Materials and Methods
2.1. Materials

**Plasmid Constructs:** The AP-1 reporter plasmid [pAP1-luc] containing luciferase reporter gene driven by a basic promoter element and tandem repeats of AP-1 (7X) binding sequence was from Stratagene, USA. Expression plasmid for human c-Jun (p RSV-cJun) was originally obtained from Dr. M Karin, University of California San Diego (Smeal et al., 1989, Angel et al., 1989). The rat Fra-1 expression plasmid pRK7-Fra-1 was a kind gift from Dr. M Busslinger, Research Institute of Molecular Pathology, Vienna Biocenter, Austria (Bergers et al., 1995). The human Fra-1 promoter-reporter construct was a kind gift from Dr. SP Reddy, Johns Hopkins University, Baltimore, USA (Adiseshaiah et al., 2003).

**Cell Culture:** Dulbecco’s Modified Eagle’s Medium (DMEM), Penicillin Streptomycin solution, Amphotericin B, Trypsin-EDTA, Sodium bicarbonate, L-Norepinephrine, ATP, Angiotensin II, Escort IV transfection reagent, were purchased from Sigma Aldrich, USA unless mentioned otherwise. Tissue culture plastic ware was purchased from Nalge Nunc international, Denmark and Greiner, Germany. Lipofectamine-2000 and Fetal Bovine Serum were procured from Invitrogen Life Technologies, USA. Tissue culture grade water was prepared in the laboratory by using Quartz distillation system (Bhanu Scientific, Bangalore, India). The filter unit for medium filtration was purchased from Nalge Nunc International, Denmark and filter membranes (0.22μm pore size) were from Millipore. Rat embryonic cardiac muscle cell line H9c2 was procured from National Centre for Cell Sciences, Pune, India (originally from ATCC, USA). Phosphate buffered saline (PBS), pH 7.4 was prepared in tissue culture grade water and autoclaved.

**Antibodies:** Rabbit polyclonal antibodies for c-Jun (SC-1694), JunB (SC-46), c-Fos (SC-52), FosB (SC-48), Fra-1 (SC-605), JNK (SC-474), p38 (SC-535), phospho-JNK (SC-6254) and phospho-p38 (SC-7973) were from Santa Cruz Biotechnology Inc, CA, USA. Mouse monoclonal antibodies for phospho-ERK and polyclonal antibodies against ERK were kind gift from Dr. Vijay Kumar, ICGEB, New Delhi. Horseradish peroxidase conjugated anti-mouse (# 7076) and anti-rabbit IgG (C 172-1013) antibodies were obtained from Cell Signaling Technology, Inc. USA and Biorad Laboratories, USA respectively. Sheep anti-
rabbit antibody (Cyc3 conjugated, C 2306) was from Sigma Aldrich, St Louis, USA.

**Biochemical and Molecular Biology Reagents:** All biochemicals were procured from Sigma-Aldrich, St Louis, USA and Qualigens, India unless mentioned otherwise. All restriction enzymes and DNA modifying enzymes were procured from MBI Fermentas, Inc. USA. Taq DNA polymerase was from Klentaq. Bacterial growth media (LB and Agar) was purchased from HiMedia. Oligonucleotide primers were synthesized from Genosys, USA. RNA Isolation reagent was from Ambion, Inc. Poly dI-dC and Sephadex G-50 were purchased from Amersham Pharmacia Biotech, Inc. Pre-stained protein molecular weight marker was obtained from MBI Fermentas, Inc. USA. Pharmacological inhibitors PD98059, SB203580 and LY294002 were from Sigma, USA. Dicumarol was a kind gift from Dr. Pushkar Sharma, NII, New Delhi.

**Luciferase Assay System** was bought from Promega Corporation, Madison, USA.

**PVDF and Nitrocellulose Membranes** were purchased from Amersham Biosciences, USA and MDI, India. X-Ray Films were purchased from Kodak. All Plastic wares were from Volex, Italy. Filters (0.22µM) used for sterilizing various reagents were purchased from Millipore (MA, USA). Filter Papers were purchased from Whatman Ltd. (Madistone, England).

