChromoLC trap (200 µm x 0.5 mm) column and desalted at a flow rate of 3 µL/ min for 40 min. After desalting, the peptides were separated using an Eksigent C18 column (75 µm x 15 cm). Peptides were eluted from the column at a flow rate of 300 nL/ min using a linear step gradient of elution buffer (100% acetonitrile, 0.1% formic acid and 10 mM ammonium acetate) from 5- 30% over 100 min, 30-50% for 20 min, 50- 90% for 2 min and 90% for another 8 min. The LC eluent was analysed using the C18 column connected Nano spray pico tip (New Objectives, USA) which was coupled with the nanospray interface into the 5600TripleTOF system. Samples were analysed using a nebulising gas of 5, a curtain gas of 25, an Ionspray voltage of 2400 V, heater interface
temperature of 150ºC and declustering potential of 80V. The TripleTOF 5600 system was operated in an information dependent acquisition (IDA) mode with the (first) TOF/MS survey scan (350-1250 m/z) with 0.24 sec accumulation time. For the MS/MS product ion scan, a maximum of 10 precursor ions/ cycle were selected for fragmentation with a charge state from+2 to +5 only. The threshold precursor ion intensity/ abundance was set at more than 125 cps. Each MS/MS spectrum (100-1600 m/z) was accumulated for 0.1sec having a total cycle time of approximately 2.3sec. The acquisition was set so that former target ions were excluded for 10sec once they had undergone MS/MS fragmentation. The MS/MS spectra was acquired in the ‘high sensitivity mode’ with ‘adjust collision energy when using iTRAQ reagent’ setting.

### 3.8 Database search and analysis

**Materials**

1. Protein Pilot v4.0 software (AB Sciex, USA)
2. STRING v.10 software
3. Plotly 2.0 software

**Procedure (Maity et al., 2014)**

All the .wiff files containing MS and MS/MS spectra generated from TripleTOF 5600 for each 8plex iTRAQ experiment were submitted for database searching and quantitative analysis by Protein Pilot v4.0 software (AB Sciex). Paragon algorithm in a ‘Thorough ID’ search mode was employed against the ‘Rattus norvegicus’ database for the identification of proteins. The search parameters allowed modifications by IAA at cysteine residues, 8-plex peptide iTRAQ labelling of the N termini of peptides and of the side chains of lysine. 1% global protein level FDR (false discovery rate) was applied for protein identification.

The shortlisted protein accession numbers were submitted to UniProtKB for ‘ID MAPPING/RETRIEVAL’. This generated a list of 255 proteins out of 285 with UniProt id and respective gene names. Out of the remaining 30, 25 proteins were mapped to UNIPARC. 5 proteins were unmapped. The gene name list of 111 proteins that showed altered expression out of the 255 proteins was submitted to STRING v10 for the generation of predicted protein interaction network using the high (0.7) mode as the threshold. The interaction view was saved in high resolution .png format and then converted to .tiff image for representation. Further, STRING v10 was also used to perform GO and KEGG pathway enrichment analyses with the Benjamini-Hochberg corrected FDR being used to represent the processes enriched. From the molecular function classification and biological process classification, a summarized set of 9 functional categories were devised. Plotly 2.0 was used to generate the heat maps of all the 285 proteins
that were considered for the different experimental conditions to visualise the trend of alteration of the proteins.

### 3.9 Western blot analysis

**Materials**-

1. Protein extracts from cells and tissues

2. SDS-Polyacrylamide gel:
   - Resolving gel preparation recipe (for 10mL)
   - Recipe for (gel %) 6% 7.5% 10% 12.5% 15%
     - 40% Acrylamide (mL) 1.5 1.875 2.5 3.125 3.75
     - 1.5M Tris-Cl pH-8.8 (mL) 2.5 2.5 2.5 2.5 2.5
     - Sterile water (mL) 5.8 5.425 4.8 4.175 3.55
     - 10% SDS (μL) 100 100 100 100 100
     - 10% APS (μL) 100 100 100 100 100
     - TEMED (μL) 8 6 4 4 4
   - Stacking gel 5 % (for 4.0 mL)
     - 40 % Acrylamide 0.5mL
     - 0.5M Tris-Cl (pH6.8) 0.5mL
     - ddH₂O 2.8mL
     - 10% SDS 40.0μL
     - 10% APS 40.0μL
     - TEMED 4.0μL

