Section 5.
Chapter 2

Regulation of ceruloplasmin by catecholamines and its molecular mechanism
5.1 Abstract:

Ceruloplasmin (Cp) is a 132-kD copper containing protein implicated in iron metabolism due to its ferroxidase activity. Several other functions as oxidant, as antioxidant, in nitric oxide metabolism and as amine oxidase are also attributed to Cp. Accordingly, Cp was reported to be regulated by cellular iron status, hypoxia, and oxidants as well as by antioxidants. However, despite containing an amine oxidant activity Cp regulation by any biogenic amine including catecholamines have never been explored. Present study, demonstrates that epinephrine (EPI) and norepinephrine (NE) can induce Cp expression in hepatocarcinoma cell line HepG2 on transcriptional level by differential mechanisms. Earlier study has revealed the role of AP-1 response element (APRE), located about 3.7 kb upstream of Cp translation start site in Cp promoter, in up-regulation of Cp expression in HepG2 cells. It was found that both EPI and NE increased Cp expression mediated by Cp-APRE as analyzed by reporter gene assays and EMSAs. Interestingly, evidences showed that Cp-APRE is sufficient for up-regulation of Cp by NE, however, EPI, in addition to APRE also employs a C/EBP binding site, located about -700 bp upstream of Cp translation start site for Cp regulation. Use of different adrenergic subtype antagonists revealed that NE mediated regulation of Cp expression was due to activation of β1-AR, while, EPI may induce Cp expression by activation of β2-AR. These studies potentially signify the amine oxidase activity of Cp, as plasma Cp may plays a protective role against toxicity generated by catecholamines by oxidation.
5.2 Introduction:

Ceruloplasmin (Cp) is a 132 kD plasma glycoprotein consisting of 1046 amino acids with a carbohydrate content between 7% and 8% (Takahashi et al., 1984; Sato and Gitlin, 1991). It is a member of the multi-copper oxidase family of enzymes that includes ascorbate oxidase and the laccases (Messerschmidt and Huber, 1990). Cp contains 6 tightly bound and 1-loosely bound copper atoms per molecule and accounts for 95% of total plasma copper in a healthy adult (Fox et al., 1995). The X-ray crystallographic study of human Cp (Zaitsev et al., 1999) revealed that it is comprised of six cupredoxin-type domains arranged in a triangular array and three of the six integral copper ions form a trinuclear cluster at the interface of domains 1 and 6. Remainder copper ions are arranged in three mononuclear sites, one each in domains 2, 4 and 6. The arrangements of the trinuclear centre and the mononuclear copper ion in domain 6 in Cp molecule are same as that found in ascorbate oxidase (Messerschmidt et al., 1992; Lindley et al., 1997) and other members of the laccase family (Nakamura and Go, 2005), strongly suggesting its role as an oxidase in the plasma. Autosomal recessive mutations of the Cp gene in humans result in aceruloplasminemia leading to age-dependant iron accumulation in different parts of the brain (Miyajima et al., 1987; Miyajima et al., 2001; Hellman and Gitlin, 2002) and other visceral organs (Kono et al., 2006; Dunaief et al., 2006). Cp knockout mice also show slow iron accumulation in CNS and other tissues (Harris et al., 1999; Patel et al., 2002; Jeong and David, 2006), confirming the essential role of Cp in iron homeostasis.

There are two known forms of ceruloplasmin: (a) the secreted form, predominantly synthesized by the liver (Weissman et al., 1966) and activated macrophages (Mazumder et al., 1997) and (b) the membrane-linked glycosylphosphatidylinositol (GPI) -anchored form, predominantly found in the brain (Patel and David, 1997). Secretory and GPI-anchored forms are produced by alternate splicing of Cp mRNA (Patel et al., 2000). Evidences showed that in brain, astroglial cells express GPI-anchored Cp on their surface which plays an essential role in brain iron metabolism as it is involved in iron efflux from cells in the CNS (Patel et al., 2000; Jeong and David, 2003). Both forms of ceruloplasmin have been detected in retinal cells and lymphocytes (Chen et al., 2003; He et al., 2007; Banha et al., 2008).
Chapter 2

