Part I

Hypertrophic and Apoptotic Doses of Norepinephrine Induce Different Repertoire of Jun and Fos Proteins
Part I

In higher eukaryotes, gene specific transcription factors are highly evolved and are functionally diversified by multiple mechanisms such as tissue-restricted expression (such as MyoD family), generation of spliced variants (CREB family), selection of dimeric partners (bZip, bHLH family) and intracellular shuttling (NF-kB, Forkhead). Since the discovery of Jun as a transcription factor in late eighties, the numbers of proteins it can form dimers with are ever expanding and their roles in differential gene expression have been of increasing interest. Jun can heterodimerize with a diverse family of bZip proteins such as Fos, CNC (Cap’n’collar, Vinson et al., 2002), ATF 2/3, and c-Maf. There are nine members in the Fos family i.e., Fos, FosB, Fra-1, Fra-2, hcp34067, ATF3, JDP2, SNFT and BATF, of which the first five come under one subgroup characterized by a distinctive arrangement of amino acids (acidic, basic and hydrophobic) in their leucine zipper region (Chinenov and Kerppola, 2001; Vinson et al., 2002). Fos proteins also can form dimers with Jun and several other members of the bZip family such as CNC and small Maf. However, Jun is presumably the most preferred dimeric partner of Fos (i.e., Fos, FosB, Fra-1, Fra-2) and vice versa. The Jun-Fos dimers are collectively called AP-1 which bind to the consensus DNA element TRE (TGA^G/cTCA, Hess et al., 2004) and apparently regulate different (but not necessarily exclusive) repertoire of genes. AP-1 proteins are often simultaneously expressed in a given cell type and are regulated by similar mechanisms (phosphorylation/ dephosphorylation etc). Thus the molecular mechanisms by which different AP-1 proteins recognize their respective target genes are of immense interest.

The primary objective of this study is to use cardiac myoblast as a model system for understanding the molecular mechanism by which different AP-1 complexes are engaged in differential gene expression. In cardiac myocytes (and also in the intact myocardium) induction of AP-1 have been documented by disparate agonists like mechanical stretch, pressure-volume over load, neuroendocrine modulation (Angiotensin II, catecholamine, endothelin I etc), purinergic stimulation etc. I thus tested the tenets that since the downstream consequences of stimulation by these agonists are divergent; the AP-1 activities induced under respective conditions are likely to be different. Furthermore, although AP-1
activities are regulated at multiple levels (and thereby contribute to their functional divergence), formation of different dimeric complexes is the primary mode of divergence of AP-1 function. I thus decided to compare the composition of AP-1 activities under two distinct conditions i.e., upon exposure to low dose NE (2 μM) that induces cardiac myocyte hypertrophy (and proliferation for H9c2 cardiac myoblasts) and upon exposure to high dose NE (100 μM) that results in apoptosis. During the past decade, number of approaches has been taken for understanding the functional divergence of different AP-1 proteins. For eg, a number of target genes for different AP-1 proteins were identified in fibroblasts by monitoring the changes in temporal expression of various Jun-Fos proteins during the progression of cell cycle (Kovary and Bravo, 1991; Kovary and Bravo, 1992). Gene knockout and over expression studies have also revealed number of hitherto unknown functions of different AP-1 proteins. In a recent study, by using a novel approach of covalently linked (through a flexible polypeptide) Fos-Jun proteins, their contributions in cellular transformation were analyzed (Bakiri et al., 2002). Nevertheless, fundamental limitations of ablation (siRNA, gene knock out etc) and overexpression studies are that none of those conditions truly maintain the normal cellular context where different Fos/Jun members select their preferred partners and engage with cognate promoters. An alternative approach thus has been used here, where the profile of different Fos-Jun members were analyzed in cardiac myoblasts under two different conditions of adrenergic stimulation. It was argued that, since both low and high doses of NE treatment induces AP-1 but still lead to distinct consequences (cell proliferation and apoptosis respectively as detailed in the following section), the composition and target genes of AP-1 that initiate the two events are likely to be different. Therefore, by analyzing the repertoire of Jun-Fos proteins, following low dose and high dose NE treatment might provide the tools for probing of their target genes located further downstream and understand the logic of their functional specificity.

3.1. Results

3.1.1. Divergent Agonists induce AP-1 Activity in H9c2 Myoblasts

Upon treatment with divergent pathophysiological agonists, cardiac myocytes induce AP-1 activities followed by respective responses (Paradis et al.,
Whether or not similar responses exist in H9c2 myoblasts, especially under adrenergic stimulation, is yet to be reported. Though the primary objective of this study was to investigate the role of AP-1 in inducing hypertrophic and apoptotic programmes by NE (in H9c2 myoblasts), it was imperative to see whether H9c2 myoblasts were amenable to the induction of AP-1 by diverse agonists implicated in cardiac function. A series of agonists (with divergent physiological effects) were thus selected and tested for their effects on H9c2 cardiac myoblasts as assayed by the induction of AP-1. H9c2 cells were grown on 100mm culture dishes and kept in serum free medium for 24 hrs (synchronized by serum deprivation) followed by treatment with NE (2 μM and 100 μM as hypertrophic and apoptotic doses respectively, (Ph D thesis, Manveen K Gupta, Special Centre for Molecular Medicine, JNU, 2005; Simpson, 1985), Angiotensin II [100nM as hypertrophic agonist, (Sadoshima and Izumo, 1995)], ATP [100 μM as antihypertrophic agonist, (Zheng et al., 1996)]. Following treatment, nuclear extracts were prepared at different time points and analyzed for the induction of AP-1 activity by gel mobility shift assay. As shown in Fig 1A, all these agonists induced AP-1 activities albeit with differences in the kinetic as well as the extent of induction. NE (both at 2 μM and 100 μM) induced AP-1 activities within one hour of treatment. Furthermore, while 2 μM NE showed a modest induction of AP-1 (~ 4.0 fold in four hours), 100 μM showed more robust induction (~ 6.5 fold in four hours). On the other hand, both 100 nM Angiotensin II and 100 μM ATP were moderate (~3-4 fold) inducers of AP-1 and induction by Angiotensin II was more sustained (till 24 hours, Fig 1A & 1C). To demonstrate the authenticity of the AP-1 complex, competition experiments were performed with 50-100 fold molar excess of unlabelled self (TRE consensus sequence from Collagenase promoter and AP-1 sequence from Angiotensin II promoter) and non-self (100 fold molar excess SP-1 consensus sequence) oligonucleotides. As expected, the unlabelled AP-1 DNA (but not the SP-1 DNA) strongly competed out the AP-1 complex (Fig 1B).

Further, analysis of the induction pattern of c-Jun and c-Fos, two well studied and prototype members of the AP-1 family were done by immunoblot analysis. As
Fig.1(A). Norepinephrine, Angiotensin and ATP induce AP-1 activity in H9C2 myoblasts. Cells were treated with 2 μM and 100 μM NE, 100 nM Angiotensin II and 100 μM ATP for various time periods as indicated. Eight microgram (upper panel, left) and six microgram (lower panel, left) of nuclear extract were assayed for AP-1 binding activity by gel mobility shift assay. The representative data from two independent experiments is shown. For redundancy, only the part of the autoradiogram having AP-1 binding activity is shown and for clarity, representative lanes (2μM NE treatment) is shown on the right panel.
Fig. 1(B). Competition analysis was done with 50 and 100 fold molar excess of unlabelled AP-1 (Collagenase TRE lanes 2 and 3, Angiotensin II AP-1 sequence, lanes 4 and 5) and SP-1 (100 fold molar excess, lane 6) oligonucleotide. (C) Densitometric analysis of AP-1 complexes is shown. Analysis was done in Fuji phosphorimager (model, FLA 5000).
shown in Figure 2, in agreement with the electrophoretic mobility shift assay (shown in Fig 1), all those agonists induced c-Jun within one hour. As expected, the extent of induction was different for different agonists in the following order: Angiotensin 1µM (~3.14 fold) > Serum 10 % (~3 Fold) > Angiotensin 100nM (~2.62 fold) > NE 100 µM (~2.59 fold) > ATP 100µM (~2.3 fold) > NE 2µM (~1.76 fold). Similarly, c-Fos was also induced by Serum (~8.3 fold) > Angiotensin 100nM (~5.9 fold) > NE 2uM (~2.4 fold) (Fig. 3). These experiments thus demonstrate that H9c2 myoblasts respond to different extracellular agonists by inducing AP-1 albeit with subtle differences in the kinetic and the extent of induction. This is thus in agreement with the general observation that early responsive genes differently interpret the signal inputs from various extracellular agonists (Balmanno and Cook, 1999; Cook et al., 1999; Murphy et al., 2004). These results also demonstrate that H9c2 myoblasts are functionally close to primary cardiac myocytes in responding to those agonists and induce AP-1 as an immediate early response (Paradis et al., 1996; Takemoto et al., 1999; Clerk et al., 2002; Frantz et al., 2003; Taimor et al., 2004). Notably, the differences in the levels of induction of Jun & Fos proteins and the AP-1 binding is likely to be a reflection of the relative abundance of various AP-1 proteins and their posttranslational modifications which significantly contribute to the formation of functional dimers and transcriptional activities (Bakiri et al., 2002; Hazzalin and Mahadevan, 2002).