**Enhanced Chemiluminiscence (ECL) detection reagent** was purchased from Santa Cruz Biotechnology, Inc. USA.

**Radiochemicals:** γ-³²P ATP and α-³²P-dCTP was purchased from Amersham Biosciences and BRIT, India.

**Double Stranded Oligonucleotides** harboring the AP-1 (Collagenase TRE: TGACTCA) for gel mobility shift assay was obtained from Stratagene, USA. AP-1 (Angiotensin II TRE sequence) was custom synthesized from Sigma, USA. Phosphorothioate modified antisense and sense oligonucleotides for fra-1 were custom synthesized from Sigma, USA.
2.2. Methods

2.2.1. Cell Culture

H9c2, rat cardiac myoblasts were cultured as monolayers in DMEM containing 10% FBS, Glutamine (2 mmol/L), Penicillin (100 U/ml), Streptomycin (100 μg/ml) and Amphotericin B (2.5 μg/ml) in humidified, 5% CO₂ containing incubator at 37°C to 60-85% confluence. Upon reaching confluence, cells were sub-cultured in the ratio 1:4 with 0.025% trypsin-EDTA solution (Gabai et al., 2000).

2.2.2. Agonist Treatment

H9c2 cells were grown to 70% confluence, incubated with serum free medium for 24 hours and treated with Norepinephrine (2 μM and 100 μM), Angiotensin II (100 nM and 1 μM), and ATP (100 μM) for required time periods and processed for respective experiments. The dishes in which norepinephrine was added were supplemented with 100 μM ascorbic acid to prevent oxidative degradation (Simpson, 1983).

2.2.3. Preparation of Chemically Competent DH5α Cells

_E. coli_ strain DH5α was streaked on a LB-Naladixic Acid (25 μg/ml) plate (and grown overnight at 37°C. One single colony was inoculated into 25 ml LB media (with Nalidixic Acid, 25 μg/ml) and grown overnight at 37°C with shaking at 200 rpm. Five hundred micro litre of the overnight culture was added to 100 ml LB media (without antibiotic) and grown at 37°C with shaking for 2-3 hours at 200 rpm. When the culture attained an OD₆₀₀ of 0.4-0.5; cells were chilled on ice for 2 hours, centrifuged at 3600g for 15 minutes at 4°C and the supernatant was discarded. Cells were resuspended in 100ml of chilled Trituration Buffer (100 mM CaCl₂, 70 mM MgCl₂, 40 mM CH₃COONa) and incubated on ice for 45 minutes. The cells were then collected by centrifugation at 1800g for 15 minutes at 4°C and the pellet was resuspended in 5ml of ice-cold Trituration buffer containing 15% glycerol and stored in aliquots of 300 μl. at -80°C.
2.2.4. Transformation of Competent DH5α Cells with Plasmid DNA

Three to five nano-gram of plasmid DNA or equivalent quantity of ligation reaction was added to 100 µl of competent cells, mixed well by gentle tapping and incubated on ice for 30 minutes. Heat shock was given for 90 seconds at 42°C followed by a rapid chilling for 5 minutes. LB medium (900 µl) was added and the transformation mix was allowed to revive for 1 hour at 37°C. Appropriate volume of the transformation culture plated on LB agar plate supplemented with appropriate antibiotic. Plates were incubated at 37°C for 12-16 hours until the colonies were apparent.