The principal function attributed to Cp is that of the plasma ferroxidase activity catalyzing conversion of Fe$^{2+}$ to Fe$^{3+}$ ion that attributes for its antioxidant property as well as required for iron efflux. Ceruloplasmin can function as an antioxidant, since Fe$^{2+}$ catalyzes the generation of hydroxyl radical, the most reactive of the reactive oxygen species (ROS) via the Fenton reaction and due to its ferroxidase activity Cp converts toxic ferrous iron to its nontoxic ferric form (Osaki, 1966). By doing so, Cp also facilitating iron loading to transferrin and ferritin that only bind ferric iron. Since, it is suggested that only ferrous ion can cross the plasma membrane, whereas only ferric ion can be taken up by transferrin, thus by oxidizing ferrous to ferric and increasing iron loading into transferrin, Cp can create an iron gradient that in turn helps in iron release from the cells (Osaki et al., 1966; Lindley et al., 1997; Harris et al., 1999; Hellman and Gitlin, 2002). On the other hand several other reports have shown that ceruloplasmin facilitates iron uptake in iron deficient cells and it was also observed to be regulated by cellular iron content like other iron homeostasis proteins (Mukhopadhyay et al., 1998; Attieh et al., 1999; Qian et al., 2001). Cp also exhibits pro-oxidant activity due to its 7th loosely bound surface Cu atom and can oxidize low density lipoprotein (LDL) in vitro as well as in presence of vascular endothelial and smooth muscle cells (Mukhopadhyay et al., 1996; Mukhopadhyay et al., 1997). Also, elevated levels of Cp have been shown to be linked with development of atherosclerosis and other cardiovascular diseases (Fox et al., 2000). Cp is an acute-phase protein as its concentration in plasma is increased during infections, which suggests its role in inflammation and bactericidal activities (Mazumder et al., 1997; Mazumder et al., 2005). Another series of structural studies located the binding sites for azide (an inhibitor), and various amine substrates including biogenic amines (Zaitsev et al., 1999).

The amine oxidase activity of Cp is known since 1950s when Holmberg and Laurell first measured oxidase activity of Cp using an amine p-phenylenediamine (PPD) as substrate (Holmberg and Laurell, 1951). Later it was shown that Cp can also oxidize biogenic amines (Fellman et al., 1962; Richards et al., 1983) including catecholamines. Still, until now, significance of amine oxidase activity of Cp in plasma with respect to biogenic amines has never been explored. Stress hormones EPI and NE are biogenic amines that might regulate iron homeostasis by up-regulating transferrin receptor 1 (TfR1) expression (in liver and skeletal muscle cells) as
described in previous chapter. Due to its essential role in iron homeostasis and oxidizing amines Cp can serve as a potential target to be regulated by catecholamines EPI and NE. Therefore, to elucidate the effect of catecholamines on Cp expression, a study was carried out in hepatocarcinoma cell line HepG2, as Cp is primarily synthesized by liver, as well as it expresses adrenergic receptors on their surface making it an appropriate model to study Cp expression by catecholamines.

Earlier studies showed the regulation of ceruloplasmin at the transcriptional level involving several cis-acting elements present at the promoter region of Cp gene (Mukhopadhyay et al., 2000; Das et al., 2007; Conley et al., 2005). In the current study it was observed that catecholamines, epinephrine and norepinephrine can upregulate the Cp synthesis by increasing its transcription. Earlier work has provided evidences of presence of an activator protein-1 response element (APRE) at about 3.7 kb away from translation start site in Cp gene (Das et al., 2007) and increased binding at this Cp-APRE was observed by redox copper (Das et al., 2007). In current study, it was observed that both EPI and NE can increase the binding at Cp-APRE that was sufficient for NE mediated Cp expression. However, unlike NE, EPI was also observed to upregulate Cp promoter activity in a chimera reporter construct which was devoid of Cp-APRE.