3.1.2. NE induces c-Jun but not JunB

As the primary objective of this study was to investigate the molecular basis of induction of hypertrophic and apoptotic programmes (in cardiac myoblasts under adrenergic stimulation) by AP-1, it was subsequently investigated whether there are any differences in the constituents of AP-1 under these two conditions. H9c2 myoblasts when treated with low dose NE (2-10 µM) undergo hypertrophic response (proliferation) and further increase in NE concentration (100 µM) results in apoptosis (PhD thesis submitted by Manveen K Gupta, Special centre for Molecular Medicine, JNU, 2005). Notably, numerous studies (with other cell types) have shown that difference in the dimeric composition of AP-1 complexes often contributes to their ability to target different
Fig. 2. Induction of c-Jun by various agonists. H9c2 myoblasts were treated with the respective agonists. (A) One hour after treatment, total cellular extracts were prepared and 125 µg protein was analysed by immunoblot with c-Jun antibody. To ensure equal protein loading, twenty microgram of the same extract was run on a separate gel and stained with CBB (A, lower panel). (B) Fold increases in c-Jun (39 kD) level were measured by densitometric analysis in Fuji FLA 5000 phosphorimager. The representative data from two independent experiments is shown.
Fig. 3. Induction of c-Fos by various agonists. H9c2 myoblasts were treated with respective agonists for the indicated time periods. (A) Total cellular extracts were prepared and 200 μg protein was analysed by immunoblot with c-Fos antibody. (B) Fold increases in c-Fos (62 kD) level were measured by densitometric analysis by Fuji FLA 5000 phosphorimager. The representative data from two independent experiments is shown.
genes. H9c2 cells were thus treated with low and high doses of NE (2 μM and 100 μM as the hypertrophic and apoptotic doses respectively) and immunoblot blot analyses were performed using specific antibodies against Jun, JunB, Fos, FosB, and Fra-1. As shown in (Fig. 4), NE at 2μM induced c-Jun up to ~2.1 fold with a peak at 4hour while at 100μM NE the induction was up to ~ 4 fold with a peak at 2 hours. Also under both conditions, c-Jun level remained above the control till the eight hour time period analyzed (Fig. 4). Notably, in agreement with gel mobility shift assay (Fig 1), the extent of induction of c-Jun by 100 μM NE was more as compared to that by 2 μM NE. For a comparison, analysis of the kinetic of induction of c-Jun by Serum (10%) was also done. As shown in Fig. 5, Serum treatment showed a consistent induction of c-Jun till 8 hours (the last time point analyzed). Amongst two other members of the Jun family, JunB is also induced in cardiac myocytes by various agonists (Black et al., 1991; Brand et al., 1993; Zheng et al., 1996; Xia et al., 1998). Following mitogenic stimulation in fibroblast cells, JunB specifically modulate the expression of a number of cell cycle regulatory genes (Andrecht et al., 2002). I thus analyzed the induction of JunB by NE (2 μM and 100 μM) and as shown in Fig 6, neither doses of NE induced Jun B till 4 hours. Notably, primary myocytes upon treatment with phenylephrine, leads to induction of JunB, further exemplifying the existence of distinct signaling cascades for inducing hypertrophy (Santalucia et al., 2003). Notably, in unstimulated H9c2 cardiac myoblasts, while there was a fairly detectable basal level of Jun (Fig 2, 4 & 5), that of JunB was barely detectable.

3.1.3. Shift in the Expression of Fos Family Proteins with the Increase in Norepinephrine Concentration

Subsequently analysis of the induction of various Fos family members (c-Fos, FosB and Fra-1) by NE was carried out. As shown in Fig 7, c-Fos showed an early induction by either doses of NE, reaching a maximum within one hour. Other studies, including those with primary cardiac myocytes have also shown that c-Fos is one of the earliest AP-1 family member to be induced (Webster et al., 1993; Cook et al., 1999). Furthermore, while 2 μM NE resulted in ~2.5 fold induction of c-Fos, 100 μM NE induced it by ~1.82 fold. Moreover, c-Fos induced by 100 μM NE sustained at a lower level till 4 hours while that induced by 2 μM
Fig. 4. Kinetic of induction of c-Jun by NE (2 and 100 μM). H9c2 myoblasts were treated with NE 2 μM and 100 μM for the indicated time periods. Total cellular extracts were prepared and 150 μg protein was analysed by immuno-blotting with c-Jun antibody (Fig. A, NE 2 μM and Fig. B, NE 100 μM). Serum treatment was done as a positive control (Fig. A, lane S). Non-treated control at 8hr also is shown (8C). To ensure equal protein loading, twenty μg of the same extract was run on a separate gel and stained with CBB (lower panel, Fig. A&B). Fold increases in Jun level was measured by densitometric analysis by Fuji FLA 5000 phosphorimager. The representative data from two independent experiments is shown.
Fig. 5. Kinetic of induction of c-Jun by serum (10%). H9c2 myoblasts were treated with 10% serum for the indicated time period. (A) Total cellular extracts were prepared and 150 μg protein was analysed by immuno-blotting with c-Jun antibody. (B) Fold increases in Jun level were measured by densitometric analysis by Fuji FLA 5000 phosphorimager. The representative data from two independent experiments is shown.
Fig. 6. Induction of JunB NE (2 and 100 μM). Cells were treated with NE 2 μM and 100 μM for 1-4 hours. (A) Total cellular extracts were analysed (150 μg) by immunoblot analysis with JunB antibody. Extract from serum treated cells were used as a positive control (lane S). To ensure equal protein loading, 15 μg of the same extract was run on a separate gel and stained with CBB (lower panel). (B) Fold increases in JunB (42 kD) level were measured by densitometric analysis in Fuji FLA 5000 phosphorimager. The representative data from two independent experiments is shown.
**Fig. 7. Kinetic of induction of c-Fos by NE (2 and 100 μM).** H9c2 myoblasts were treated with NE 2μM and 100 μM for the indicated time periods. Total cellular extracts were prepared and 150 μg protein was analysed by immuno-blotting with c-Fos antibody (Fig A, NE 2 μM and Fig B NE 100 μM). Serum treatment was done as a positive control (lane S). To ensure equal protein loading, 20 μg of the same extract was run on a separate gel and stained with CBB (Fig A&B, lower panel). Fold increases in Fos level was measured by densitometric analysis by Fuji FLA 5000 phosphorimager. The representative data from two independent experiments is shown.
NE diminished after 2 hours. As expected, serum stimulation resulted in more robust induction of c-Fos, presumably due to the stimulation of multiple receptors. Contrary to c-Fos, the kinetic of induction of FosB and Fra-1 were different and reciprocal. As shown in Fig 8, Fos B was strongly induced by low dose NE, with a peak induction of 3.59 fold within 2 hrs followed by a sustained level (2.5 fold) till 4hrs. On the other hand, 100 µM NE was a moderate inducer of FosB with a peak induction of 2.2 fold within an hour followed by a decline below the basal level by four hours. Significantly, a reciprocal pattern of induction was observed in case of Fra-1 (Fig 9 & 25). Fra-1 was primarily induced by the high dose NE with a delayed kinetic of activation (~ 1.7 fold at 4th hour). Amongst different Fos proteins, Fra-1 is characterized by a delayed response though the relevance is not known yet (Cook et al., 1999; Burch et al., 2004; Murphy et al., 2004). Notably, both FosB and Fra-1 showed multiple bands reflective of multiple phosphorylation status as reported before (Cook et al., 1999; Casalino et al., 2003; Vial and Marshall, 2003; Murphy et al., 2004). It was also tested whether the induction of Fra-1 is a characteristic of only adrenergic signaling and thus analysed whether it is induced by 10% serum. As shown in Fig.10 serum treatment also led to a delayed induction of Fra-1. Since 100µM NE treatment causes cell death while serum treatment leads to cell proliferation or survival it is yet to be seen what is the role of Fra-1 induced under these conditions. Nonetheless, in a recent study, transcript profiling (micro-array) of experimentally induced heart failure models has also shown induction of Fra-1(Wellner et al., 2005). Also according to a recent study, transgenic mice overexpressing Fra-1 under JunD/- background undergoes dilated cardiomyopathy associated with increased cardiomyocyte apoptosis (Ricci et al., 2005). These results taken together, illustrate that various Jun/Fos proteins (comprising different AP-1 complexes) are induced prior to the implementation of hypertrophic and apoptotic programs by NE in cardiac muscle cells. It remains to be seen how different AP-1 complexes presumably target different sets of genes and elicit respective effects. Also, it remains to be seen how FosB and Fra-1 promoters differentiate between the apparently overlapping signal transduction pathways induced by low and high dose NE.
Fig. 8. Differential induction of FosB by 2 and 100 µM NE. Cells were treated with NE 2 µM and 100 µM for 1-4 hours. (A) Total cellular extracts were analysed (150 µg) by immunoblot analysis with FosB antibody. To ensure equal protein loading, 20 µg of the same extract was run on a separate gel and stained with CBB (A, lower panel). (B) Fold increases in FosB (40 kD) level were measured by densitometric analysis by Fuji FLA 5000 phosphorimager. The representative data from two independent experiments is shown.
Fig. 9. Differential induction of Fra-1 by NE 2 and 100 μM NE. H9c2 myoblasts were treated with NE 2 μM and 100 μM for 1-4 hours. (A) Total cellular extracts were analysed (150 μg) by immunoblot analysis with Fra-1 antibody. To ensure equal protein loading, 20 μg of the same extract was run on a separate gel and stained with CBB (A, lower panel). (B) Fold increases in Fra-1 (46 kD) level were measured by densitometric analysis by Fuji FLA 5000 phosphorimager. The representative data from three independent experiments is shown.
Fig. 10. Induction of Fra-1 by serum. H9c2 myoblasts were treated with serum (10%) for the indicated time periods. (A) Following treatment, total cellular extracts were prepared and analyzed (100 μg) by immunoblotting with Fra-1 antibody. (B) Fold increases in Fra-1 level were measured by densitometric analysis on a Fuji FLA 5000 phosphorimager. The representative data from two independent experiments is shown.
3.1.4. Induction of Fra-1 by 100μM NE is at the Level of Transcript

AP-1 family members are primarily induced at the level of transcription. It was thus tested whether the induction of Fra-1 by NE is also at the level of transcripts. H9c2 cells were treated with both low and high doses of NE and the total RNA was isolated 1-4 hours and Northern analysis was performed with rat Fra-1 cDNA probe. Two mRNA transcripts of fra-1, ~1.8kb and ~3.5kB were observed (Fig.11). Induction of two different human fra-1 transcripts (presumably due to differential splicing) in bronchial epithelial cells has also been reported recently (Zhang et al., 2004). In agreement with the Western analysis, transcript analysis also showed that with increasing doses of NE, the extent of induction of fra-1 also increased with a peak of 2.2 fold by 1hr for the 1.8 kb transcript and a peak of 2.3 fold for the 3.5 kb transcript by 4hr.