2.2.5. Plasmid DNA Isolation (Mini Preparation)

Single colony culture was grown in 2 ml LB medium with appropriate antibiotic (such as 100 µg/ml ampicillin) at 37°C overnight with vigorous shaking. The culture was then pelleted at 5000 rpm for 5 minutes; the supernatant was drained out by leaving about 50 µl of LB. The cell pellet was then resuspended in the remaining LB by vortexing followed by the addition of 150 µl of solution II (0.2 N NaOH, 1% SDS). The suspension was mixed by inversion and incubated on ice for 5 minutes. Three hundred micro litres of ice-cold solution III (5 M Potassium acetate 60 ml, glacial acetic acid 11.5 ml, H₂O 28.5 ml) was added, incubated on ice for 5 minutes and then centrifuged for 5 minutes at 13,000 rpm. The supernatant was transferred in to a fresh tube and the plasmid DNA was precipitated by adding 900 µl of 100% ethanol. After incubation at room temperature for 15 minutes, DNA was collected by centrifugation at 13,000 rpm for 15 minutes. The pellet was washed with 70% ethanol, dried and resuspended in 10-20 µl of sterile distilled water or TE.

2.2.6. Isolation of Plasmid DNA by Alkali Lysis Method (MIDI PREP)

Plasmid DNA was isolated essentially as described by Sambrook et al., (1989). One milli litre inoculum of E coli (DH5α) cells containing the desired plasmid was added to 100ml LB medium containing appropriate antibiotic. The culture was incubated at 37°C overnight in shaker incubator at 220 rpm. Cells were collected by centrifugation at 5000 rpm for 15 minutes at 4°C and the pellet was resuspended in 2ml of ice cold Solution I (25 mM Tris-HCl (pH 8.0), 10 mM
EDTA, 50mM Glucose) and incubated on ice for 10 minutes. Four milli litre of freshly prepared Solution II (0.2 N NaOH, 1% SDS) was added, mixed gently by inverting the tubes 3-4 times and incubated at room temperature for 10 minutes. Three ml of ice cold Solution III (3 M Potassium acetate, pH 5.2) was added, mixed thoroughly and incubated on ice for 10 minutes. Cell debris was removed by centrifugation at 13,000 rpm for 15 minutes at room temperature. The supernatant was transferred to fresh GSA bottles, 0.6 volume of isopropanol was added and the tube was incubated at room temperature for 15 minutes. Centrifugation was carried out at 13,000 rpm for 15 minutes and the supernatant was drained out. The pellet was washed with 70% ethanol, dried and resuspended in 1ml of TE with RNAse (final concentration of 1μg/ml). Following incubation at 37°C for 20 minutes, the DNA was deproteinated with phenol chloroform (1:1 vol/vol), precipitated by ethanol and finally resuspended in 500 μl of TE.

2.2.7. Spectrophotometric Estimation of Nucleic Acids

The quantity and purity of plasmid was determined by measuring absorbance at 260 nm and 280 nm. The concentration of ds DNA was calculated by taking the Abs_{260} = 1 = 50 μg/ml. The concentration of ss RNA was calculated by taking the Abs_{260} = 1 = 40 μg/ml and the concentration of ss DNA was calculated by taking the Abs_{260} = 1 = 33 μg/ml.

2.2.8. Restriction Digestion

Restriction digestion was carried out in the appropriate buffer according to the manufactures instructions (MBI Fermentas, Inc. USA).

2.2.9. Ligation Reaction

Thirty-nano gram of restriction digested and purified insert DNA was ligated with 10 ng of linearized vector DNA in 20μl volume in 1X Ligase buffer and 5U of T4 DNA ligase (MBI Fermentas). Reactions were carried out at 16°C overnight.

2.2.10. Uniform end Labelling by Random Primers

DNA fragments purified from the Agarose gels were labeled with α^{32}P-dCTP by random primer labeling according to the manufacturer’s protocol (MBI Fermentas). The unincorporated nucleotides were removed by passing through a
Sephadex G50 spun column. The purified probe was denatured in boiling water bath for 5-7 minutes and then chilled on ice for 5 minutes prior to hybridization.

2.2.11. Polymerase Chain Reaction:

Polymerase chain reaction was carried out in 50μl volume with the following ingredients:

- 10X Buffer: 5μl
- dNTP Mix (10mM): 1μl
- Primer Mix (50ng/μl): 1.5μl
- Template: 50pg
- Klen Taq DNA polymerase: 1U
- Water: Up to 50μl.