3. 1X Tris-Glycine electrophoresis buffer: (for 1L):
   - Tris Base : 3.02 g (25 mM)
   - Glycine (Electrophoresis Grade) pH 8.3 : 18.8 g (250 mM)
   - 10 % SDS : 10mL

4. Protein Loading buffer (for 1mL):
   - LaemmLi buffer (BIO-RAD Laboratories, CA) : 950μL
   - β-mercaptoethanol (Sigma, MO) : 50μL

5. 1X Transfer Buffer (for wet transfer): (for 1L):
   - 25mM Tris Base - 3.03g
   - 192mM Glycine (Electrophoresis Grade) pH 8.3 -14.41g
6. Polyvinylidene fluoride (PVDF+) membrane (BIO-RAD Laboratories, CA, USA)

7. 1X Tris Buffered Saline (TBS) : (for 1L):
   - Tris : 3.028 g (25mM)
   - NaCl : 8.775 g (0.15M)

8. 1X TBS and 0.1% Tween-20 (v/v) [TBST]
   - 1X TBS : 500mL
   - Tween-20 : 500μL

9. 5% Milk TBST (w/v)
   - Non fat dry milk (BIO-RAD Laboratories, CA, USA) : 5g
   - 1X TBST : 100 mL

10. 5% BSA TBST (w/v)
   - BSA : 5g
   - 1X TBST : 100mL

11. Primary and secondary antibodies (Table 1)

12. Enhanced chemiluminescence (Immobilon Western chemiluminescence reagent, Millipore)
   - Luminol Reagent
   - Oxidizing Reagent

13. X-ray film (Kodak® Biomax™ MR, Sigma-Aldrich, Inc.; MO)

14. Developer and Fixer (Manual Medical X-Ray, Kodak, USA)

Table 1: Specification for antibodies and their dilutions

<table>
<thead>
<tr>
<th>ANTIBODY</th>
<th>CATALOGUE #</th>
<th>COMPANY</th>
<th>DILUTION</th>
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<tr>
<td>GRP 78</td>
<td>ab21685</td>
<td>Abcam</td>
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<tr>
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<td>------------------</td>
<td>------</td>
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<tr>
<td>(H+L) Secondary Antibody, HRP conjugate</td>
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</tr>
</tbody>
</table>

**Procedure**

20-50µg of protein for each experiment was fractionated by 6 - 15% SDS-PAGE, depending on the size (Kilo Dalton; kDa) of the protein of interest, at 30mA current. Fractionated total protein was transferred to methanol activated PVDF+ membrane at 100V for 1h using BIO-RAD Mini PROTEAN® 3 transfer apparatus which was subsequently blocked with 5% milk TBST for 1hr and 30 minutes at room temperature. The membrane was incubated with primary antibodies diluted in 5% BSA-TBST (Table 1) overnight at 4ºC. The membrane was then washed thoroughly with 1X TBST for three times, 10 minutes each, followed by incubation with Horseradish Peroxidase (HRP) -conjugated secondary antibodies diluted in 5% milk TBST for
2h at room temperature (Table 1). After that the membrane was washed thrice with 1X TBST for 15 minutes each. Final wash was done with 1X TBS for 10 minutes. Immunoreactive bands were visualized using enhanced chemiluminescence. Equal volumes of enhanced luminol reagent and oxidizing reagent were mixed to yield 0.125mL of solution/ cm² of the membrane and the membrane was incubated with this chemiluminescence substrate for 2 minutes at room temperature in dark. Then the membrane was exposed to X-ray film ranging for 10 seconds to 1 minute and developed using Developer and Fixer in dark. The blots were scanned and quantitated using GelDoc XR system and Quantity One® software version 4.6.3 (BioRad, Hercules, CA). Ribosomal protein L32 (RPL32) was used as loading control.