Earlier studies have shown the role of CCAAT/enhancer-binding proteins (C/EBPs) in trans-activation of multiple acute-phase genes as well as in modulation of genes that are involved in energy metabolism by its binding to specific sequences in the 5’flanking regions of these genes (Poli et al., 1990; Akira et al., 1990; Baumann et al., 1992; Agrawal et al., 2001). Also, binding of C/EBP to the rat Cp promoter has been shown previously (Bingle et al., 1993). Thus, considering the possibility of the involvement of C/EBP in the activation of Cp, a search for a consensus C/EBP binding element was carried out that revealed one putative C/EBP binding site in proximal region of Cp promoter and its role in EPI mediated Cp regulation was investigated.

In present study, it has been shown that catecholamines EPI and NE up-regulate Cp expression in human hepatocarcinoma cell line HepG2 at the transcriptional level, though, utilizing different mechanisms. This difference in EPI and NE response could be due to differential binding of these hormones to specific subtypes of adrenergic receptors expressed by liver cells.
5.3 Results:

5.3:1 Induction of Cp expression by catecholamines EPI and NE in HepG2 cells:

Cp, a plasma glycoprotein, plays essential role in iron homeostasis due to its ferroxidase activity. Cp also exhibit amine oxidase activity and can be a potential target to be regulated by catecholamines EPI and NE as they are the major biogenic amines in the circulation. Therefore, HepG2 cells were treated with increasing concentrations (0-10 μM) of EPI or NE and after incubating for 16 h, Cp levels were detected in conditioned media by immunoblot analyses. Both the catecholamines increased Cp expression in HepG2 cells with a maximal 4 fold increase for EPI and 3.5 fold increase for NE (Fig. 1A-1B) as detected by densitometric analyses. To understand the mechanism of catecholamine-mediated Cp regulation, sub-confluent HepG2 cells were treated with increasing doses of EPI and NE and total RNA was isolated after 12 h for the RT-PCR analyses. A steady state induction in Cp transcript was observed with increasing doses of both EPI and NE while no change in β-actin transcript was detected (Fig. 2A-2B). Time course analysis revealed that a marginal increase in Cp transcript could be detected as early as 4 h with 10 μM NE treatment that was further increased at least up to 16 h (Fig. 2C), as analyzed by RT-PCR.

5.3:2 Catecholamines increase Cp expression by increasing its transcription:

