MATERIALS AND METHODS
2.1. Materials

**Constructs and plasmids:** NF-kB reporter plasmid [(NF-kB)$_6$-TK-luciferase] was a kind gift from Dr. Rakesh K Tyagi, Special Centre for Molecular Medicine, JNU, New Delhi. The FKHR-promoter reporter plasmid [pGL3b-FOXOBD-luciferase] (Brunet et al., 1999) was a kind gift from Prof. G.C. Grosveld, Department of Genetics, St Jude Children's Research Hospital, Memphis, USA. Wild type expression vector for FOXO1 [pcDNA3-FKHR] and its dominant active mutant (FKHR-AAA) (Galili et al., 1993; Eric et al., 1999) were kind gifts from Prof. W. Bai. The Foxo1-GFP fusion plasmid [pEGFP-FK-WT] was a kind gift from Prof. Terry G Unterman, University of Illinois, Chicago, USA.

**Cell culture:** Dulbecco's Modified Eagle's Medium (DMEM), Penicillin Streptomycin solution, Amphotericin B, Trypsin-EDTA, Sodium bicarbonate, L-Norepinephrine, ATP, Isoproteronol, Escort IV Transfection Reagent were purchased from Sigma unless mentioned otherwise. Tissue culture plastic-ware was purchased from Nunc, USA and Greiner, Germany. Lipofectamine-2000 transfection reagent and Fetal Bovine Serum was procured from Invitrogen Life Technologies, California, USA. Tissue culture grade water was prepared in the laboratory by using Quartz Distillation system (Bhanu Scientific, Banglore, India). The filter unit for medium filtration was purchased from Nunc and filter membranes (0.22 μm 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, pH 7.4, was prepared in tissue culture grade water and autoclaved.

**Antibodies:** Rabbit polyclonal antibodies for Caspase-3 (sc-7148), PARP (sc-7150), Bcl-2 (sc-783), Bax (sc-20067), Phospho-ERK (sc-7383), ERK (sc-94), Phospho-JNK (sc-6254), JNK (sc-474), Phospho-p38 (sc-739), p-38 (sc-535), IkBβ (sc-946), P50 (sc-7178) were bought from Santa Cruz Biotechnology Inc, CA, USA. Antibody for FKHR (#9462), Phospho-FKHR (Ser 256, #9461S), Phospho-Akt Ser (473, #9271S), IkBa (#9242), NF-kB (p65, #3034) were purchased from Cell Signaling Technology, USA. NF-kB (p50, IMG 442) was from Imgenex India, Horseradish peroxidase conjugated anti-mouse (#7076) and anti-rabbit IgG antibodies were obtained from Cell Signaling Technology, Inc and
from Bio-Rad, USA respectively. Sheep anti-rabbit Cyc3 (#C2306) antibody was purchased from Sigma, USA.

**Biochemicals** were procured from Sigma-Aldrich, St Louis, USA and Qualigens, India unless mentioned otherwise. Poly dI-dC and Sephadex G-50 were purchased from Pharmacia Biotech Inc. Pre-stained protein molecular weight marker was obtained from MBI Fermentas, USA.

**Luciferase assay system** was purchased from Promega Corporation, Madison, USA.

**PVDF and Nitrocellulose** membrane for western blot was purchased from Amersham Biosciences, USA and MDI, India.

**Enhanced Chemiluminescence (ECL)** detection reagent was purchased from Amersham Biosciences, USA.

**Radiochemicals**: γ-P\(^{32}\) ATP was purchased from Amersham Biosciences and BRIT, India.

**Double stranded oligonucleotides** used in gel mobility shift assays were either purchased from Promega Corporation, USA for AP-1 (Collagenase TRE 5’-CGCTTGATGAGTCAGCCGAA-3’); Banglore Genei for NF-\(\text{AB}\): (5’-AGTTGAGGGGACTTTCCCAGGC-3’); or custom synthesized from Sigma, USA, for FKHR: (5’-GATCTCATGTTGTGTTACTTAAGGGATC-3’); NRF2: (5’-AATTGCTGACTCAGCATTAC-3’[MARE])

## 2.2. METHODS

### 2.2.1. Cell Culture

**H9c2 Myoblast culture**

H9c2 myoblasts were cultured and maintained in DMEM (high glucose) medium reconstituted with 10% heat inactivated fetal bovine serum containing 100 units/ml penicillin, 100 μg/ml Streptomycin and 2.5 μg/ml Amphotericin B, at 37°C in humidified chamber with 5% CO\(_2\) (Bonavita et al., 2003). Upon reaching confluence (in 2-3 days), monolayer of cells were washed in PBS, treated with 0.025% trypsin-EDTA solution and sub-cultured (1:4 ratio). After reaching 70% confluence cells were kept in serum free medium overnight followed by treatment
with required agonists (NE (2 μM and 100 μM), H₂O₂ (200 μM), Isoproterenol (100 μM), Insulin (100 nM) for requisite time periods.