3.1.5. Nuclear Localization of Fra-1 Induced by 100 μM NE

In addition to transcriptional activation and post-translational processing (phosphorylation), Fra-1 activity is also modulated at the level of nucleocytoplasmic trafficking by MAPKinases (Burch et al., 2004). I then confirmed differential induction of Fra-1 by indirect immunofluorescence assay. H9c2 cells were treated with 2 μM and 100 μM NE and fixed in methanol at different time points. Fixed cells were stained with Fra-1 antibody followed by Cy3 conjugated secondary antibody. As shown in Fig 12A, while 2 μM NE treatment resulted in a low intensity induction of Fra-1, 100 μM caused a delayed but strong induction. This result was therefore in full agreement with the western blot analysis described above. A dose dependent study was performed next with increasing doses of Norepinephrine as shown in figure 12B. An increase in Fra-1 nuclear localization was found in a dose dependent manner. The maximum localization was found at 500 μM dose analyzed (Fig 12B). Furthermore, induced Fra-1 was primarily localized in the nucleus as expected. Notably, control cells also had a background level of nuclear localized Fra-1 and upon treatment; majority of the cells but not all the cells had increased Fra-1 expression. According to a recent study in C10 mouse type II alveolar cells when ERK activities (induced by oxidative stress) were inhibited by pharmacological inhibitor (U01126), only a fraction but not all the cells responded by inducing cyclin D1 (Burch et al., 2004).
Fig. 11. Induction of fra-1 transcript by NE. H9c2 myoblasts were treated with NE 2 μM and 100 μM for 1-4 hours. Total RNA (20μg /lane) was isolated, blotted, and hybridized with 32P-labeled rat fra-1 cDNA probe. Fold increases in 3.5 kb transcript (Bottom, Left panel) and 1.8 kb transcript (bottom, right panel) level were measured by densitometric analysis of the autoradiogram by Fuji FLA 5000 phosphorimager. The representative data from two independent experiments is shown.
Fig. 12a. Immunolocalisation of Fra-1 after NE treatment. H9c2 cells were plated on glass coverslips, synchronised by serum deprivation for 24 hours, and then stimulated with NE 2 and 100 μM. Cells were fixed at the indicated time periods and stained for Fra-1 (red). Nuclei were stained with DAPI (blue). The representative data from two independent experiments is shown.
Fig. 12b. Immunolocalisation of Fra-1 after increasing doses of NE treatment. H9c2 cells were plated on glass coverslips, synchronised by serum deprivation for 24 hours, and then stimulated with increasing doses of NE as indicated. Cells were fixed after four hours and stained for Fra-1 (red). Nuclei were stained with DAPI (blue). The representative data from two independent experiments is shown.
It thus appears that in a given time not all the cells in a population are responsive to agonist induction (100 µM NE in the present case). In agreement, I also have observed that upon treatment with 100 µM NE, a small population of cells always survives after 60 hours (when majority of the cells perish). However, the basis of the existence of a subpopulation of cells not responding to the agonist is not understood.

3.1.6. Differential Induction of Fra-1 and FosB by NE In Vivo

It was finally investigated whether the differential induction of FosB and Fra-1 (respectively by 2 µM and 100 µM NE) as observed in H9c2 myoblasts is also reproducible in the *in vivo* context. Adult rats were injected intraperitoneally (i.p) with three different doses of NE (0.05 mg/Kg bodyweight, 0.2 mg/Kg bodyweight, 2.5 mg/Kg bodyweight (Hannan et al., 1993) and immunohistochemical analysis of the myocardium was done at 2 hour and 4 hour time periods. As shown in Fig 13, a dose responsive induction of c-Jun was visible with medium and high doses of NE. Notably, only a sub-population of cells was Jun positive. In agreement with the Western blot data, JunB was not induced with 0.2 mg/Kg body weight dose analyzed (Fig 17). Similar analysis with c-Fos showed that only the highest dose of NE tested (2.5 mg/Kg body weight) induced c-Fos significantly by 4 hour (Fig 14). It is a possibility that at low and moderate doses, the level of induction of Fos was below the detection level as the control tissue also did not show any background level of Fos. Like Jun, induction of Fos also had a scattered pattern indicating that only a subset of cells were responsive to the NE stimulation (Gonzalez-Cuello et al., 2004). Finally, in agreement with the western blot data, FosB and Fra-1 showed a reciprocal pattern of induction (Fig 15, 16). While the lowest dose of NE (0.05 mg/Kg body weight) showed the maximum level of induced FosB, Fra-1 was most prevalent with the highest dose. Also a uniform staining throught out the myocardium was observed for Fra-1. Haematoxylin-Eosin stained sections showed that the integrity of the myocardium was normal.
Fig. 13. Photomicrograph of c-Jun immunoreactivity in the heart after 2 and 4 hours of norepinephrine administration. After a single i.p. injection of saline or norepinephrine (NE 0.05 mg/Kg, NE 0.2 mg/Kg, NE 2.5 mg/Kg respectively) rat hearts were removed and immunostained for c-Jun. Results are representative of two independent experiments.
Fig. 14. Photomicrograph of c-Fos immunoreactivity in the heart after 2 and 4 hours of norepinephrine administration. After a single i.p. injection of saline or norepinephrine (NE 0.05 mg/Kg, NE 0.2 mg/Kg, NE 2.5 mg/Kg respectively) rat hearts were removed and immunostained for c-Fos.
Fig. 15. Photomicrograph of FosB immunoreactivity in the heart after 2 and 4 hours of norepinephrine administration. After a single i.p. injection of saline or norepinephrine (NE 0.05 mg/Kg, NE 0.2 mg/Kg, NE 2.5 mg/Kg respectively) rat hearts were removed and immunostained for FosB. Results are representative of two independent experiments.
Fig. 16. Photomicrograph of Fra-1 immunoreactivity in the heart after 2 and 4 hours of norepinephrine administration. After a single i.p. injection of saline or norepinephrine (NE 0.05 mg/Kg, NE 0.2 mg/Kg, NE 2.5 mg/Kg respectively) rat hearts were removed and immunostained for Fra-1. Results are representative of two independent experiments.
Fig. 17. Photomicrograph of JunB immunoreactivity in the heart after 2 and 4 hours of norepinephrine administration. After a single i.p. injection of saline or norepinephrine (NE 0.2 mg/Kg) rat hearts were removed and immunostained for JunB (Upper panel). Hematoxyline-eosin stained sections are shown in the lower panel.
3.1.7. NE 100uM Results in a Higher Transcriptional Activation of AP-1

Following stimulation by respective agonists, AP-1 activities are regulated at multiple levels viz., gene transcription, mRNA stability, post translational modifications by various kinases and phosphatases, protein stability and selection of dimeric partners (Short and Pfarr, 2002; Gao et al., 2004). Furthermore, various Jun/Fos proteins have different transactivation potential (eg, Jun B and Fra-1 are weaker transactivators than Jun and Fos respectively). Our studies as described above have demonstrated that following low dose and high dose NE treatment, different Fos proteins are induced and the total DNA binding activities induced by 100 µM NE is higher than that by 2 µM NE (Fig 1). It was thus imperative to know what were the relative transactivation potential of various AP-1 complexes induced by NE (2 µM and 100 µM) at different time points. To address this issue, H9c2 cells were transfected with a (TRE)-7-Basic-promoter-Luciferase reporter plasmid (Stratagene, USA) and the luciferase activities were estimated at various time points post treatment (with NE). As shown in Fig 18, such analysis demonstrated that the AP-1 reporter activity initially increased till 1 hour following which it remained at a steady level (till eight hours tested). Also, in agreement with the DNA binding and Western blot assays, the luciferase reporter activity were higher in case of 100 µM NE than that of 2 µM NE. Since, the activities found at latter time points (4-8 hours) were presumably the total activities accumulated over the said period, it appears that the AP-1 complexes appearing at later time points were less active than that at the beginning. Moreover, AP-1-reporter activity was the total activities contributed by different AP-1 complexes at any given time point and it is not possible to assay which of them was more active than the other. So, it appears that at latter time point when FosB and Fra-1 were induced (by 2µM and 100µM NE respectively), the AP-1 complexes were less active than those in the early time point (comprising Jun and Fos). As expected, serum treated cells showed a more robust induction of AP-1 reporter activity.

3.2. Discussion

In eukaryotes, the specificity of gene expression in an enormously complex genetic background is achieved by multiple strategies including selection of
Fig. 18. Induction of transcriptional activation after NE 2 μM and NE 100 μM treatment for the indicated time periods. Cells were transiently transfected with 1 μg of the indicated pAP-1 Luc reporter construct. Forty eight hours after transfection cells were treated with norepinephrine 2 and 100 μM for the indicated time periods. The relative reporter activity per μg protein was calculated, and data are expressed as fold induction compared to control. Data represent mean values (+/-) standard deviation of three independent experiments.
dimeric partners by various transcription factors. All major families of eukaryotic transcriptional regulators viz., MADS box family (Mef2, SRF), bHLH family (E12/E47, MyoD), NFKB family (Rel, p65, p50), bZip (CREB, Jun, Fos) are characterized by differential formation of dimeric complexes with gene specific activities. Such attainment of functional diversity with evolution is further exemplified by the observation that while in mammals many of these transcriptional regulators have multiple isoforms and homologues (Drosophila has fewer counterparts). In this context, the possible dimers of AP-1 proteins are enormously large and their functional targets are ever elusive. To have an insight into the biochemical basis of partner selection and its consequences in binding to target DNA, structural biologists have compiled the predicted stabilities of various leucine zipper dimers and had arrived at a rational classification of 53 bZip proteins identified in the human genome. Based on such analysis, the most preferred dimeric partners of the Jun proteins are the Fos family members (Vinson et al., 2002). Such conclusion is thus in agreement with the experimental evidence (antibody super shift assays etc) that the Jun-Fos proteins coexist in various AP-1 complexes. However, super-shift assays are largely semi-quantitative and the sensitivity/efficacy may differ from one antibody to another, thereby leading to misleading conclusions. Therefore it was rationalized that if differences amongst AP-1 activities induced under two different conditions (as it is likely from the downstream consequences) is due to the formation of different dimers (in addition to other mechanisms such as recruitment of coactivators, mutual antagonism etc.), it will be apparent from the systematic analysis of various Jun, Fos proteins induced under specific conditions. Although AP-1 proteins are early responsive transcription factors primarily induced at the level of transcription, their activities are modulated at multiple levels (Short and Pfarr, 2002; Gao et al., 2004). I thus preferred to monitor their modulation at the protein level (by Western analysis) so that the contribution of other regulatory components is also taken into account.

The levels of AP-1 proteins in differentiated/ quiescent cells are normally low and it is induced rapidly after specific stimulation. Amongst various signaling kinases, ERK, JNK, p38 MAPK, JAK, PKA and CaMK have been implicated in the induction of AP-1 (Whitmarsh and Davis, 1996; Han et al., 1997; Masuya et al., 1999; Kato et al., 1997; Joo et al., 2004; Pursiheimo et al., 2002). It has also been
suggested that being immediate early genes, AP-1 members have the ability to differentiate the strength and duration of the induced signals and respond accordingly (Cook et al., 1999; Murphy et al., 2004). Therefore, before undertaking a comprehensive study of various AP-1 proteins induced by NE in cardiac myoblasts, the kinetic and extent of induction of AP-1 by a number of agonists i.e., Angiotensin II, NE and ATP were analysed. The rationale for the selection of these agonists were that all of them transmit their signals through the G protein coupled receptors but still have distinctive biological effects (as described in the results section). G protein coupled receptors are highly versatile in terms of the ligands but they mediate biological effects with remarkable speed and specificity (Hur and Kim, 2002). It was observed that, each of these agonists showed a distinct kinetic and extent of AP-1 induction thereby exemplifying the sensitivity of the respective signaling as well as the assay conditions (gel mobility shift assay and Western blot analysis). It is also notable that amongst all the agonists tested, serum was the most potent inducer of Jun/AP-1 presumably due to the stimulation of multiple receptors, further supporting the argument that multiple signals can be integrated quantitatively at the level of AP-1 induction.