Cycling condition was as follows:

1. 95°C for 10 minutes — Denaturation
2. 95°C for 1 minute — Denaturation
3. 57.5°C for 30 seconds — Annealing
4. 72°C for 1 minute — Extension

Step 2-4 35 times.
72°C for 10 minutes — Final Extension

Primers used for PCR Reactions:

Forward primer: 5'-AGCTCAAGCTTATGACTGCAAAGATG-3'
Reverse primer: 5'-CCACCTAGGTCTCTCTTCTCTCTTAAAATG-3'

2.2.12. Agarose Gel Electrophoresis of DNA

Agarose gel electrophoresis was done as described in Sambrook et al., 1989. Routinely 0.5 X TBE (0.045 M Tris-borate, 0.001 M EDTA, pH 7.9) buffer was used and Ethedium bromide was added to the gel (final concentration of 0.5 μg/ml) prior to casting the gel. For most of the purposes 0.8% agarose gels were used.
2.2.13. Nuclear Extract Preparation

H9c2 cardiac myoblasts (70%-80% confluence in 100 mm dishes), after appropriate treatments, were washed twice in ice cold PBS and lysed in 1 ml of buffer containing (20 mM HEPES (pH 7.9), 20% glycerol, 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X100, 0.2 mM PMSF, 1 mM DTT, 4 µg/ml Leupeptin, 10 µg/ml Aprotinin and 2 µg/ml Pepstatin) on ice. Homogenate was centrifuged at 4°C for 15 minutes at 2000rpm. The nuclear pellet was resuspended in 50 µl of Buffer B (20 mM HEPES (pH 7.9), 20% glycerol, 500 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X100, 0.2 mM PMSF, 1 mM DTT, 4 µg/ml Leupeptin, 10 µg/ml Aprotinin and 2 µg/ml Pepstatin) and incubated on ice for 45 minutes with intermittent tapping. The homogenate was then centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatant was aliquoted and snap frozen at -80°C (Sindhu et al, 2004).

2.2.14. Protein Estimation

Protein concentrations in the extracts were estimated by modified Bradford method (Bradford, 1976; Kruger, 1994). Appropriate aliquots of protein were diluted in 0.15M Nacl (100µl), One milliliter Bradford Reagent was added and the OD was recorded after 5 minutes at 595nm in a spectrophotometer (Cary, Eclipse).

2.2.15. End labeling Reaction

For the preparation of double stranded oligonucleotide probes for gel mobility shift assay, 10ng of respective double stranded oligonucleotides were incubated with 20µCi γ³²P ATP (6000 Ci/m mole, Amersham), 2 units of T4 polynucleotide kinase in 1X polynucleotide kinase buffer (Epicentre) in 20ul reaction at 20°C overnight. The labeled DNA was purified by passing through Sephadex G50 spun column to remove unincorporated nucleotides. Normally, the DNA was eluted in 100 ml volume that contained 20,000- 40,000 CPM per microlitre (1-2 µl was used for gel mobility shift assay per reaction).

2.2.16. Gel Mobility Shift Assay

Gel mobility shift assay was done essentially as described by (Sindhu et al., 2004). DNA-protein binding reactions were carried out in 40µl binding buffer containing
20 mM HEPES (pH 7.9), 5% glycerol, 60 mM NaCl, 1.5 mM MgCl₂, 1.0 mM EDTA, 1.0 mM DTT, 1 µg poly dI-dC, 32P-labelled oligonucleotide harboring the AP-1 binding site (40,000 cpm) and 6 to 8 µg of nuclear protein on ice for 40 minutes. Competition reaction was carried out using 50 and 100-fold molar excess of unlabeled DNA. Protein-DNA complexes were fractionated on 8% polyacrylamide gel with 0.5X TBE at 200V for 3 hours at 4°C. The dried gel was visualized either by autoradiography or in a phosphorimager (Fuji, FLA 5000). The images were processed and specific bands were quantitated by densitometry wherever required. The oligonucleotides used for the gel mobility shift assay were (1) AP-1 (Collagenase TRE): 5’-CGC TTG ATG AGT CAG CCG GAA-3’ and (2) AP-1 (Angiotensin II): 5’-CAC ATT TTG AGT CAG CCA CAG-3’.