**Neonatal Primary rat cardiomyocyte culture**

The primary myocytes were isolated from neonatal rats hearts (1-2 days) according to the method described in Claycomb et al., 1981. In brief, 2-3 hearts were finely minced in the ice cold cell buffer containing 8.10 g/l NaCl, 0.40 g/l KCl, 0.60 g/l Na₂HPO₄·H₂O and 0.90 g/l glucose. The minced hearts were treated with 0.5 mg/ml collagenase and 0.25 mg/ml hyluronidase (in cell buffer) for 15 min at 37°C. The cell debris obtained from the first digestion was discarded and subsequent digests (30 minutes each for 3-4 times) were collected in 5ml cell buffer and kept on ice. The pool of cells was finally centrifuged at 1000 rpm for 10-15 minutes, washed twice in cell buffer and finally resuspended in DMEM with 10% FBS followed by plating in 100 mm dish. Primary myocyte culture was then incubated in CO₂ incubator at 37°C for two hours to enable the attachment of fibroblasts (panning). The supernatant was then transferred to another dish for two more rounds of panning. Finally, the enriched cardiomyocytes were plated in dishes of appropriate size and grown for 48 hours prior to any treatment.

**2.2.2. Transient Transfection**

Transient transfection of H9c2 myoblasts (with various reporter plasmids and expression vectors) was done in 35 mm dishes with sub-confluent cells either with Lipofectamine 2000 (Life Technologies, USA) or Escort IV transfection reagent (Sigma, USA) according to the manufacturer’s instructions. One to two microgram of plasmid DNA was routinely used per 35 mm dish. Cells were incubated in transfection reagents in serum-antibiotic free medium for 8-10 hrs. Subsequently, cells were re-fed with fresh medium and kept for next 12 hours. Finally, cells were again kept in serum free medium for 12 hours followed by the requisite agonist treatments. The cells were either observed under microscope for fluorescence (GFP) or processed for luciferase reporter activity after 6-8 hours (36-48 hours post transfection).
2.2.3. Preparation of competent DH5α cells

E. coli strain DH5α was streaked on a LB-Nalidixic Acid (25 μg/ml) plate and grown overnight at 37°C. One isolated colony was inoculated into 25 ml LB media (with Nalidixic Acid 50 μg/μl) and grown overnight at 37°C with shaking at 200 rpm. Five hundred microgram 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 OD of 0.4-0.5, cells were chilled on ice for 2 hours, centrifuged at 3600 g for 15 minutes at 4°C and the supernatant was discarded. Cells were resuspended in 100 ml 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 1800 g for 15 minutes at 4°C and the pellet was resuspended in 5 ml of ice-cold Trituration buffer containing 15% glycerol and stored in aliquots of 300 μl at -80°C.

2.2.3. Transformation of competent DH5α cells with plasmid DNA

Three to five nano-gram of plasmid DNA 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 culture was allowed to revive for 1 hour at 37°C. Hundred microlitre of the culture was then plated on LB agar supplemented with appropriate antibiotic (50 μg/ml Ampicillin or 50 μg/ml Kanamycin). Plates were incubated at 37°C for 12-16 hours until the colonies were apparent.

2.2.4 Plasmid DNA isolation (Mini Preparation)

Single colony culture was grown in 2 ml LB medium with appropriate antibiotic (such as 50 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.