3.10 Haematoxylin and eosin staining of cardiac tissue sections

Materials-

1. Karnovsky’s fixative
   - Composition for 55mL solution
     - Paraformaldehyde- 2g
     - Sodium hydroxide- 1M (1/2 drops)
     - 25% Glutaraldehyde- 10mL
     - 0.2M Cacodylate buffer (pH 7.4)- 20mL

2. 10% KCl solution

3. 1 X PBS

4. Graded Alcohol (30%, 50%, 70%, 90%, absolute)

5. Cedar wood oil

6. Paraffin

7. L-blocks, Glass plate

8. Mayer’s Albumen

9. Xylene

10. Haematoxylin

11. Eosin

12. Vectashield (Vector Laboratories, USA)
Procedure

The isolated heart from all the groups (n= 5) was transferred to a petridish containing 1X PBS, as early as possible. Then the heart was perfused with 10% KCl to arrest at diastole. After that the heart was perfused with 1X PBS (chilled). The heart was then kept in Karnovsky’s fixative at 4°C till tissues became whitish.

The tissues (from fixatives) were first kept in 30% alcohol for 30 min with two changes. Then they were dehydrated with 50% alcohol for 30min with two changes. The tissues were then kept in 70% alcohol for overnight. On the next day the tissues were dehydrated through 90% and absolute alcohol (each for 30min with two changes) respectively. Finally they were immersed in Cedar wood oil and were kept for around two weeks till the tissues appeared transparent. All steps were done at room temperature. The next step was the removal of dehydrant from tissue. For that tissues were taken out from the cedar wood oil and then immersed in xylene for 10 min. The tissues were then changed in fresh xylene and kept for 10min.

After the tissues have been dehydrated and cleared, they were transferred to molten paraffin, kept in paraffin bath (60°C), for 30 min for infiltration. Then they were transferred to second change of fresh paraffin for 30 min. A rectangular cavity was made with copper L-blocks’ over a glass plate. The molten paraffin was poured into the cavity slowly up to the brim of the block. A hot scalpel was inserted inside the paraffin-filled cavity and stirred sidewise slowly to get rid of air bubbles, if any. The paraffin bed was allowed to cool down and harden keeping the upper layer, still liquid, due to continuous stirring of hot scalpel. Finally, a tissue piece was taken out from the paraffin bath and inserted to the paraffin-filled cavity slowly, with the cut surface facing down. The whole set up was allowed to cool down, taking caution not to disturb it. After the tissue got hardened (paraffin becoming opaque) the L-blocks were detached to take the block out. The block with the tissue was trimmed in proper shape for sectioning.

The tissue was cut longitudinally at 4μm thickness with help of a manual rotary microtome (Spencers, India). The paraffin sections were floated over 37°C water bath and heat stretched over a hotplate and adhered to clear, ultrathin glass slides (Riviera, India) previously coated with Mayer’s albumen.

Paraffin embedded sections were dewaxed in xylene and rehydrated in graded ethanol series to water inside Coplin jars.

Xylene1 - 5 min Xylene2 - 5 min. Absolute ethanol - 5 min 95% ethanol - 5 min. 70% ethanol - 10 min 30% ethanol - 3 min dH2O - 3 min.
Haematoxylin solution was added dropwise on the tissue sections on the slides and incubated for 30sec- 1min. The haematoxylin stain was matured by the process of mordanting. For mordanting, the slides with after haematoxylin incubation were dipped in tap water till the stained sections became blue from brown colour.

The sections were the dehydrated in graded ethanol series- 50% ethanol- 5min, 70% ethanol – 10min. Consequently the slides were dipped in Eosin solution for 30sec- 1min. Thereafter, excess eosin was removed by briefly washing the slides with 95% ethanol (few dips). The slides were then immersed in absolute ethanol for 3min; excess alcohol wiped off from the edges of the slides and then mounted with Vectashield. After drying, the slides were observed under a bright filed microscope.