Amongst three Jun family members, c-Jun has been extensively studied and attributed to disparate functions such as cell proliferation, differentiation, apoptosis and survival in various cell types including cardiac myocytes. Till recently (when this work was organized) relatively less was known about the other two relatives of Jun (JunB and JunD) in general and in the context of cardiac myocytes in particular. Available literature suggested that in cardiac myocytes, while JunB is induced by pressure over load, isoproterenol, ATP, electrical stimulation (Xia et al., 1998; Zheng et al., 1996; Brand et al., 1993; Black et al., 1991) JunD is not induced by isoproterenol and its level goes down in myopathic heart (Brand et al., 1993; Pollack et al., 1997). With that background in this study I had analyzed only the induction of JunB but not JunD. Nevertheless, very recently it has been demonstrated that JunD -/- mouse develop less adaptive hypertrophy in heart (after mechanical pressure overload) and cardiomyocyte-specific expression of JunD results in spontaneous ventricular dilation and decreased contractility (Ricci et al., 2005). Interestingly, it was observed that although neither doses of NE induces JunB, it is strongly induced by serum. Since
hypertrophy can be of different types with distinct gene expression profiles (Kon et al., 2005), it is likely that JunB plays a role in mediating certain hypertrophic response (elicited by other agonists) but not in all. Another possibility is, although others have documented the induction of JunB mRNA by multiple agonists (i.e., cardiac myocytes), it might not be induced at the protein level. Taken together, it appears that during NE induced hypertrophy and apoptosis, Jun but not JunB is the major constituent of the AP-1 activities induced by respective doses. However, in contrast to Jun, the induction profile of two Fos related proteins were different at two different doses of NE. While immediately after treatment (2 μM and 10 μM NE), c-Fos was transiently induced for initial two hours, the kinetic induction of FosB and Fra-1 were reciprocal. H9c2 cells had basal detectable levels of both FosB and Fra-1. Upon treatment with NE, while FosB level increased (up to 3.6 fold) for lower dose, it showed lesser induction by 100 μM NE. In contrast, Fra-1, was modulated in a different way. In case of lower dose of NE, its level gradually diminished below the control level over four hours. With the higher dose, there was a decrease in Fra-1 at the first hour and then it slowly picked up with time with a peak at 4 hours. Although such changes in Fra-1 level was moderate (~1.5 fold), it was corroborated by the indirect immunostaining of cell treated with 100 μM NE at different time points and also with increasing doses of NE. Furthermore, a moderate increase in fra-1 transcript in the context of induction in pulmonary epithelial cells by diesel exhaust particles (DEP) has also been reported by others (Zhang et al., 2004). Although both FosB and Fra-1 have been implicated in both cell proliferation and differentiation, recent studies have implicated Fra-1 in reduced tumorigenesis and increased apoptosis in glioma cell (Shirsat and Shaikh, 2003). Eventhough the molecular mechanisms of cardiac hypertrophy have been extensively investigated (Olson, 2004), the mechanisms of induction of apoptosis are relatively poorly understood and are of immense clinical relevance. I thus further tested the induction of Fra-1 by NE at the RNA level and in agreement with the protein analysis; 100μM was more effective in inducing fra-1 transcript levels (~1.8Kb and ~3.5 Kb). Since expression of AP-proteins are modulated at multiple levels such as mRNA synthesis, translation and stability (of both the mRNA and the protein), the exact contribution of each of these steps in inducing Fra-1 by NE (100 μM) needs further investigation. Finally,
the differential induction of Fra-1 and Fos B were established at *in vivo* context. Rats injected with increasing doses of NE largely mimicked the kinetic and extent of induction of Jun and Fos (increased induction with increasing dose and time), JunB (no induction), FosB (induction with only the lower dose) and Fra-1 (increased induction with increasing NE dose) observed in cultured myoblasts. Notably, in each case the respective proteins were nuclear localized (as expected) and the responses was limited to a subpopulation of cells (except for Fra-1), the significance of which is yet to be understood. Nonetheless, it has often been observed that under various pathological conditions, at a given time point, only a subpopulation of myocytes undergo apoptosis (and/or hypertrophy). Taken together, this study for the first time has identified two distinctive members of the Fos family in the context of myocyte hypertrophy and apoptosis under adrenergic stress. It is thus pertinent to know (i) the mechanisms by which the adrenergic signals selectively induce those two close members of the Fos family (ii) although it is likely that FosB is a mediator of hypertrophic response it remains to be seen whether Fra-1 is pro-apoptotic or pro-survival? These questions are being addressed in the following sections.
Part II

High Dose Norepinephrine Induces Fra-1 by Sustained Activation of ERK
In cardiac myocytes, a plethora of physiological (endocrine, paracrine and autocrine) stimuli induces various signaling cascades with distinctive consequences. While some of these kinases (such as PKA) phosphorylate sarcomeric proteins and proteins involved in Ca\(^{2+}\) flux (thereby augmenting cardiac function), others such as PKC, MAPK, PI3- kinase, GSK-3β and Janus kinases (JAK) are involved in alterations in gene expression machinery with distinct physiological consequences such as hypertrophy, apoptosis and survival. Three major MAPKs, viz., ERK (Extracellular signal-Regulated Kinase), p38 and JNK (c-Jun NH2-terminal kinase) thus play pivotal roles in transmitting extracellular signals from the cell surface receptors targeted by Angiotensin II, Epinephrine, Norepinephrine and Endothelin to the nucleus and eliciting cognate responses (Bogoyevitch et al., 1996; Esposito et al., 2002; Markou and Lazou, 2002). Amongst three MAPKinasas, ERK is generally attributed to hypertrophic (and survival) response while JNK and p38 are attributed to both hypertrophic as well as stress responses, based on the context (Baines and Molkentin, 2005). Once activated, p38, JNKs, and ERKs phosphorylate a number of transcription factors such as c-Jun, ATF, CREB, SRF, GATA, NFAT, Fos etc., resulting in the specific alteration in gene expression (Hazzalin and Mahadevan, 2002). Moreover, ERK, JNK and p38 kinases also act as nodal points of diverse signaling cascades and presumably work as the integrators of disparate signaling (Baines and Molkentin, 2005). In addition to ERK, PI3-Kinase/Akt pathways also contribute to the survival signals (van Empel and De Windt, 2004). Therefore, while each of these Kinases mediates specific signaling events, their ability to coordinate with each other further defines the ultimate outcome (Liang and Molkentin, 2003).

Although AP-1 proteins are modulated at multiple levels (transcription, mRNA stability, protein translocation and stability), the primary mode of activation of *jun* and *fos* are at the level of transcription and all the three MAP Kinases have been implicated in this process (Whitmarsh and Davis, 1996; Han et al., 1997). Following synthesis, AP-1 proteins are also phosphorylated by JNK and ERK that is a prerequisite for their transcriptional activities (Derijard et al., 1994; Monje et al., 2003). Amongst other members of the AP-1 family, FosB and JunD are also phosphorylated by ERK (Rosenberger et al., 1999) but the kinases that activate
Fra-1 are largely unknown. Nonetheless, phosphorylation of Fra-1 by ERK is required for its stability (Vial and Marshall, 2003).

Duration of MAPK activation is also a key determinant of cell fate (Cook et al., 1999). While low-level activation of the Raf-MEK-MAPK pathway promotes cell proliferation, persistent and high-level activation of MAPKinase promote cell cycle arrest, cell differentiation or apoptosis (Traverse et al., 1994; Marshall, 1995; Chen et al., 1996; Sewing et al., 1997; Stanciu et al., 2000; Mansouri et al., 2003). It is believed that various members of the AP-1 family have the ability to differentially interpret subtle differences in the strength and duration of MAPK signals (Balmanno and Cook, 1999), thereby leading to quantitative differences in gene expression.

Adrenergic stimulation of cardiac myocytes leads to the induction of multiple kinases viz., ERK, JNK, p38 MAPK, PI3-Kinase, PKA and PKC (Bisognano et al., 2000; Communal et al., 2000; Rohde et al., 2000; Remondino et al., 2003; Iwai-Kanai and Hasegawa, 2004)). However, how these kinases contribute to the downstream gene regulatory events is not understood yet. The present study was aimed at understanding how two different concentrations of NE (2 μM & 100 μM) induce distinct signaling cascades leading to the differential induction of AP-1 (as manifested by the differential induction of FosB and Fra-1). However, in view of emerging role of Fra-1 in apoptosis and existing knowledge of FosB in cell cycle progression (hypertrophy, in the present context), this study was restricted to analyse the signaling cascades that induce Fra-1.

4.1. Results

4.1.1. While 2μM NE Causes Transient Activation of ERK, 100μM NE Leads to its Sustained Activation

Number of laboratories have documented that while transient induction of MAPKinases lead to cell proliferation, sustained and more robust induction may lead to either cell differentiation or apoptosis (Traverse et al., 1994; Marshall, 1995; Chen et al., 1996). We thus tested whether similar phenomenon contributes to the differential induction of AP-1 by NE (FosB by 2 μM NE and Fra-1 by 100 μM NE respectively). H9c2 myoblasts were kept in serum free medium for 24 hrs
and treated with NE (2 μM and 100 μM) for eight hours and cell lysates were assayed for activation of ERK1/2 by Western blot analysis using antibody against phosphorylated as well as non-phosphorylated ERK1/2. As shown in Fig.19 (A & B), while 2 μM NE only transiently induced ERK (2.3 fold in 30 minutes and subsequently subsiding), 100μM NE resulted in more sustained induction (reaching a maximum of 2.3 fold in 2 hour and remained well above the control till eight hours). Notably, while p42 ERK (and not p44-ERK) was the predominant form induced by NE (both doses), both p44 and p42 were strongly induced by serum (10%). Taken together, it appears that both low and high dose NE (acting through α-adrenergic receptor and through both α- and β-adrenergic receptors respectively) induce only p42 ERK, which thus have the ability to interpret the differences in the signals emanating from those receptor subtypes, by a mechanism that is yet to be investigated.

For a comprehensive analysis, the induction of Akt, another pro-survival Kinase, was also monitored. Various cell types under apoptotic stimuli often activate Akt (via PI3 kinase pathway) as an effort to survive, (Chesley et al., 2000; Coffey et al., 2005). Activated Akt thereafter induces a number of transcription factors like AP-1 (pro-survival in a given context) while attenuating others like FOXO (pro-apoptotic) (Bois and Grosveld, 2003; Li et al., 2004; Stitt et al., 2004). As shown in Fig. 20 low dose NE (2 μM) does not activate Akt, but with increase in concentration (100μM) induced Akt (~ 1.7 fold by 2 hour) though it was not as robust as by 100 nM insulin treatment for 2hr (used as a positive control). Taken together, with increasing doses of NE, the pro-survival kinases like ERK and Akt were augmented either by increased duration (ERK) or by increased strength (Akt).