Earlier studies have shown that Cp can be regulated at transcriptional level in response to various stimuli (Das et al., 2007; Mukhopadhyay et al., 2000; Seshadri et al., 2002) by activating cis-acting elements in its promoter region. To elucidate, whether the increase in Cp expression by catecholamines is due to increased transcription, Cp promoter activity was analyzed using the chimera reporter construct containing -4774 bp long Cp 5’flanking promoter region cloned upstream of luciferase gene in pGL3 basic vector, to direct its transcription (Mukhopadhyay et al., 2000; Das et al., 2007). Sub-confluent HepG2 cells were first transfected with Cp-4774-LUC construct and then treated with 10 μM of EPI and NE for 16 h. Luciferase assay was performed in cell lysates and about 5- and 3.5-fold increase in Cp promoter activities were detected with EPI and NE, respectively, in comparison to untreated cells (Fig. 3A).
Figure 1. Regulation of Cp expression by catecholamines in hepatic cells. A, HepG2 cells were incubated with EPI (0–10 μM). After 16 h of incubation conditioned media were subjected to immunoblot analyses using anti Cp antibody (upper panel). B, Similarly, HepG2 cells were incubated with NE (0-10 μM). After 16 h, immunoblot analysis of Cp was performed with the conditioned media (upper panel). All the blots were stained with Coomassie Blue to verify uniform loading of all samples (middle panels). Densitometric analyses are represented as the mean ± SD of three independent observations (lower panels).
Figure 2. Increase in Cp mRNA expression by catecholamines in HepG2 cells. HepG2 cells were incubated with EPI (0-10 μM) (A) and 0-10 μM of NE (B) for 12 h and RT-PCR analysis was performed using Cp specific (upper panel) and β-actin specific (lower panel) primers. C, HepG2 cells were untreated or treated with 10 μM of NE and total RNA was isolated at 4, 8 and 16 h. RT-PCR analysis was performed using Cp specific (upper panel) and β-actin specific (lower panel) primers. The figure shows representative results selected from at least three independent experiments.
The result indicates the presence of cis-acting sequence(s) mediating response of stress hormones within the 4774 bp 5' flanking region of human Cp promoter. To identify cis-acting elements necessary for hepatic expression of the ceruloplasmin gene, shorter fragments of the ceruloplasmin 5' flanking region were cloned upstream of the promoter less luciferase gene in pGL3 basic vector: namely Cp-3701-LUC, Cp-3639-LUC and Cp-800-LUC, as described previously (Das et al., 2007). HepG2 cells at 50% confluence were transfected with these constructs and then treated with 10 μM EPI and NE for 16 h. Luciferase assays revealed that EPI induced Cp-3701-LUC activity by more than 4-fold while NE could increase Cp-3701-LUC activity about 3-fold (Fig. 3B). Furthermore, 10 μM EPI increased luciferase activity in Cp-3639-LUC and Cp-800-LUC about 2.4 and 2.1-fold, respectively (Fig. 3B), while NE failed to induce any luciferase activity in these constructs (Fig. 3B). Previously, it was reported that the Cp gene contains a functional APRE (5'-CTGACTGA-3') between -3684 to -3677 bp upstream of translation start site in Cp promoter that is involved in redox copper mediated increase in Cp expression (Das et al., 2007). To investigate the role of Cp-APRE in catecholamine-mediated Cp expression sub-confluent HepG2 cells were transfected with Cp-APRE-LUC construct containing AP-1 responsive element cloned upstream of luciferase gene in pGL3 basic vector or with a construct containing the mutant Cp-APRE cloned upstream of luciferase gene in pGL3 vector (Das et al, 2007) and then treated with 10 μM EPI and NE for 16 h. In comparison to approx. 3-fold increase for the wild-type, the EPI and NE mediated increase in luciferase activity was completely blocked in the mutated Cp-APRE construct (Fig. 3C), indicating an essential role of Cp-APRE in catecholamine-mediated induction of Cp.

### 5.3.3 Catecholamines induce binding at the Cp-APRE:

To further elucidate the role of APRE in catecholamine-induced Cp expression, nuclear extracts were prepared from 10 μM NE treated or untreated HepG2 cells and subjected to electrophoretic mobility shift assays (EMSAs) using 32P-labeled 24bp long Cp-APRE probes. Result showed increased binding at Cp-APRE sequence in NE treated samples in comparison to control (Fig. 4A, lane 1 and 2). The specificity of the band was confirmed with cold competition assay using unlabeled wild type and mutant Cp-APRE probes. Result showed the loss of binding with 30x and 100x molar
Figure 3. Catecholamines regulate Cp expression by transcriptional mechanism. A, Top, the indicated -4774 bp long ceruloplasmin 5’flanking region, ligated upstream of the luciferase (LUC) gene in pGL3 basic vector was co-transfected into HepG2 cells with a plasmid containing SV40-β-galactosidase reporter gene. After recovery, cells were treated with medium only, 10 μM EPI and NE for 16 h. Luciferase activity in cell extracts was measured and normalized for β-galactosidase activity. B, Similarly, Chimeric pGL3-basic vectors containing -3701 bp, -3639 bp and -800 bp long Cp 5’flanking regions were transfected into HepG2 cells (along with a β-galactosidase plasmid). After recovery, cells were untreated or treated with 10 μM EPI and NE for 16 h. Luciferase activity in cell extracts was measured and normalized for β-galactosidase activity. C, Cp 5’flanking region containing the wild type (Cp-APRE-WT) and mutated (Cp-APRE-mut) sequence of APRE cloned in pGL3 reporter vectors were transiently transfected (along with a β-galactosidase plasmid) into HepG2 cells, separately. After recovery, cells were incubated with (0-10 μM) EPI and NE for 16 h. Luciferase activity in cell extracts was measured and normalized for β-galactosidase activity. All the results are representative of ± SD of three independent experiments performed in triplicates.
excess of unlabeled wild-type Cp-APRE-WT while 30x and 100x molar excess of mutant Cp-APRE-mut failed to do so (Fig. 4A, lane 3 and 4 vs. lane 5 and 6). Similarly, EMSAs were performed by incubating the nuclear extracts isolated at different time points from EPI (10 μM) treated HepG2 cells with $^{32}$P labeled Cp-APRE and a time dependent increase in binding at Cp-APRE was detected with EPI treatment (Fig. 4B, lane 1, 2 and 3). Dose dependent increase in binding at Cp-APRE was also observed in EPI treated HepG2 cells kept for 8 h of incubation, as analyzed by EMSA (Fig. 4B, lane 4, 5 and 6). The specificity of the band was confirmed by cold competition assay using wild type and mutant Cp-APRE unlabeled probes and it was found that incubation with 30x and 100x molar excess of unlabeled wild type Cp-APRE-WT probe with the nuclear extracts could block the appearance of band while 100x concentration of mutant Cp-APRE-mut sequence failed to do so (Fig. 4C, lane 3 and 4 vs. lane 5). These results indicate that both catecholamines can induce binding at Cp-APRE in Cp promoter region in order to increase Cp expression.