3.11 Immunofluorescence study of cardiomyocytes

**Materials**

1. 1X PBS
2. 4% Paraformaldehyde
3. 0.1% Triton X-100 in 1X PBS
4. 2% BSA, 0.1% Na-azide solution
5. Primary and Secondary antibodies
6. Vectashield [with DAPI] (Vector Laboratories, USA)

**Procedure**

Cardiomyocytes plated on laminin coated slides were fixed in 4% paraformaldehyde, dissolved in 1X PBS, for 10 minutes and then permeabilized with 0.1% Triton X-100 in 1X PBS for 30 minutes. The cells were then blocked with 2% BSA and 0.1% sodium azide for 1hr at room temperature. Subsequently cells were incubated with monoclonal antibodies [Anti Desmin antibody, Table 1, dilution- 1:300] diluted in 1X PBS overnight at 4°C within a moist chamber, followed by washing with 1X PBS for three times (15 minutes each). Alexa fluor® secondary antibodies diluted in 1X PBS [Alexa Fluor® 488 (Molecular Probes), dilution- 1:300] were used to incubate cells against primary antibody for 2 hours in dark at room temperature. The slides were extensively washed with 1X PBS for three times, 30 minutes each. After mounting with Vectashield [with DAPI] (Vector Laboratories, CA), cells were visualized under confocal laser scanning microscope (IX81, Olympus, Singapore).
3.12 Filter Trap assay for Desmin aggregates

Materials-

1. Lysis buffer
   - Composition
     - Tris-HCl (pH 8.8)- 50mM
     - NaCl- 100mM
     - MgCl₂- 5mM
     - Non idet P-40 (NP-40)- 0.5% (w/v)
     - 1mM EDTA containing protease inhibitor cocktail

2. Resuspension buffer
   - Composition
     - Tris-HCl (pH 8)- 20mM
     - MgCl₂- 15mM
     - DNAseI- 0.5mg/mL

3. Bradford reagent

4. 1X SDS PAGE sampling buffer (Biorad, CA)

5. Nitrocellulose membrane- pore size 0.25mm

6. SDS buffer
   - Composition
     - Resuspension buffer + 0.1% (v/v) SDS

7. Primary and secondary antibodies (Table1)

8. Immobilon™ Western chemiluminescence HRP substrate (Millipore, USA)


Procedure

Filter-trap assay was performed as described previously with slight modification (Mitra et al., 2014; Bohl et al., 2009). In brief, the myocardial tissue was homogenized in lysis buffer and centrifuged at 15000g for 10 min. The pellet fraction was resuspended in a resuspension buffer and protein concentration was estimated by Bradford Assay. 10 µg of protein extract for each sample was boiled in 100 µL of 1X SDS- PAGE sampling buffer for 5 min. The volume of the
sample was made up to 200 µL with the resuspension buffer and loaded on a nitrocellulose filter (pore size 0.25mm), using a dot blot apparatus. The membrane was then washed with 300µl of the SDS buffer with added 0.1% SDS, three times on the apparatus. The membrane was further incubated overnight at 4ºC with polyclonal primary antibody to Desmin (Abcam, USA) at a dilution of 1:1000 followed by HRP conjugated secondary antibody (ThermoFisher Scientific, USA) at a dilution of 1:4000 for 1hr. Immunoreactive spots were visualized using Immobilon™ Western chemiluminescence HRP substrate (Millipore). The blots were scanned and quantified using GelDoc XR system and Quantity One software version 4.6.3 (Bio-Rad, CA). 60S ribosomal protein L32 (RPL32) was used as loading control.

3.13 Calpain1 inhibitor and siRNA treatment of cardiomyocytes

Materials-

1. ALLN (N-Acetyl-Leu-Leu-norleucinal) [Sigma Aldrich, USA] dissolved in DMSO

2. Calpain1 specific siRNA (Flexitube siRNA, catalogue no. SI01495088, Qiagen, USA)

3. Non specific siRNA (AllStars Negative Control siRNA, catalogue no. SI3650318, Qiagen)

4. HiPerfect Transfection Reagent (Qiagen)

Procedure

ALLN (Sigma) [dissolved in DMSO] was administered at a final concentration of 50µM (Chae et al., 2007) in serum and glucose supplemented medium to neonatal rat cardiomyocytes during 8hr and 16hr reoxygenation schedules after a hypoxic treatment of 5hr. Control and hypoxic cardiomyocytes were administered DMSO without ALLN.