4.1.2. Only High Dose of NE Activates JNK and p38 MAPK

JNK and p38 MAPKinases (stress activated MAPKinases) are generally considered to be the mediators of a wide spectrum of stress signals in various cell types (Petrich and Wang, 2004; Baines and Molkentin, 2005). In cardiac muscle, activation of JNK and p38 has been documented under various pathophysiological contexts (including adrenergic stimuli) but their role (either pro- or anti-) in hypertrophy and apoptosis remains a matter of continued debate (Baines and
Fig 19. Kinetic of induction of p-ERK by NE H9c2 myoblasts were treated with 2µM and 100 µM NE for the indicated time period. Total cellular protein (100 µg) were then analyzed by immuno-blotting with phospho specific anti-ERK antibody (A & B, upper panel). Blots were then stripped, and the total level ERK, was also determined by the respective antibody (A & B lower panel). Data shown above is one of two independent experiments that yielded similar results. As a positive control, cells were treated with 10% serum for 1 h (S). 8 hour control is shown separately (8C).
Fig. 19C. Fold increases in p-ERK level were measured by densitometric analysis by Fuji FLA 50 phosphorimager and plotted accordingly.
Fig 20. Kinetic of induction of p-Akt by NE. Total cellular extracts were prepared after treatment with 2 μM and 100 μM NE for the indicated time period. (A) Total cellular protein (100 μg) were analyzed by immuno-blotting with phospho specific anti-Akt antibody. As a positive control, cells were also treated with Insulin for 1 h. (B) Fold changes in p-Akt level was measured by densitometric analysis in a Fuji FLA 5000 phosphorimager.
Molkentin, 2005). Furthermore, although both p38 and JNK have been extensively studied in the context of AP-1 induction in general and the activation of Jun (by JNK) in particular, their contribution to the induction of Fra-1 is largely unknown. It was thus imperative to know the kinetic of induction of p38 and JNK (by NE) and their contribution to that of Fra-1. Western analysis of extracts from NE treated cells for p-JNK and p-p38 showed their induction by only 100 μM NE (Fig 21 & 22) Antibodies that cross reacting with both p46 and p54 JNK showed a sustained induction of only p46 JNK by 100 μM NE till 8 hour (the last time point tested) with a peak induction of ~2 fold by 4 hour. However, the induction of JNK was moderate as compared to that by heat shock (44°C for 15 minutes) which resulted in a robust induction [UV irradiation for 20 minutes was also a moderate inducer and TNF alpha (50ng/ml) was ineffective]. Similarly, only 100 μM (but not 2 μM) NE treatment showed a moderate but sustained induction of p-p38 with a peak induction of ~1.4 fold by 30 minutes that was remained above control till 8 hours (Fig 22).

4.1.3. Transcriptional Activation by AP-1 is Primarily Mediated by the ERK/PI3-Kinase Pathways

We thereafter analyzed the role of above mentioned kinases (activated by 100μM NE) in modulating AP-1 activities further downstream. H9c2 myoblasts were transiently transfected with an AP-1-reporter plasmid [(TRE)_7-Luc] and treated with NE (2 μM and 100 μM respectively) along with the MEK inhibitor PD098059 (50 μM), PI3-Kinase inhibitor LY 294002 (25 μM), JNK inhibitor Dicumarol (50 μM) and p38 kinase inhibitor SB 203580 (20μM) [all inhibitors were added one hour prior to the addition of NE]. As shown in Figure 23, while 2μM NE only moderately induced AP-1-promoter activity, 100μM NE showed much stronger activation (~ 2 fold) (in agreement with data shown in Fig.18). Amongst various inhibitors tested, AP-1 activity induced by 2μM NE was substantially inhibited (reduced to ~50% of basal activity) by LY294002, weakly inhibited (restoring it to the basal level) by PD098059, while Dicumarol did not have any effect and SB203580 rather moderately activated it. On the other hand, in case of the AP-1 activity induced by 100μM NE, it was strongly inhibited (reduced to ~75% of basal activity) by LY294002 and moderately inhibited (by
Fig. 21. Kinetic of induction of p-JNK by 2 μM and 100 μM norepinephrine treatment. (A) Total cellular extracts were prepared after respective treatments and were analysed (200 μg for Panel A) and (150 μg for Panel B) by immuno-blotting with phospho specific anti-JNK antibody (A & B, upper panels). Extracts from cells treated with UV irradiation (20 minutes), TNF α (50ng/ml) and heat shock (44°C for 15 minutes, lane HS, Fig A) were used as positive controls. Serum treatment for one hour was also done for a comparison (lane S, Fig B). Blots were stripped, and levels of total content of JNK, was also determined (A & B, lower panels). Data shown above is a representative of two independent experiments giving similar results.
Fig 21C. Fold increases in p-JNK level were measured by densitometric analysis by Fuji FLA 5000 phosphorimager and plotted accordingly.
Fig. 22. Kinetic of induction of p-p38 by Norepinephrine. Total cellular extracts were prepared after NE treatment (2 μM and 100 μM respectively). Hundred and fifty microgram protein was analyzed by immuno-blotting with phospho specific anti-p38 antibody (A & B, upper panel). Extracts from cells were treated with UV irradiation (20 minutes), TNF α (50ng/ml) and heat shock (44°C for 15 minutes, lane HS, panel A) were used as positive controls. Extracts from serum treated cells were also used for a comparison (lane S, panel B). Blots were stripped, and the total content of p38 MAPK, was determined using the respective antibody (A & B, lower panels). Data shown above is one of the two independent experiments that yielded similar results.
Fig 22 C. Fold increases in p-p38 MAPK level were measured by densitometric analysis by Fuji FLA 5000 phosphorimager and plotted accordingly.
Fig. 23. PI3-Kinase, ERK, p38 and JNK pathways contribute to the transcriptional activation of AP-1 by Norepinephrine. H9C2 myoblasts cells were transfected with 1.0 microgram of a AP-1 promoter-reporter plasmid (described in Fig 18. Chapter 1). Forty eight hours after transfection and one hour prior to NE treatment, cells were treated with respective vehicles (Veh) or inhibitors of various signaling kinases. Eight hours after, cell lysate was prepared and assayed for luciferase activity. Data were derived from of 3 independent experiments and mean value +/- standard deviation was plotted. For further details please see Materials and Methods and Results.
restoring it to the basal level) by PD098059, dicumarol and SB203580. Since AP-1 reporter activity is a collective reflection of the relative abundance as well of dimeric compositions of various AP-1 complexes, it is thus concluded that all three MAPKinas (ERK, JNK and p38) and PI3 kinase contribute to the basal as well as inducible (by NE) AP-1 activities. Furthermore, since transactivation potentials of different AP-1 proteins (in addition to the induction of respective genes) are also modulated by other kinases, it is difficult to estimate the relative contributions of each of these kinases in this process.

4.1.4. Fra-1 Requires ERK Pathway for its Expression and Stabilisation

Our results as described above indicate that AP-1 activities induced by high dose NE involves ERK, JNK, p38 and PI3-Kinase although the relative contribution of each was not clear as many of these pathways are mutually synergistic or antagonistic. Thereafter, contribution of these kinases towards the induction (by 100μM NE) of Fra-1 was tested. H9c2 myoblasts were treated with respective pharmacological inhibitors for one hour prior to the addition of NE (100 μM). Four hours after NE treatment, total cellular extracts were prepared and immunoblot analysis was done for Fra-1. As shown in Fig 24, while treatment with PD098059 (50μM, MEK inhibitor) and dicumarol (50 μM, JNK inhibitor) substantially diminished the level of Fra-1, LY294002 (50 μM, PI3-Kinase inhibitor) and SB 203580 (20 μM, p38 MAPK inhibitor) only weakly reduced the Fra-1 level. Further analysis of the effects of PD098059 on the kinetic of induction Fra-1 (protein level by immunoblot) showed a gradual decline. Also, all the three bands appearing due to its multiple phosphorylation (a characteristic of Fra-1 protein, Vial and Marshall, 2003) diminished with increasing time (Fig.25). Notably, in cells treated with 2μM NE and in untreated control, PD 098059 also had caused a reduction in Fra-1 level, thereby indicating a role of ERK in basal expression of Fra-1 as well (note that there was a basal but detectable level of p-ERK in untreated cells). Notably similar assay with the JNK inhibitor was not done (though it had shown inhibitory effect on Fra-1 in Fig.24) since the addition of Dicumarol has detrimental effect on cells as evident from the cell morphology seen under microscope after 4 hours.
Fig. 24. Role of PI3-kinase and MAPKinas in inducing Fra-1: Cells were treated with PI3 kinase inhibitor LY294002 (25 μM), p38 MAPK inhibitor SB203580 (20 μM), JNK inhibitor (Dicumarol, 50 μM) and MEK inhibitor PD98059 (50 μM) one hour prior to NE (100 μM) treatment. After four hours cell extracts were prepared and assayed for Fra-1 level by immunoblot. Serum treatment for 4 hour was done as a positive control. Fold increases in FRA-1 level was measured by densitometric analysis of the autoluminogram by Fuji FLA 5000 phosphorimager. Data shown above is one of two independent experiments that yielded similar results.
Fig. 25 A&B. Induction (and stabilisation) of Fra-1 by NE (2 μM and 100 μM) is mediated via ERK. Thirty minutes prior to NE treatment, cells were treated with MEK inhibitor PD 098059 (50 μM). Serum treatment (4 hour) was done as a positive control. One hundred and fifty microgram of total cellular protein were assayed for the Fra-1 level by immunoblot analysis (A & B).
Fig. 25C. Fold changes in FRA-1 level was measured by densitometric analysis in a Fuji FLA 5000 phosphorimager. Data shown above is one of the two independent experiments that yielded similar results.
4.2. Discussion

In eukaryotes, extracellular stimuli are transduced to the nucleus via multitudes of signal transduction pathways some of which are well understood and others are presumably not known yet. Mechanisms by which various signal transduction pathways achieve specificity as well as divergence have been a subject of immense interest. As an example, hundreds of G-protein coupled receptors utilize a common second messenger i.e., cAMP, but still achieve disparate biological effects by a mechanism that is not fully understood yet. Emerging evidences suggest that specificity of signal transmission, its amplitude and duration is achieved by distinctive interactions among various second messengers, kinases and phosphatases.