2.2.17. Preparation of Whole Cell Lysate for Western Analysis

Cell lysates were prepared according to Bakiri et al, (2002). Adhering cells were washed with ice cold PBS, scrapped and collected by low speed centrifugation (2000 rpm) at 4°C. Cells were lysed in ice-cold buffer containing 50 mM Tris (pH 7.6), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 0.5 µg/ml Leupeptin, 0.5 µg/ml Pepstatin, 0.5 µg/ml Aprotenin and 1 mM Phenyl Methyl Sulfonyl Fluoride (PMSF). During p-MAPK and p-AKT experiments the cell extracts were prepared in the above mentioned buffer supplemented with 1 mM Sodium Orthovanadate and 10 mM Sodium Flouride. Cell lysates were then centrifuged at 13,000 rpm (at 4°C) and the supernatants were saved at -80°C.

2.2.18. Immunoblot Analysis

Equal quantities of protein samples (whole cell lysates, 100 -150 µg) were resolved by Sodium Dodecyl Sulphate/Polyacrylamide Gel Electrophoresis (SDS/PAGE; 10% Acrylamide) as described in Sambrook et al., 1989. Part of the protein gel (selected based on the molecular weight of the protein of interest) were sliced out and transferred to Nitrocellulose/PVDF membranes in Towbin’s buffer (25 mM Tris, 192 mM glycine and 20% methanol). Following transfer equal protein loading was confirmed by staining the membranes with Ponceau Red (Amresco). The membranes were washed with Tris-buffered-saline (TBS: 10 mM Tris pH 7.4, 150 mM NaCl) and blocked for 1 h at 37°C with 5 % (w/v) skimmed
milk powder in TBS containing 0.05% Tween-20 (TBST). Blots were incubated overnight at 4°C with respective primary antibodies at appropriate dilutions in TBST supplemented with 0.5% (w/v) skimmed milk powder. Next day the blots were extensively washed with TBST and incubated for 2 h at 37°C with respective secondary antibody (at appropriate dilutions) in TBST supplemented with 0.5% (w/v) skimmed milk powder. Following removal of the secondary antibody, blots were extensively washed and developed using the Enhanced Chemiluminescence detection system (ECL). Whenever necessary, the signals were quantified densitometrically in phosphorimager (Fuji, FLA 5000). When stripping was required, the membranes were incubated with 62.5mM Tris-HCl (pH 6.8), 2% SDS, 0.1M 2-mercaptoethanol for 1 hour at 56°C and washed extensively with TBS before being subjected to reblocking and probing (Rebollo et al., 2000). The remaining part of the gels (which was not transferred) containing separated proteins were visualized by Coomassie brilliant blue staining (0.1% W/V CBB dissolved in 25% V/V methanol and 10% V/V acetic acid) whenever required.

2.2.19. Isolation of RNA

Cells were washed in ice cold Phosphate buffered Saline (PBS) and 1 ml of RNA Wiz (Ambion, 1 ml per 100 mm dish) was added. The lysate was then incubated at room temperature for 5 minutes and 0.2 volume of chloroform was added followed by a brief but vigorous shaking. Following incubation at room temperature for ten minutes, the mixture was centrifuged at 10,000g for 15 minutes at 4°C. The upper aqueous phase was carefully removed in a clean RNAse free tube and RNAse free water was added (0.5X-starting volume). One starting volume of isopropanol was then added and the mixture was incubated at room temperature for 10 minutes. Generally, the samples were stored at -80°C at this step. Prior to use, the samples were centrifuged at 10,000g for 20 minutes at 4°C to pellet down the RNA. The pellet was then washed with 70% ethanol (ice cold) followed by centrifugation at 10,000g for 5 minutes at 4°C. The pellet was air dried and resuspended in appropriate amount of RNAse free water.
2.2.20. Northern Blotting