Cardiomyocytes which had undergone hypoxiawere transfected with either siRNA against Calpain1 (Catalogue no. SI01495088, to block the expression of Calpain1 or negative control siRNA (AllStars Negative Control siRNA, Catalogue no. SI3650318) using HiPerFect Transfection Reagent as per manufacturer’s protocol. Briefly, 256 ng siRNA was diluted in 100 µL culture medium without serum. 20µL HiPerFect Transfection Reagent was added to the diluted siRNA and mixed by vortexing. The mixture was incubated for 10 minutes at room temperature (15- 25°C) to allow the formation of transfection complexes. Then, the complexes were added drop-wise onto the cells in 60mm culture plates containing 4mL culture media. The plate was gently swirled to ensure uniform distribution of the transfection complexes. The cells were incubated with the transfection complexes under their normal growth conditions for 8h and 16h of reoxygenation time points.
3.14 ATP assay

**Materials**-

1. ATP releasing buffer

2. ATP determination kit (Molecular Probes®; Life Technologies)

**Procedure**

Ventricular tissue samples from the infarct region as well as cultured cardiomyocytes were lysed in ATP releasing buffer (Trichloroacetic acid- 1% and EDTA- 4mM) and ATP levels were measured using ATP determination kit (Molecular Probes®; Life Technologies) as per manufacturer's protocol. ATP content was calculated as nanomolars of ATP per microgram of protein.

3.15 Blue Native PAGE of cardiac tissue homogenate and in-gel activity assay

**Materials**-

1. Homogenization buffer
   - Composition
     - 1X PBS
     - Protease inhibitor cocktail

2. Aminocaproic acid buffer (pH 7.0)
   - Composition
     - Aminocaproic acid- 1.5M
     - Imidazole- 50mM

3. Anode buffer (pH 7.0)
   - Composition
     - Imidazole- 25mM [pH of the solution to be adjusted to 7 with HCl]

4. Cathode buffer (pH 7.0)
   - Composition
     - Tricine- 50mM
     - Imidazole/HCl- 7.5mM [Imidazole/HCl is an Imidazole solution where the pH has been adjusted to 7 with HCl]
5. Blue cathode buffer

- Composition
  - Cathode buffer + 0.02% (w/v) of 5% Coomassie Blue G solution

6. 5% Coomassie Blue G solution

- Composition for 50mL stock solution
  - 5% weight/ volume
  - Aminocaproic acid solution- 750mM
  - Imidazole- 50mM
  - EDTA- 0.5mM

7. 10% Dodecyl maltoside

- Composition
  - Resuspended 1gm of Dodecyl maltoside powder in 5mL of sterile water to obtain a 20% (w/v) stock solution. 500µL of 20% stock solution diluted in 1mL of sterile water to obtain 10% (w/v) Dodecyl maltoside solution.

8. In gel activity assay buffers (Table 2)

9. Precast Bis-Tris gradient native gel- 4%- 13% (Biorad, CA)

**Procedure**

Preparation of cardiac tissue homogenate and blue native gradient gel electrophoresis was done as described earlier (Diaz et al., 2009; Wittig et al., 2007). Briefly, 50mg ventricular tissue from the infarct zone of different experimental groups was homogenised in a small volume (100-200µL) of 1X PBS with added protease inhibitors at 4°C. The homogenate was centrifuged at 600g for 10 min at 4°C to remove debris. The supernatant was collected and total protein content was determined by Bradford assay. To 200µg of protein, solubilisation buffer (1.5M aminocaproic acid + 50mM Bis-Tris) was added to bring the final volume of the solution to 100µL. To this solution, 12µL of 10%w/v lauryl maltoside was added and incubated on ice for 20 min. The solution was then centrifuged at 21,130g for 30 min at 4°C and the supernatant was collected. 6µL of Coomassie blue G250 was added to the supernatant and loaded into the well of a precast Bis-Tris gradient native gel (4-13%, Biorad) at 40µg of protein per lane. The cathode chamber of the Criterion gel electrophoresis tank (Biorad) was filled with blue cathode buffer (Tricine-Imidazole with added Coomassie G250) while the anode chamber was filled with anode buffer (Imidazole). The gel was run at 4-7°C at 100 volts till the dye had reached
2/3rd of the gel. The blue cathode buffer was then changed for the clear cathode buffer (without Coomassie G250) and the gel run till the dye had reached the end of the gel.