A large number of signaling kinases such as PKC, PKA, PKB, ERK, JNK and p38 MAPKs have been implicated in the induction of AP-1 in various cellular contexts (Whitmarsh and Davis, 1996; Han et al., 1997; Kato et al., 1997; Pursiheimo et al., 2002; Li et al., 2002; Cataisson et al., 2003). Nevertheless, induction of AP-1 proteins are primarily regulated by MAPKinase pathways and at least some signaling kinases such as PKC and PI3-Kinase feed to the ERK pathway and thereby mediate their effects. Analysis of the regulatory regions of AP-1 genes have shown unique assemblies of cis-regulatory elements (such as SP-1, SRF, CRE etc.), distinctively contributing to their patterns of expression (Hazzalin and Mahadevan, 2002). How stimuli as diverse as UV radiation to mechanical stretch are integrated at the level of above mentioned signaling kinases and induce distinct sets of AP-1 genes are largely unknown (Peverali et al., 2001; Li et al., 2002; Mitchell et al., 2004; Tanos et al., 2005). Furthermore, AP-1 genes are also under auto-regulatory loops that modulates their own transcription (Deng and Karin, 1994; Berger and Shaul, 1998; Murakami et al., 1999) How different members of the AP-1 family integrate the input signals and generate distinctive expression profile is an emerging area of interest (Hazzalin and Mahadevan, 2002).

In cardiac myocytes, induction of MAPKinases viz., ERK, JNK and p38 have been documented under various conditions such as mechanical stretch, pressure-volume over load, neuroendocrine stimuli.; (Frey and Olson, 2003; Petrich and
Wang, 2004; Baines and Molkentin, 2005). In this context, our observation that two different doses of NE differentially induce the members of the MAPKinase family i.e., ERK, JNK, p38-Kinase and PI3-Kinase exemplifies how signals emanating from multiple adrenergic receptors (α, β1 and β2) are integrated further downstream. In metazoan cells cross talk between various signaling cascades is a highly evolved mechanism of signal augmentation (Bourguignon et al., 2005; Hendriks et al., 2005), signal attenuation (Williamson et al., 2005) and signal sustenance (Kamata et al., 2005; Knodler et al., 2005). Although sustained activation of various MAPKinases have often been associated with cell cycle arrest and apoptosis, it is not known yet whether it is a general mechanism of triggering cell death (Wang et al., 2004; Chen et al., 2005). The consequences of transient versus sustained activation of various MAPKinases are also context dependent. While in hepatocytes expressing hepatitis B virus X protein, sustained induction of p38 and JNK is associated with the expression of pro-apoptotic FasL and TNF-α receptor (Wang et al., 2004), in osteoclasts, pro-apoptotic effects of 17 beta-estradiol are related to the sustained activation and nuclear localization of ERK, presumably engaging it in the transcription of pro-apoptotic genes (Chen et al., 2005). The biochemical mechanism (s) by which a transient signal is converted into a sustained one is largely unknown and is of emerging interest (Price et al., 2004). Taken together, it will be of future interest to understand how signals transduced by α- and β-adrenergic receptors are integrated at the level of ERK, JNK and p38 and modulate their amplitude and durations thereby causing a transition from hypertrophic to apoptotic phenotype.

In cardiac myocytes (and also in the myocardium in vivo) induction of ERK, JNK and p38 have extensively been documented in the context of their proposed roles in inducing hypertrophy and apoptosis (Liang and Molkentin, 2003; Petrich and Wang, 2004; Baines and Molkentin, 2005). However, modulation of individual MAPKinases during transition from hypertrophic to apoptotic phenotypes and its connection to downstream gene expression events is largely unknown (Bueno and Molkentin, 2002; Liang and Molkentin, 2003; Petrich and Wang, 2004). In this context, activation of JNK, p38-Kinases and sustenance of p-ERK by 100 μM NE gain significance. Activated MAPKinases phosphorylate and thereby activate a
number of transcription factors like ATF-2, Elk-1, Ets-1, MEF-2, c-Jun, p53, SRF and NFAT-4 involved in downstream gene expression. Although the scope of the present study was restricted only to the AP-1 proteins as the downstream targets of induced MAPKinases, it is thus presumed that with increasing doses of NE, multitudes of other transcriptional regulators are also activated and thereby contributes to the subsequent events. Thus, it will be of future interest to analyze the contributions of each of those subtypes of MAPKinas in modulating various transcriptional regulators further downstream.

Notably, although the results described above (Fig.23) exemplified that the downstream AP-1 activities are connected to all three upstream MAPKinases and PI3- Kinases, available assays such as AP-1 promoter-reporter analysis and gel mobility shift assay could not differentiate between various AP-1 activities (gel mobility-super shift assay was not equally efficient for all the Fos/Jun proteins and thus could not be used for further clarifications). Thus it was not possible to address how each member of the Fos/Jun family are regulated by various kinases upstream. It is also presumed that the synergistic and antagonistic interactions between various pathways might also contribute to the complexity of the process. Nevertheless, establishing a direct link between the induction of ERK and that of Fra-1, has addressed this issue at least partially. During the recent years, number of laboratories have implicated sustained activation of ERK to the activation, stabilization and trafficking of Fos family members (mainly Fos and Fra-1, Murphy et.al., 2002; Murphy et.al., 2004). It will thus be of future interest to know how the regulatory sequences present in the upstream region of the fra-1 gene can discriminate between transient and sustained ERK activities and gets modulated accordingly. Moreover, it will also be relevant to know how fosB expression is inversely regulated under respective conditions. Experiments in these directions are currently under progress by other researchers in the laboratory.
Part III

While Jun has a Strong Pro-Survival Function, Overexpression of Jun and Fra-1 Do not Enhance Cell Survival
Gene expression research is aimed at deciphering the signaling cascades that activate transcription factors which in turn modulate the downstream targets. Mechanism by which sequence specific DNA binding proteins (transcription factors in the present context) recognize their cognate sequences is an emerging area of structural biochemistry. Hundreds of homeodomain proteins recognize their cognate sequences with distinct specificity arising due to subtle differences in the amino acid sequences present in their DNA binding domains (homeodomain) (Kornberg, 1993) Since the discovery of Jun and Fos proteins in late eighties, the number of constituents of AP-1 complexes as well as the repertoire of target genes they regulate have significantly expanded. Nevertheless, in spite of the diversity of the target genes and their roles in cell function, the DNA elements recognized by different AP-1 (and related) dimers are highly invariant (Vinson et al., 2002). The canonical AP-1 sequence targeted by different Fos Jun dimers is defined as TGA\textsuperscript{C/G}TCA (TRE). Therefore, how different AP-1 complexes discriminate between various target genes, especially in a milieu where multiple AP-1 complexes are present, remains an enigma. It has been proposed that AP-1 proteins upon binding, bend the DNA that involves the nucleotides adjacent to the core binding sites and thereby those flanking nucleotides contribute to their binding specificities (Ramirez-Carrozzi and Kerppola, 2001; Chinenov and Kerpola, 2001; Ramirez-Carrozzi and Kerppola, 2003).

Over the past decade diverse techniques of molecular biology such as transcript analysis (Northern blot and micro array), promoter-reporter analysis (by co-transfection assays using promoter-reporter constructs with expression vectors for Jun/Fos proteins), and gene/transcript ablation (by antisense oligonucleotide, gene knockout and RNAi mediated inactivation of various Fos/Jun proteins) have been attempted to identify the role of AP-1 proteins and their target genes. However, such assays have often led to dichotomous results. As an example, induction of ANF gene is considered to be a hallmark of hypertrophy and the upstream regulatory regions of ANF promoter contain AP-1 binding sites. However, coexpression of various Jun/Fos proteins with the ANF promoter reporter constructs showed both activation and repression based on the experimental contexts (McBride and Nemer, 1993; Kovacic-Milivojevic and Gardner, 1995).
Such ambiguous results may however be attributed to the less appreciation (during early nineties) of role of coactivators and other chromatin modifying enzymes in promoter engagement of transcription factors. Similarly, gene knock out studies in vivo has also revealed that a wide role of various Jun/Fos proteins in cell cycle regulation (both progression and arrest), cell differentiation (Keratinocytes, T Cells, Bone cells etc), induction of apoptosis (Neurons, Fibroblasts, Photoreceptor cells, Keratinocytes etc) and tumor progression. Further more, the role of individual AP-1 proteins in those events might be highly context specific (for example, while Fos is pro apoptotic in photoreceptor cells and hepatocytes it is anti-apoptotic in neurons). Therefore, to define a role for AP-1 proteins in general and Fra-1 in particular (in cardiac myocytes), it might be more rational to establish first whether it is pro or anti apoptotic in the present context and then to identify the potential target (genes) and understand their regulation in a specific context. In view of the scope of the present study, it was restricted only to understand whether Jun andFra-1 play pro- or anti-apoptotic roles in H9c2 myoblasts under adrenergic stress. Data shown in the following section establishes a pro survival function of Jun while that of Fra-1 remains ambiguous.