5.3.4 Epinephrine induces binding of C/EBP to Cp 5’flanking region:

As described in the above section unlike NE, EPI increased the activity of both Cp-3639-LUC and Cp-800-LUC chimera constructs that were devoid of APRE indicating the role of additional cis-acting element(s) in response to EPI in hepatocytes. Thus to identify the region functionally important in EPI mediated Cp expression, additional transfections were performed with constructs generated by further deletion of -800bp 5’flanking region of Cp promoter and cloned upstream of luciferase gene in pGL3 basic vector: namely Cp-768-LUC, Cp-700-LUC and Cp-600-LUC containing 768, 700 and 600bp long 5’flanking region of Cp promoter, respectively. HepG2 cells were transfected with these constructs and then treated with 10 μM epinephrine for 16 h. Luciferase assay showed about 2.3-fold induction in both Cp-768-LUC and Cp-700-LUC constructs by EPI, whereas, EPI failed to alter luciferase expression of Cp-600-LUC construct (Fig. 5A), suggesting the presence of EPI sensitive cis-acting element within -700 to -600 bp 5’flanking region of Cp gene.

The region was further analyzed for sequence homology for known cis-acting elements using nucleotide data base. Between -700 and -650bp a region of partial homology to the C/EBP binding site was identified. The greatest homology of this region (85 %) exists between -677 and -660bp away from Cp translation start site. No
Figure 4. Specific binding of catecholamine-stimulated transcription factor complex to Cp-APRE. EMSAs were carried out to determine the Cp-APRE complexes. A, HepG2 cells were untreated and treated for 8 h with 10 μM NE and nuclear extracts were prepared. The 32P-labelled 24bp long probe containing the Cp-APRE was also pre-mixed with unlabelled wild-type Cp-APRE (lane 3 and 4) and mutated Cp-APRE (Cp-APRE-mut) (lane 5 and 6) probes in 30x and 100x molar excess before addition to the nuclear extracts. B, HepG2 cells were treated with 10 μM EPI for different time periods (0, 4 and 8 h) and with different doses of EPI (0-10 μM) for 8 h and nuclear extracts were subjected to EMSAs as described in the text. C, Specificity of the binding was determined with 30x and 100x molar excess of cold wild type Cp-APRE-WT (lane 3 and 4) and a 100x molar excess of Cp-APRE-mut (lane 5). Probe-bound complexes were resolved by 5% non-denaturing PAGE and visualized by autoradiography. The figure shows representative results selected from at least three independent experiments.
other sequences homologous to characterized cis-acting elements were identified in functionally defined region of the Cp gene.