After the electrophoresis had been completed the gels were separately incubated in specific in-gel assay buffers to assess the activity of the mitochondrial complexes I, II, III, IV and V. The compositions of the assay buffers are given in a tabular form below:

**Table 2: In gel activity assay buffers**

<table>
<thead>
<tr>
<th>Complex</th>
<th>Buffer composition</th>
<th>Method</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex I</td>
<td>0.1M Tris-HCl (pH 7.4), 1mg/mL NBT (Nitroblue tetrazolium), 0.14 mM NADH</td>
<td>Incubation of the gel in the assay buffer at room temperature with gentle shaking</td>
<td>Development of blue-purple colour</td>
</tr>
<tr>
<td>Complex II</td>
<td>50mM Phosphate buffer (pH 7.4), 84mM Succinic acid, 0.2mM Phenazine methosulfate, 2mg/mL NBT, 4.5mM EDTA, 10mM KCN</td>
<td>Incubation of the gel in the assay buffer at room temperature with gentle shaking</td>
<td>Development of blue-purple colour</td>
</tr>
<tr>
<td>Complex III &amp; IV</td>
<td>DAB (3,3’-diaminobenzidine), 50mM Phosphate buffer, cytochrome C, sucrose</td>
<td>Incubation of the gel in the assay buffer at room temperature with gentle shaking</td>
<td>Development of brown colour</td>
</tr>
<tr>
<td>Complex V</td>
<td>35mM Tris, 270mM glycine, 14mM MgCl2, 5mM ATP, 0.2% Pb(NO3)2</td>
<td>Incubation of the gel in the assay buffer at 37°C with gentle shaking</td>
<td>Development of a white colour</td>
</tr>
</tbody>
</table>

### 3.16 ROS activity assay

**Materials**

1. DCFDA (2”,7”-dichlorofluorescin diacetate) ROS assay (Abcam, USA)
Procedure

ROS was estimated from tissue lysates using a DCFDA (2',7'-dichlorofluorescin diacetate) based assay (Abcam). Equal amounts of tissue from the infarct and remote regions of all groups (0 day MI, 30 and 120 days post MI) and from control rats were lysed in 1X lysis buffer to yield protein extracts whose concentrations were determined by Bradford assay. Equal amounts of protein extracts were used to determine ROS activity in a microplate reader at excitation/emission wavelengths of 485nm/535 nm following assay protocol.

3.17 RNA isolation from cardiac tissue

Materials-

1. TRIzol Reagent (Invitrogen™, CA)
2. Chloroform
3. Isopropanol
4. 75% Ethanol
5. 1X TE buffer

- Composition
  - Tris-HCl (pH 7.5)- 10mM
  - EDTA- 1mM

Procedure

Heart tissues taken from ventricular region of adult rats were snap frozen in liquid nitrogen and then homogenized in TRIzol Reagent. All the samples were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. 0.2mL of chloroform/mL of TRIzol Reagent was added. Tubes were then shaken vigorously by hand for 15 seconds and were incubated at room temperature for 2-3 minutes. Samples were centrifuged at 12,500 rpm for 15 minutes at 4°C. Uppermost clear aqueous phase was transferred to a fresh tube. Precipitation of RNA from aqueous phase was done by mixing with isopropyl alcohol. 0.5mL of isopropanol, per mL of TRIzol Reagent, used for the initial homogenization was added. After invert mixing, samples were incubated at room temperature for 10 minutes and then centrifuged at 12,500 rpm for 10 minutes at 4°C. RNA wash was done by adding 75% Ethanol. RNA pellet was collected by centrifuging at 10,000 rpm for 5 minutes at 4°C. RNA pellet was air dried and was dissolved in 50μl of 1X TE buffer. For proper mixing tubes were tapped gently and were incubated at 58°C for 10 minutes. Quantification of the RNA was done
by spectrometric reading at 260nm and purity was checked at 260/280nm using UV-vis Spectrophotometer (Eppendorf, Germany) using the following equation:

\[
\text{RNA Concentration} = \text{OD} \times 40 \times \text{df} \quad \text{[OD= optical density; df (Dilution Factor) = Total vol. / Added sample vol.]}\]

### 3.18 Reverse Transcription

**Materials**-

1. Isolated RNA

2. Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen Life Technologies)
   - 10 X RT-PCR buffers
   - RNase Inhibitor
   - MuLV RT Enzyme