5.1 Results

5.1.1 Different AP-1 Complexes May Simultaneously Occupy the Same Target Site

In a nuclear milieu, specificity and affinity of different AP-1 complexes towards their target site(s) is influenced by the presence of other regulatory proteins such as CREB, Maf, JDP (bZip family) and Menin (a non-bZip tumor suppressor protein). Furthermore, the promoter engagement of a particular AP-1 complex may also be influenced by the transcription factors occupying the adjoining sequences in the enhanceosome (Maniatis et al., 1998). Since the long term objective of this study is to understand the molecular mechanisms by which different AP-1 complexes (induced by 2 μM and 100 μM of NE in the present context) recognize their target sequences and thereby modulate the cognate genes, it was imperative to test how respective AP-1 complexes differentiate between the target sequences.
Available information suggests that chromatin immuno-precipitation (Chip) is the assay of choice to answer such questions. However, it is feasible only when the target promoter is known and thus is not feasible in the present case. As an alternative, antibody super-shift analysis was attempted. Cells were treated with 2 μM and 100 μM NE respectively and gel mobility shift assay were performed with the nuclear extracts using radiolabeled TRE (Collagenase promoter). The AP-1 complexes thus formed were thereafter analyzed by super-shift assay using antibody against Jun, Fos, FosB and Fra-1. Contrary to the expectation, however, none of the antibodies except Jun and Fra-1 showed any visible shift in the AP-1 complexes (Fig. 26A and 26B). Furthermore, the super-shift shown by Fra-1 appeared to be weak and presumably partial. Such limitations in super-shift assay have also been experienced by others and are attributed to the inability of the antibodies to recognize the native form of binding proteins (Novak and Paradiso, 1995; Yamamoto, 1997; Osborn et al., 1999). Thus a second strategy i.e., limited proteolytic analysis was attempted thereafter. Limited proteolytic digestion is based on the principle that if a DNA protein complex is subjected to a low concentration of a proteolytic enzyme (like Chymotrypsin in the present case), the domains other than the DNA binding domain will be digested first (being extended outward) and a smaller complex, characteristic of the residual DNA binding domain still bound to the cognate sequence would appear. Based on the digestion pattern thus generated, the extent of heterogeneity (arising due to the binding of closely related proteins) in a given DNA protein complex can be ascertained (Fujita et al., 1992; Wood et al., 1998; Yoneda et al., 1999; Ayala et al., 2002). Such analysis of the AP-1 complexes formed by nuclear proteins prepared from cells treated with 2 μM and 100 μM NE and radiolabeled TRE (Collagenase promoter) did not reveal any appreciable difference in profile (Fig. 27A). To reiterate this observation, similar analysis was also undertaken with the AP-1 sequence found in the Angiotensin II promoter (implicated to its induction by hypertrophic stimuli) and as shown in Fig. 27B a subtle difference in the profile of the AP-1 complex was observed. Unlike with Collagenase TRE, the Angiotensin II AP-1 site formed two discrete but closely migrating complexes (identified by arrows). However, the chymotrypsin digestion profile of the AP-1 complex generated by Ang II TRE was largely similar to that of the Collagenase
Fig. 26A. Antibody super-shift analysis of AP-1 complexes induced by NE: Nuclear extracts were prepared from cells treated with 2 μM and NE 100 μM NE for two hours. Gel mobility shift analysis was done with eight microgram of nuclear extracts with AP-1 probe both in absence (left panel) and in presence (right panel) of rabbit polyclonal antibody against Jun. Antibody (1 μl) was added to binding reaction ten minutes before the addition of probe. Retention of a small amount of radioactivity in the wells of antibody plus lanes indicate a partial supershift.
Fig. 26B. Antibody super-shift analysis of AP-1 complexes induced by NE:
Nuclear extracts were prepared from cells treated with 2 μM (left panel) and 100 μM (right panel) NE for two hours. Gel mobility shift analysis was done with eight microgram of nuclear extracts and AP-1 probe both in absence (left panel) and in presence (right panel) of rabbit polyclonal antibody against FosB, Fra-1 and Fos. Antibodies (1 μl) were added to binding reaction ten minutes before the addition of probe. A partial supershift was seen only with Fra-1 antibody and extracts from 100μM NE treated cells.
Fig. 27A. Limited proteolytic cleavage of AP-1 complexes: (Left panel): Nuclear extracts from H9c2 cells treated with 2 μM NE for the indicated time period were incubated with radio-labelled TRE (collagenase promoter) for 40 minutes on ice followed by the addition of 10 ng of Chymotrypsin. The reactions were then incubated for 5 minutes (Lanes 2, 5, 8, 11) and 10 minutes (Lanes 3, 6, 9 and 12) at room temperature and resolved on non-denaturing polyacrylamide gel in 0.5 X TBE. Undigested lanes were numbered as 1, 4, 7 and 10. For a comparison Serum treated extracts were also digested for 10 min (Lane 13).

(Right panel) Nuclear extracts from 100 μM NE treated cells for the indicated time periods were digested similarly with chymotrypsin for 5 minutes (Lanes 2, 5, 8, 11) and 10 minutes (Lanes 3, 6, 9 and 12) followed by non-denaturing polyacrylamide gel electrophoresis. For a comparison Serum treated extracts were also digested for 5 and 10 minutes (Lane 14 and 15). Undigested lanes were shown as 1, 4, 7, 7 and 13.
Fig. 27B. Limited proteolytic cleavage of AP-1 complexes. Nuclear extracts from H9c2 cells treated with 100 uM NE were incubated with radiolabelled AP-1 oligonucleotide (containing AP-1 binding site from Angiotensin II promoter) for 40 minutes on ice followed by the addition of 10 ng of chymotrypsin. The reactions were then incubated for 5 minutes (Lanes 2, 5, 8, 11) and 10 minutes (Lanes 3, 6, 9 and 12) at room temperature and resolved on non-denaturing polyacrylamide gel in 0.5 X TBE. For a comparison serum treated extracts were also digested similarly (lanes 14 and 15). Undigested lanes are shown as 1, 4, 7, 10 and 13.
TRE (Fig 27B). Taken together it appears that both super-shift and limited proteolysis were not sensitive enough to determine the composition of AP-1 complexes bound to various TRE sequences. Nonetheless, it is likely that at a given condition (at least in vitro), a particular TRE sequence is likely to be concurrently occupied by multiple AP-1 dimers.

5.1.2 Human fra-1 Promoter is Differentially Induced by NE

As shown in Fig 18 (Chapter 1), a promoter-reporter construct containing a heptamer of AP-1 binding sites ([TRE]$_7$=-TATA-Luc) is induced by NE in a dose dependent manner (100 μM > 2 μM). Since levels of induction of AP-1 binding activity is also dose responsive (Fig 1, Chapter 1), it appears that, synthetic AP-1 promoters are activated in a dose responsive manner rather than a dimer specific manner (Jun:FosB versus Jun:Fra-1 in the given context). This observation is also in agreement with the in vitro binding data shown in Fig.26 - 27. However, numerous studies have also shown that different AP-1 proteins have gene specific functions (Hess et al., 2004). To address this discrepancy, the modulations of human fra-1 promoter by low and high doses of NE were tested. In epithelial cells, human fra-1 is induced by a wide spectrum of environmental toxicants such as tobacco smoke, silica and asbestos (Adiseshaiah et al., 2003). It has thus been hypothesized that following initial stimulation (by smoke etc.), AP-1 complex consisting of Jun:Fos activates the fra-1 promoter as its down stream target and thereby sustains the AP-1 activity). Human fra-1 promoter contains five TRE elements (spanning over -861 bp upstream) mediating its induction by various stimuli. It was thus likely that in H9c2 cells, human fra-1 promoter reporter construct would be preferentially activated by high dose NE (as it occurs for endogenous fra-1). H9c2 cells were thus transfected with the human fra-1 promoter reporter constructs (hfra-I-861-Luc and hfra-I-371-Luc, kind gifts from Dr. S. Reddy, Department of Environmental Health Sciences, The Johns Hopkins University, Baltimore, USA) and the luciferase reporter activities were measured after 4 hours of treatment with 100 μM NE. As shown in Fig.28, NE increased the reporter activity driven by the hfra-I-861-Luc that was compromised upon deletion of the promoter sequence to -371 bp (hfra-I-371-Luc), thereby indicating a role of three TRE elements present in the region between -861 to -371.
Fig. 28. Induction of human fra-1 promoter by 100 μM NE: Cells were transiently transfected with 1μg (per 35 mm dish) of the indicated human fra-1 promoter reporter plasmids hfra-1-861-luc and hfra-1-371-luc. Thirty six hours after transfection cells were treated with 100 μM norepinephrine for three hours. The reporter activity per μg protein was calculated and expressed as fold induction compared to control (hrfa-1-861-luc). Serum treatment was done as a positive control. Data shown above is an average of two independent experiments.
Fig. 29. Differential induction of human Fra-1 promoter by NE: Cells were transiently transfected with 1μg of hfra-1-861-luc plasmid and thirty six hours after transfection cells were treated with 2 and 100 μM norepinephrine for the indicated time periods. The relative reporter activity per μg protein was calculated and data expressed as fold induction compared to untreated control. Data shown here is an average of two independent experiments showing similar results.
(Adiseshaiah et al., 2003). When the effects of both doses of NE were tested, there was an initial decrease (2 hours), in luciferase level that was followed by a moderate increase in 4 hours (for both low and high doses of NE). However, contrary to the pattern of induction of endogenous fra-1 (100 μM > 2 μM), hfra-1-871-Luc was induced more by 2 μM NE than by 100 μM NE (Fig.29). Available literature suggests that the arrangements of various regulatory sequences in the human fra-1 gene are different from that in rat fra-1 (upstream regions vis à vis intronic region) although both are AP-1 responsive (Bergers et al., 1995). Nonetheless, such differential induction of human fra-1 promoter by NE (2 μM > 100 μM) exemplified that in the natural context, an AP-1 containing promoter can respond to specific AP-1 complexes rather than the total AP-1 level. However, for substantiating this conclusion, further analysis with the sequence (TRE) specific mutants are required (for more comprehensive analysis, the rat fra-1 and fosB promoters are currently being cloned in the laboratory).

5.1.3 While c-Jun is a Pro-Survival Factor, Overexpression or Attenuation of Fra-1 Does not Influence the Apoptotic or Survival Events

Based on the cellular context, Jun might have both pro- (neurons and fibroblasts) and anti-apoptotic effects (hepatocytes) (Hess et al., 2004). In cardiac myocytes, induction of Jun has been documented under multiple conditions but its role in apoptosis has not been investigated. To determine the role of Jun in this context, a dominant negative mutant of Jun was constructed. The activation domain of Jun (consisting of three subdomains) spans over first ~200 amino acids from the amino terminus followed by the leucine zipper and the DNA binding domains. A recombinant cDNA was thus constructed where the first 194 amino acids of Jun were replaced by the ~26 kD Green fluorescence protein (GFP, in frame with the DNA binding domain) and the fusion protein was confirmed to be nuclear localized after transient transfection (Fig.30 & 31). This recombinant protein is thus likely to dimerize with the counter part of Jun (presumably Fos at the beginning and FosB or Fra-1 at latter times based on NE concentration) and target cognate genes but not activating it as it lacks the activation domain. When this recombinant protein was expressed in H9c2 cells, the recipient cells remained normal till in serum containing medium. However subsequent incubation in serum free medium (for treatment with NE), resulted in extensive cell death in 12 hours,
Fig. 30. Detailed structure of the Jun Dominant negative mutant: The nucleotide sequence of the Jun ORF is shown. The sequence corresponding to aa 1-6 and 195-332 is marked in blue and is present in the mutant while the intervening sequence (aa 6-194) is marked red and is absent. The corresponding amino acid sequence is shown below with the same colour representation. The Recombinant cDNA lacking nucleotides corresponding to aa 6-194 was generated by PCR amplification of a mutant cDNA (lacking aa 6-194, Angel et al., 1989; Goswami et al., 2001) by forward and reverse primers shown above and below the sequence (where the complementary sequence is marked blue and adjoining sequence is marked black). The PCR product was digested by HindIII and BamHI and the digested DNA was inserted into HindIII & BamHI Digested GFP expression vector (pEGFP N1, Clonetech) in frame with GFP at the amino terminus.
Fig. 31. A. Nuclear localisation of GFP-DNM-Jun. Schematic presentation of the GFP expression vector used for the construction of GFP-DNM-Jun. Multiple cloning site of the same is also shown (Lower Panel). B. Cos-7 cells were transfected with GFP-DNM-Jun. After 24 hours nuclear localisation was observed under fluorescent microscope. CTD: Carboxy terminal domain; DB (LZ): DNA binding domain with leucine zipper;
Fig. 32. Jun is a prosurvival factor for H9c2 myoblasts. H9c2 myoblasts were transfected with GFP-DNM-Jun or GFP-Vector alone (2 µg per 35 mm dishes) Twenty four after transfection cells were treated with serum free medium for another 12 Hours. Extensive cell death was seen in GFP-DNM-Jun transfected cells upon serum withdrawal.
thereby indicating a strong pro-survival role of Jun (Fig 32). Since recombinant GFP-DNJun expressing cells did not survive in serum free medium, the consequences of treatment with NE could not be ascertained.