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

Plasmid DNA was isolated essentially as described by Sambrook et al., 1989. One milliliter inoculum of DH5α cells containing the desired plasmid was added to 100 milliliter LB medium containing the appropriate antibiotic. The culture was then incubated at 37°C overnight in a shaker incubator. Cells were then collected by centrifugation at 5000 rpm for 15 minutes at 4°C. The pellet was resuspended in 2 ml of ice cold Solution I (25mM Tris-HCl [pH 8.0], 10mM EDTA, 50mM Glucose) and incubated on ice for 10 minutes. Four ml of freshly prepared Solution II (0.2N 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 (5M Potassium acetate 60ml, glacial acetic acid 11.5 ml, H2O 28.5 ml) was added, mixed thoroughly and incubated on ice for 10 minutes. Centrifugation was carried out at 13,000 rpm for 15 minutes at room temperature. Supernatant was transferred (avoiding the cell debris) 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 discarded. The pellet was washed with 70% ethanol, dried and resuspended in 1ml of TE containing RNase (final concentration of 1 μg/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.

2.2.6. Spectrophotometric estimation of nucleic acids

The quantity and purity of nucleic acids in solution was determined by measuring absorbance at 260 nm and 280 nm. The concentration of ds DNA was calculated by taking the Abs260 = 1 = 50 μg/ml. The concentration of ss RNA was calculated
by taking the $\text{Abs}_{260} = 1 = 40 \mu g/ml$ and the concentration of ss DNA was calculated by taking the $\text{Abs}_{260} = 1 = 33 \mu g/ml$.

### 2.2.7. 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 $\mu g/ml$) prior to casting. For most of the purposes 0.8% agarose gels were used. The gel was visualised under the UV transilluminator for DNA samples.

### 2.2.8. End labelling reaction:

Ten nanogram of double stranded oligonucleotide [(AP-1,NF-kB, FKHR,NRF2{MARE})] was incubated with 20 $\mu$Ci $[\gamma^{32}\text{P}]$ATP (6000 Ci/ mole), T4 Polynucleotide Kinase (1 unit) in 1X Polynucleotide Kinase buffer containing 70 mM Tris-Cl pH 7.6, 10 mM MgCl2, 50 mM DTT in 20 $\mu$l reaction at 20°C overnight. The labelled DNA was purified by passing through Sephadex G-50 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 ml was used for gel mobility shift assay per reaction).

### 2.2.9. 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 A containing (20 mM HEPES pH 7.9, 20% glycerol, 10 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.1% Triton-X 100, 0.2 mM PMSF, 1 mM DTT, 4 $\mu$g/ml Leupeptin, 10 $\mu$g/ml Aprotenin and 2 $\mu$g/ml Pepstatin) on ice. Homogenates were centrifuged at 2000 rpm at 4°C for 15 minutes. The nuclear pellet was resuspended in 30 $\mu$l 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-X 100, 0.2 mM PMSF, 1mM DTT, 4 $\mu$g/ml Leupeptin, 10 $\mu$g/ml Aprotenin and 2 $\mu$g/ml Pepstatin) and incubated on ice for 45 minutes with intermittent tapping. The homogenates were then centrifuged at 10,000 rpm at 4°C for 15 minutes. The supernatant containing nuclear extracts were then aliquoted into (10 $\mu$l each) and snap frozen at –80°C (Sindhu et al., 2004).
2.2.10. Protein Estimation

Modified Bradford method (Bradford, 1976; Kruger, 1994) was used for the estimation of protein concentration for nuclear and total cell extracts. Appropriate aliquots of proteins were diluted to 100 µl and 1ml of Bradford Reagent was added. After 5 minutes, OD was recorded at 595 nm in a Spectrophotometer (Cary Eclipse).

2.2.11. Gel Mobility Shift Assay

DNA protein binding reaction was carried out in 40 µl 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 dl-dC, γ³²P-labelled probe (40,000 cpm) and 6-8 µg nuclear extract on ice for 40 minutes as described in Sindhu et al., 2004. Competition reaction was carried out using 100-fold molar excess of unlabeled DNA. Protein-DNA complex were fractionated on 8% polyacrylamide gel with 0.5 X TBE at 200 V for about 3 hours at 4°C. The dried gel was developed either on X-ray film or in phosporimager, Fuji FLA 5000.

2.2.12. FACS Analysis

After appropriate treatment (H9c2 cells grown in 60 mm dishes), cells were collected by trypsinization followed by centrifugation at 2000 rpm at 4°C (for adherent cells) and by centrifugation of the culture medium (for floating cells). The pellet was suspended in 500 µl ice-cold PBS followed by the addition of 5 milliliter chilled ethanol (added drop by drop). The cells were then stored at -20°C until use. Cells were then pelleted at 2000 rpm, evenly resuspended in 500 µl PBS followed by adding 5 µl serum (FBS) and incubated at room temperature for 30 min. The cells were then centrifuged at 2000 rpm and pelleted. The cell pellets were then resuspended in 400 µl PBS containing 20 µl of 1mg/ml propidium iodide and 5 µl of Dnase free RNase (1mg/ml) and incubated in dark at 37°C for 1 hr. Reading was taken within 2 hours for flow cytometry (BD Biosciences).