Northern blot was essentially done as described by Sambrook et al., (1989). RNA samples were prepared by mixing 20 μg of RNA in 9μl H2O, 4μl of 5X MOPS buffer, 7μl formaldehyde and 20μl of formamide. Samples were incubated at 65°C for 15 minutes, chilled on ice and briefly centrifuged. Two microlitres of loading dye (50% glycerol, 1 mM EDTA pH 8, 0.25% Bromophenol blue and 0.25% Xylene Cyanol) and 1μl Ethidium Bromide (0.5 μg/ml) was added and loaded on to a 1.2% formaldehyde agarose gel. Electrophoresis was carried out at 80V for ~3hours in 1X MOPS buffer (0.02 M MOPS, 8 mM Sodium Acetate, 1 mM EDTA). Following electrophoresis, quality of RNA was ascertained by visualizing under UV transilluminator and the gel was rinsed twice in autoclaved water (10 minutes each) to facilitate the removal of formaldehyde. The gel was then equilibrated in 10 X SSC (1.5 M NaCl and 0.15 M Sodium Citrate) for 10 minutes followed by transferring the RNA on to Nylon membrane (N+) by overnight capillary transfer (Sambrook et al., 1998). Next morning, the membrane was rinsed in 2X SSC and the RNA was cross-linked to the membrane in a UV cross linker (Stratagene). Pre-hybridization was done in solution containing 0.5 M Sodium phosphate (pH 7.2), 7% SDS, 1 mM EDTA (pH 8.0) and 100 μg/ml sheared denatured salmon sperm DNA at 60°C for 4 hours following which denatured uniformly labeled cDNA probe (random labeled, 10^6 cpm/ml final) was directly added to the pre-hybridisation solution. Hybridisation was then carried out overnight at 65°C. Filters were washed sequentially in 2X SSC, 0.1% SDS; 1X SSC, 0.1% SDS; 0.5X SSC, 0.1%SDS and 0.2X SSC, 0.1% SDS (10 minutes each) at 60°C. Filters were air-dried and exposed to imaging plate or X-ray film. The image was developed either by phosphorimager (Fuji, FLA 5000) or X-ray developer.

2.2.21. Immunocytochemistry

Cells were grown to 70% confluence on glass cover slips in 35 mm dishes, incubated in serum-free medium for 24 hours, and stimulated with required agonists. At appropriate time points, cells were fixed with chilled methanol for 5 minutes, air dried, permeabilized with 0.1% Triton-X 100 in PBS (pH 7.4) for 30 minutes and then blocked with 3% BSA (1 hour at 37°C). Fixed cells were then
incubated with primary antibody (1:100 dilution) in 0.3% BSA in Phosphate buffered saline containing 0.05% Tween 20 (PBST) overnight at 4°C. After three washes with PBS, samples were incubated with Cy3 conjugated sheep anti rabbit secondary antibody (diluted in PBST with 0.3 % BSA to 1:200) for 1 h at room temperature in dark. Cells were finally washed thrice in PBS and finally incubated with 0.5 μg/ml DAPI in PBS (to identify the nuclei). Cells were again washed in PBS and the cover slips were mounted in 80 % glycerol containing PBS on glass slides and examined under fluorescence microscope coupled to a video camera (Nikon, TE300).

### 2.2.2. Animals and Treatments

Male Sprague Dawley rats (200-300g body weight, 2-3 weeks of age) were housed four to five per cage in animal house where normal rat food and water was available. Rats were injected with either saline (as control) or 3 different doses of norepinephrine i.e., 0.05mg/kg body weight, 0.2 mg/Kg body weight, 2.5 mg/ kg bodyweight intra-peritoneally.