Although the induction of fra-1 has been documented in various cellular contexts, its target genes are largely unknown (Burch et al., 2004) and at least under certain conditions, it can compensate for c-Fos (Fleischmann et. al., 2004). Nevertheless, Fra-1 has been attributed to certain cellular events like bone matrix formation (Eferl et. al., 2004), cellular transformation (Tchernitsa et al., 2004) and retinal apoptosis (Wenzel et al., 2002). However, function of Fra-1 in cardiac muscle cells is completely unknown. I thus tested the role of Fra-1 in the context of apoptosis induced by NE. To this purpose, two different approaches were taken i.e., overexpression of Fra-1(by Fra-1 expression vector) and attenuation its of transcript by antisense oligonucleotides. H9c2 cells were transfected transiently with an expression vector for fra-1 (pRK7-fra-1, a kind gift from Busslinger M, Vienna Biocentre, Vienna) alone or in conjunction with that for c-jun (pRSV-jun). Following transfection, cells were treated with 100 µM NE and the cell viability were assayed after 36 hours. As shown in Fig 34, over expression of Fra-1 alone had a moderate (~ 20 %) stimulatory effect on H9c2 myoblast survival and also it had shown a moderate protecting effect from NE mediated apoptosis. On the other hand, Jun did not influence the survival of H9c2 cells either alone or with Fra-1. Furthermore, at a latter time points (48 - 60 hours), when the extent of cell death was more, the protective effects of Fra-1 was less apparent (data not shown). Similar analysis with antisense Fra-1 oligonucleotides also did not show any appreciable difference between the treated and untreated sets (Fig.33). Taken together, it appears that Fra-1 might play a protective (but ultimately it becomes nullified by other factors) following NE treatment, however, it fails to provide any absolute protection when expressed in abundance.

5.2 Discussion

Induction of transcription factor activity is followed by a sequence of events (such as binding to dimeric partners, other coactivators, and chromatin modifying enzymes) that leads to its engagement to the cognate genes. Although biochemical, molecular and genetic approaches have significantly contributed to
Fig. 33. Antisense Fra-1 oligonucleotide does not prevent cell death by NE: H9c2 cells were treated with antisense (5 μM, 5’-GAAGTCTCGGTACAT-3, phosphorothioate modified) and corresponding sense-oligonucleotides (5 μM, 5’-ATGACTGCAAAGATG-3’) oligonucleotides for fra-1 along with treatment with 100 μM NE. Cells were replenished with the oligonucleotides every 12 hours till 24 hours. Cell survival was assessed by the MTT reagent after 36 hours and expressed relative to control (untreated) cells.
Fig. 34. H9c2 myoblasts were transfected 2 ug (per 35 mm dish) each of Fra-1, Jun and Jun + Fra-1 expression plasmids or respective empty vectors (as controls). Twenty-four hours after transfection cells were treated with serum free medium for 16 hours and thereafter treated with NE 100μM. Cell survival was assessed by the MTT reagent 36 hours after NE treatment and expressed relative to respective control (empty vector).
our understanding of those events (and thereby the mechanisms of context specific gene expression), a comprehensive picture of gene regulatory events in a cellular milieu is a distant goal as yet. Furthermore, many of the suggested mechanisms of gene specific engagement of transcriptional regulators is restrictive to a particular tissue type rather than a universal phenomenon. For example, while under certain conditions Fra-1 can compensate for Fos, under certain others, they are mutually antagonistic (Yoshioka et al., 1995; Fleischmann et al., 2000).

The cardinal feature of the present study is the evidence that adrenergic stimuli induced by lower dose and higher doses of NE converge to two distinct members of the Fos family i.e., FosB and Fra-1. The underlying mechanisms is further exemplified by the demonstration that the duration and amplitude of ERK activity induced by 100 μM NE is the key determinant of the induction of Fra-1 (while that of Fos B is yet to be determined). Nevertheless, the essence of these observations lies at the identification of set of genes that is differentially activated by Fra-1. Paradoxically, investigation of AP-1 target genes in cardiac myocytes has often led to dichotomous results (McBride et al., 1993; Kovacic-Milivojevic and Gardner, 1995) and thus needs further investigation.

Consensus AP-1 element is a palindrome (TGACTCA) and X-ray crystal structure of the DNA-AP-1 complex suggests that both Fos and Jun make essentially identical contacts with the two half-sites (Ramirez-Carrozzi and Kerppola, 2001; Chinenov and Kerppola, 2001; Ramirez-Carrozzi and Kerppola, 2003). However, the target site for AP-1 is not absolutely symmetrical as the central base pair (C/G) creates two different overlapping half-sites (TGAc and TGAg). Base substitution studies indicated that the preference (by Fos and Jun proteins) for the half sites is influenced by the asymmetric central base pair as well as the flanking DNA sequences. Upon binding to the half sites, Fos and Jun interact with the flanking nucleotides and bend DNA in opposite directions. It is thus suggested that since the ability of DNA bending for Fos and Jun are different, based on the relative bending propensities of the flanking sequences (on both sides of the core sequence), the orientation of Fos-Jun binding on to the palindrome is determined (Ramirez-Carrozzi and Kerppola, 2001; Ramirez-Carrozzi and Kerppola, 2003). Furthermore, the orientation of Fos:Jun binding can also be influenced by their
interactions with other transcription factors binding to an adjacent regulatory element (Ramirez-Carrozzi and Kerppola, 2003). Notably, the DNA recognition preference for other AP-1 complexes (Jun:Fra-1; Jun:FosB) has not been investigated and experimentation in this regard may shed light on the molecular basis of gene specific function of other AP-1 complexes (Jun-Fra-1, Jun-FosB etc). In this context, the antibody super-shift analysis and limited proteolysis appears preliminary and more advance assays like conformationally sensitive gel electrophoresis and fluorescence resonance energy transfer might be required for addressing these questions and was not within the scope of the present study.

Although a large repertoire of AP-1 target genes have been identified over the years, primarily they are the targets of Jun-Fos dimers while few targets for Fra-1 are known as yet. Nevertheless, in cellular (and in physiological) context a number of phenotypes such as cell survival, cell death and cell differentiation has been associated with Fra-1. It is thus anticipated that a repertoire of genes selectively targeted by Fra-1 might exist but a comprehensive understanding of their identities are yet to emerge. In the context of cardiac muscle, regulatory regions of a number of marker genes for hypertrophy (such as atrial natriuretic factor and skeletal alpha actin) contain AP-1 target sites and different AP-1 members activate respective promoters in transient transfection assays. However, transiently transfected promoter reporter constructs are devoid of genomic contexts and can be targeted by transcription factors which otherwise are not involved in its regulation. Moreover, genes, which are modulated during the onset of apoptosis induced by NE, are not known yet. Taken together, the antisense and the overexpression approach to assess the contribution of Fra-1 was reasonable though it did not lead to any appreciable conclusion. Such outcome can be explained by a number of possibilities such as (i) whatever roles Fra-1 plays is achieved by the basal and the induced expression of endogenous Fra-1 that is not further augmented by the overexpression, (ii) Increased level of Fra-1 (in association with Jun) might not be able to exert their effects due to the lack of other co-regulators, (iii) it is also possible that Fra-1 play a secondary role in the apoptotic events induced by NE and thus its overexpression (or attenuation by antisense oligo nucleotide) does not affect the ultimate consequences. Alternative experiments such as siRNA or dominant negative mutant approach might be
needed for further evaluation of the role of Fra-1 in NE induced cell death (or survival).

In this context, the pro-survival function of Jun as demonstrated by its dominant negative mutant assumes significance. Since Jun is the dimerization partner of multiple Fos proteins (assuming that other members of Jun family i.e., JunD and JunB are absent in this context), suppression of Jun function by a dominant negative mutant was likely to cause a general inhibition of AP-1 activities. Dominant negative mutants of AP-1 proteins are of two different types (i) one in which the DNA binding domain is kept intact while the transactivation domain is removed. Thereby, the mutant protein binds DNA but fails to activate transcription. Such mutants have been used for suppression of both Jun and Fos functions (Dhar et al., 2004). (ii) Alternatively, the DNA binding domain is removed while keeping the transactivation domain intact. These mutants will squelch out specific coactivators and thereby suppress the function of the wild type counterpart. However, in view of the close interrelationship amongst various Jun/Fos proteins (and other bZIP members as well), any alteration in the functions of one might impair functions of others. Nevertheless amongst the three dominant mutants of AP-1 proteins (Jun, Fos and Fra-1), the one that is for Jun (TAM-67) has extensively been used for the attenuation of Jun function ex vivo (Dhar et al., 2004). Furthermore, another dominant negative Jun mutant (aa 3 - 122) has been tested in the context of cardiac myocytes and it blocks induction of hypertrophy induced by Endothelin and Phenylephrine (Omura et al., 2002). Notably, number of other studies has also revealed that TAM-67 still retained some transactivation functions (Chen et al., 2005). The possible explanation is that the transactivation domain of Jun consists of ~100 aa from the amino terminus comprising three sub-domains (Goswami et al., 2001 and Angel et al., 1989). In TAM-67, first two activation domains are deleted while the third one is retained, presumably contributing to the residual activity. In contrast to TAM-67, the dominant negative mutant constructed in this study had entire transactivation domain plus an additional 100 aa (i.e., aa 6 to 194, McBride et al., 1993; Goswami et al., 2001) deleted and the resulting mutant was highly detrimental to cell survival in serum free medium (but not in serum containing medium). In contrast, the dominant negative mutant used by Omura et al had aa 3 -122 deleted and it
blocked only hypertrophy but did affect cell survival. Taken together it appears that the N-terminus region (aa 1 to 194) of Jun might contribute in multiple ways (i.e., towards the induction of hypertrophy and cell survival, presumably via interaction with different cofactors). Therefore, further study involving the dominant negative mutants of Fos and Fra-1 in conjunction with a number of putative target genes might extend our current understanding of the role of AP-1 in cardiac muscle gene expression in general and the apoptotic events in particular.