2.2.13. DNA Fragmentation

Following agonist treatment (100 µM NE or 200 µM H₂O₂) H9c2 cells were washed in ice cold PBS before followed by lysis in 100 mM NaCl, 10 mM Tris HCl (pH 8.0), 25 mM EDTA, 0.5% SDS, 0.2 mg/ml Proteinase K (Turner et al.,...
1998). The lysates were then kept at 55°C for 16 hrs. Following RNase treatment (2μg/ml) for 3 hours at 37°C, the lysates were further purified by extraction with phenol-chloroform. The DNA (genomic) was then precipitated by 0.6 volume of isopropanol and washed with 70% alcohol before resuspending in 100 μl TE. The DNA samples were run on 1.5% agarose gel for 1-2 hours at 80 Volt and stained with ethidium bromide.

2.2.14. Nuclear condensation with DAPI staining

To visualize the nuclear morphology, H9c2 cardiac myoblasts were grown on coverslips and fixed with ice cold methanol for 5 min at 4°C. Coverslips were air dried and washed with PBS before incubating with DAPI or Hoechst dye 33258 (1 μg/ml in PBS) for 15 min at 37°C as previously reported (Kirshenbaum and Moissac, 1997). Coverslips were then thoroughly washed in PBS twice 15 min each at 37°C before mounting on glass slides and seen under UV filter in Fluorescence microscope (Zeiss) to observe nuclear morphology.

2.2.15. Cell Viability Assay

Cell survival was analyzed using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazollium bromide MTT method (Denizot and Lang, 1986) as reported previously, with minor modifications. Cells were grown in 35 mm dishes to 70% confluence and serum starved for 12 hours followed by agonist treatment. After required time period, cells were incubated with MTT reagent 1.5 mg/ml in DMEM) for 2 hour at 37°C. The 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 micro-titre plates and the optical density were read in a Bio-Rad microplate reader (Hercules, CA) at 590 nm.

2.2.16. Preparation of whole cell extracts

After the requisite agonist treatment, H9c2 cells were washed twice with ice cold PBS. Cells were then scraped in PBS and collected at 2000 rpm at 4°C. Cells were then lysed in ice-cold buffer containing 50 mM Tris pH 7.6, 400 mM NaCl, 1 mM EDTA, 1mM EGTA, 1% NP-40, 1 mM Sodium Orthovanadate, 10 mM Sodium Fluoride, Leupeptin 0.5 μg/ml, Pepstatin 0.5 μg/ml Aprotinin 0.5 μg/ml, PMSF 1mM (Bakiri et al., 2002). The cell lysates were clarified by centrifugation at
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1300 rpm for 10 min at 4°C and the supernatants were collected. Protein estimation was done by Bradford method as described above.

2.2.17. Immunoblot analysis.

Equal quantities of protein samples (nuclear or whole cell lysates) were resolved on Sodium Dodecyl Sulphate/Polyacrylamide Gel (SDS/PAGE;10% acrylamide). Part of the protein gel (selected based on the molecular weight of the protein of interest) were sliced out and transferred (Wet transfer) to Nitrocellulose/PVDF membranes in Towbin’s buffer (in 25 mM Tris, 192 mM glycine and 20% Methanol). Following transfer, the membranes were washed with Tris buffered saline (TBS: 10mM Tris pH 7.4, 150 mM NaCl) and blocked for 1 h at room temperature with 5% (w/v) skimmed milk powder in TBST (TBS containing 0.05%Tween 20). Blots were then incubated overnight at 4°C with primary antibodies in 0.5% (w/v) skimmed milk powder dissolved in TBST. Primary antibodies were then removed and the blots were extensively washed with TBST. Blots were then incubated (2 hours at room temperature) with the secondary antibodies (anti-rabbit or anti mouse antibody coupled to horseradish peroxidase; 1:1000 dilution) in 0.5% (w/v) skimmed milk powder dissolved in TBST. Following removal of the secondary antibody, blots were extensively washed and developed using the Enhanced Chemiluminescence detection system and quantified densitometrically (Fuji, FLA 5000). Immunoblots were re-probed with different antibodies after stripping membranes in 0.05% Tween-20 in 0.2 M glycine-HCl (pH2.5) for 2 hours at 80°C. To ensure equal loading (in addition to staining of part of the gel as described above) part of the protein gel that were not transferred (based on the molecular weight of the protein of interest) was also stained with CBB., nitrocellulose membranes were stained with Ponceau S after transfer and PVDF membranes were stained with CBB after developing chemiluminogram. Densitometric analysis of the ECL autoradiograph was performed using Fuji, FLA 5000.