### 2.2.23. Immunohistochemistry

Hearts were excised 2 and 4 hours post treatment. Five micron transverse sections of the myocardium were made in cryotome and mounted on to glass slides. Sections were air dried for 2 minutes and fixed immediately in chilled acetone for 10 minutes. Slides with sections were stored at -20°C till used. Endogenous peroxidase activity was removed by incubating the sections in methanolic H$_2$O$_2$ (0.5%) for 30 minutes. After rinsing in TBS (pH 7.4) the sections were permeabilised by incubating in 0.1% Triton X-100 in TBS and non-specifically blocked with 1% BSA for 1hr. The sections were then incubated with primary antibody (1:100; in TBST with 0.1% BSA) overnight at 4°C. After washing with TBS, the sections were incubated with Horseradish Peroxidase conjugated secondary antibody (1:200 dilutions in TBST with 0.1% BSA) for 1 hour. Peroxidase activity was then visualized with 3’, 3’-diaminobenzidine (DAB, 0.04 %) and H$_2$O$_2$ (0.015%) in 0.05 M TBS. The reaction was stopped in water. Sections were air dried, dehydrated in ethanol, cleared in xylene and mounted in DPX (Dibutylphthalate) and photographed in a Nikon microscope. Immunocytochemical controls for the experimental sections were incubated as
above with omission of the primary antibody (Hannan et al, 1993). Heamatoxylin-Eosin staining of the sections were done to see the integrity of myocardium.

2.2.24. Transient Transfection

Transient transfection was done in 35mm dishes. Sub-confluent cells were transfected with the reporter constructs with the help of Escort IV transfection reagent (Sigma-Aldrich, USA) and expression constructs with Lipofectamine-2000 reagent (Invitrogen Life Technologies) according to manufacturer's instructions. Normally 1-2 µg of plasmid DNA was used per dish and cells were incubated for 8-10 hrs with the DNA-transfection reagent in serum & antibiotic free medium following which complete (antibiotic and serum containing) medium was added. After 12 hours, cells were again treated with serum free medium for 24 hours followed by the requisite agonist treatments. The cells were either observed under microscope for fluorescence (GFP) or processed for luciferase reporter activity. Reporter assays were done 44- 48 hours post transfection.

2.2.25. Treatment with Antisense fra-1 Oligonucleotides

H9c2 myocytes were grown to 60-70% confluence and treated with serum free medium for 24 hours in presence and absence of antisense phosphorothioate modified oligonucleotides against fra-1 (5 µM, 5'-GAAGTCTCGGTACAT-3') as described in Urakami et al., 1997. As a control, cells were also treated with a sense oligonucleotide (5 µM, 5'-ATGACTGCAAAGATG-3', not complementary to the antisense, Urakami et al., 1997). Replenishment with fresh serum free medium with sense and anti-sense oligonucleotides were done every 12 hours. Agonist treatment was done for a period of 48 hours (in presence and in absence of both oligonucleotides) and cell survival was assayed by MTT reagent.

2.2.26. MTT Cell Survival Assay

Cells were grown in 35 mm dishes to 70% confluence and serum starved for 24 hours followed by agonist treatment. The method followed with some minor modifications has been taken from Denizot and Lang, 1986. After required time period, cells were incubated with MTT reagent [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, 1.5 mg/ml in DMEM] for 2 hour at 37°C. The
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MTT reagent was then removed and 100 µl 10% SDS in 0.01 N HCl was added. Following incubation overnight in at 37°C, the extracts were collected in 64 well microtitre plates and the optical density were read in a Bio-Rad microplate reader (Hercules, CA) at 570 nm.

2.2.27. Luciferase Reporter Assays

Transfected cells were lysed in cell lysis buffer (Promega) and the lysates were assayed for luciferase activities using the luciferase assay reagent (Promega). Readings were taken in a luminometer (Turner scientific). Normalization of transfection efficiency was done either by the estimation of total protein used for the luciferase assay (Burch et al, 2004) or by co-transfection with a β-galactosidase expression plasmid (followed by β-galactosidase reporter assay). We observed that either way it gave reproducible results.

Statistical Analysis

Experiments were done at least thrice with similar results until mentioned otherwise in the respective figure legends. Data (MTT, reporter assay etc.) represented as mean value +/- standard deviation.