3. Random Hexamer (Invitrogen™, CA)

4. Nuclease-Free Water (Promega, WI)

5. Deoxyribonucleotide (dNTP) mix (Sigma-Aldrich, Inc., MO)

**Procedure**

Each 20 μL RT reaction mix contains 1μg mRNA, 0.25 mM each of dATP, dCTP, dGTP, dTTP, 300ng of Random hexamers and nuclease free water. RNA was denatured at 65°C for 5 min and then mixture of 1 unit of MulV RT enzyme, 10X RT-PCR buffer, RNase Inhibitor was added to that on ice and then programmed at 25°C for 10 min, 42°C for 1 h and 72°C for 10 min.

### 3.19 Real time PCR

**Materials**-

1. RT reaction products

2. Power SYBR Green™ PCR Master Mix (Applied Biosystems, USA)

**Procedure**

Relative quantification of PCR-amplified products was done by real-time PCR with Power SYBR Green™ PCR Master Mix using ABI7500 (Applied Biosystems). Mitochondrial precursor tRNAs for Lysine, Leucine and Methionine and total tRNAs for Lysine, Leucine and Methionine were the genes whose expressions were studied by real time PCR and RPL32 was
used as a reference gene to normalize expression of other genes. All real time PCR reactions were done in triplicate after an initial incubation at 95 °C for 10 min and then a PCR cycle at 95 °C for 30s, 59 °C for 30s, and 72 °C for 30s for 40 cycles. Relative gene expression was quantified by comparative “ct” ($2^{-\Delta\Delta ct}$) method. The primer sequences are given below-

**Table 3**: Primer sequences of rat mitochondrial tRNA

<table>
<thead>
<tr>
<th>Mitochondrial Gene</th>
<th>Primer sequence 5’- 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor tRNA Lysine Forward</td>
<td>CCATTGTACTAGAAATAGTCCCTCTCTAA</td>
</tr>
<tr>
<td>Reverse</td>
<td>TGTGGAGATTTGTTGTCTCTAACT</td>
</tr>
<tr>
<td>Precursor tRNA Leucine Forward</td>
<td>AAGTGCTCCCAACCAATTTATG</td>
</tr>
<tr>
<td>Reverse</td>
<td>TACGCAATTACCTGGCTCTG</td>
</tr>
<tr>
<td>Precursor tRNA Methionine Forward</td>
<td>AATTCTCCGTCTACCAATACA</td>
</tr>
<tr>
<td>Reverse</td>
<td>TATGGGCCCGATAGCTTTAGT</td>
</tr>
<tr>
<td>Total tRNA Lysine  Forward</td>
<td>TTGCGAAGCTTAGAGCGTTA</td>
</tr>
<tr>
<td>Reverse</td>
<td>TGTGGAGATTTGTTGTCTCTAAACT</td>
</tr>
<tr>
<td>Total tRNA Leucine Forward</td>
<td>GAGCCAGGTAATTCGTAAGA</td>
</tr>
<tr>
<td>Reverse</td>
<td>GGATTTGAACCTCTGGGAACA</td>
</tr>
<tr>
<td>Total tRNA Methionine Forward</td>
<td>AAGGTCAGCTAACTAAGCTATCG</td>
</tr>
<tr>
<td>Reverse</td>
<td>GGGAAGGGTTTAGACCAACAT</td>
</tr>
</tbody>
</table>

**3.20 Statistical Analysis**

Results have been expressed as mean± S.E.M. of greater than three experiments. The data obtained following the different experimental conditions other than proteomics, were subjected to test for homogeneity of variance prior to application of univariate ANOVA, following the norms of generalized linear model. In all instances, the homogeneity of variance was judged through Levene’s test using SPSS 11.0 software. Following the significant value of the Levene’s test, the data was transformed as log (n+1) and applied to ANOVA with post hoc Tukey test. Results with p value < 0.05 have been considered as significant. For proteomic study, multiple comparison was carried out through Kruskal-Wallis non-parametric ANOVA with Steel- Dwass- Critchlow- Fligner multiple pair wise comparison with Bonferroni correction to constrain dispersion. Protein expressions were quantified by densitometric scanning using Quantity One software, and were normalised to 60S ribosomal protein L32 (RPL32) which was used as loading control.