2.2.18. Measurement of intracellular ROS levels.

The method has been adopted from Chandel et al., 2001 with some modifications. H9c2 cells were plated in 35 mm dishes and after attainment of ~70 confluence, cells were kept in serum free medium for 12 hours. Thirty minutes prior to agonist
treatment, DCFH-DA (10 μM final) was added. At different time points after agonist treatment, cells were lysed as described in 2.2.16 and oxidized DCFH was measured at an excitation wavelength of 500 nm and emission wavelength of 530 nm using a fluorimeter (Cary Eclipse).

2.2.19. Luciferase Reporter assays.

Cells were transiently transfected with the reporter constructs as described in 2.2.2. Six to eight hours after agonist treatment, cells were lysed in reporter lysis buffer (Promega). Lysates were then analyzed for the luciferase activities using the Luciferase Reagent Assay Kit (Promega) and readings were taken in a luminometer (Turner Scientific). For the normalization of DNA uptake β-galactosidase expression plasmid pCMV gal was co-transfected (1μg). Also in certain experiments, the normalization was done based on protein content (Burch et al., 2004) as liposome mediated plasmid transfer is characterized by uniform transfection efficiency between dishes.

2.2.20. Animals and Treatments

Male Sprague-Dawley rats (weighing 200-300g), aged 2-3 weeks were housed for 4-5 per cage in animal house where normal rat food and water was provided. Rats were injected either with saline (Control) or NE injected intra-peritoneally with at a dose of 0.05 mg/Kg body weight, 0.2 mg /Kg body weight, 2.5 mg/Kg body weight.

2.2.21. Immunohistochemistry

Hearts were excised 2 and 4 hours post treatment isolated after 2 and 4 hours. Five micron transverse sections were made in a cryotome at -20°C and were mounted on glass slides. The sections were immediately thawed, fixed in chilled acetone for 10 minutes and air dried. After rinsing in PBS (pH 7.4) the sections were permeabilized in 0.1% Triton X-100 and non-specifically blocked with 1% normal goat serum. The sections were then incubated with anti-FKHR antibody (1:100 in PBS with 0.1% BSA) overnight at 4°C. After washing with PBS, the sections were incubated with Horseradish Peroxidase conjugated antibody IgG in (1:100 dilution) for 1 hour. Peroxidase activity was developed with 3’3’-diaminobenzimide (0.04%) and H2O2 (0.004%). Sections were dehydrated in
ethanol, cleared in xylene and mounted in DPX and photographed in a Nikon microscope.

2.2.23. Immunocytochemistry

Cells were grown on glass covers slips in 35 mm dishes to 70% confluence and kept in serum-free medium for 12 hours following agonist treatment (2 μM and 100 μM of NE and 200 μM H2O2). Cells were then fixed with chilled methanol, permeabilized with 0.1% Triton-X 100 in PBS for 30 minutes and then blocked with 3% BSA for 1 hour at 37°C. Cells were then incubated with primary antibody diluted (1:100) in 0.3% BSA in PBST (PBS with 0.05%). After three washes with PBS, samples were incubated with sheep anti rabbit Cy3 secondary antibody diluted in PBST (1:200 dilution) for 1 h at room temperature. Coverslips were mounted on glass slides and examined by fluorescence microscopy (Nikon). For the detection of nuclear morphology, cells were also incubated (after incubation with secondary antibody) in 1 μg/ml Hoechst 33258 (Sigma, St. Louis, MO) in PBS for 10 min and fluorescent images of DAPI-stained nuclei were captured. (Antin et al., 1988)

2.2.24. Data analysis

All the experiments were done at least twice unless mentioned. Data were expressed as mean +/- standard deviation.