Thus, as the region from -700 to -600 demonstrated functional activities in transfection studies that includes the C/EBP consensus sequences, the interaction of nuclear proteins with this region was investigated. EMSAs were performed using $^{32}$P labeled double-stranded oligonucleotides corresponding to C/EBP binding site and nuclear extracts isolated from EPI treated and untreated HepG2 cells. In response to 10 µM EPI a time dependent increase in binding was detected (Fig. 5B, lane 1, 2 and 3) and the specificity of the band was demonstrated by competition with an excess of unlabeled DNA from the same region (Fig. 5B). Result demonstrated that 30x and 100x fold concentration of the unlabeled wild type Cp-C/EBP binding sequence (Cp-C/EBP-WT) decreased the binding (Fig. 5B, lane 5 and 6), whereas, 30x and 100x fold concentrations of unlabeled mutated Cp-C/EBP binding sequence (Cp-C/EBP-mut) could not alter the binding (Fig. 5B, lane 7 and 8). To further confirm that EPI mediated DNA-protein complex formation is consist of C/EBP, supershift analysis was performed with epinephrine treated nuclear extracts incubated with polyclonal anti C/EBPα and C/EBPβ antibodies. It was observed that both anti C/EBPα and C/EBPβ antibodies could block the complex formation (Fig. 5C) that confirms the role of C/EBP in epinephrine-mediated Cp regulation.

Earlier studies have shown that cytokine IL-6 can also induce Cp expression transcriptionally by increasing the binding at IL-6 response element located within -800 bp in Cp promoter (Seshadri et al., 2002; Conley et al., 2005), thus to elucidate whether EPI mediated Cp expression utilizes the same mechanism or not, HepG2 cells were treated with 10 µM EPI and 25 ng/ml IL-6 for 16 h and condition medium was subjected to immunoblot analysis. Fig 5D shows that EPI and IL-6 alone induced Cp expression about 4-fold while more than 7 fold of induction in Cp expression was detected when cells were treated with EPI and IL-6 together. This additive response suggests that EPI utilizes a mechanism other than used by IL-6 to regulate Cp expression in HepG2 cells.

Thus it is evident by now that EPI and NE increase Cp synthesis in liver cells using different pathways that could be the result of differential binding of EPI and NE to different subtypes of adrenergic receptors expressed by HepG2 on cell surface.
Figure 5. Determination of the additional EPI-responsive element of the Cp gene 5' flanking region by reporter gene expression and mobility shift analysis. A, Chimeras of pGL3-basic vectors containing the proximal 768, 700 or 600bp (upstream of the translation initiation site) fragments of the Cp 5' flanking region were transiently transfected into HepG2 cells (with a plasmid containing β-galactosidase to correct for transfection efficiency) as described in text. After recovery, the transfected cells were untreated and treated with 10 μM EPI for 16 h. Luciferase activity in cell extracts was measured and normalized for β-galactosidase activity. Shown are the means ± SD. B, Nuclear extracts were isolated from HepG2 cells after 0, 4 and 8 h of EPI (10 μM) treatment (lanes 1, 2, and 3) and specificity of the binding was confirmed by incubating the nuclear extracts with 30x and 100x molar excess of unlabeled wild type (Cp-C/EBP-WT) (lanes 5 and 6) and mutated DNA from the same region (Cp-C/EBP-mut) (lanes 7 and 8). The figure shows a representative result selected from at least three independent experiments.
Figure 5. Continued:
C, for supershift analysis, nuclear extracts were mixed with 1 μg of anti C/EBPα or anti C/EBPβ antibodies, 30 min before mixing with the 32P-labelled probe. D, HepG2 cells were incubated with IL-6 (25 ng/ml) for 30 min before the addition of 10 μM EPI. After 16 h of incubation, immunoblot analysis of Cp was performed (upper panel) with the conditioned medium and blot was incubated in Coomassie blue stain (middle panel). Densitometric analyses are represented as the mean ± SD of three independent observations (lowest panel).
5.3.5 Identification of cell surface receptor(s):

Catecholamines bind to cell surface adrenergic receptors on their target tissues, in order to exert their physiological effects. Different subtypes of α-AR and β-AR are differentially expressed in different tissues and cells. To identify specific adrenergic receptor(s) in HepG2 cells responsible for epinephrine mediated up-regulation of Cp, HepG2 cells were treated with subtype specific antagonists prazosin (10 μM) and propranolol (10 μM) that block the activation of α-AR and β-AR, respectively, 30 min prior to EPI (10 μM) treatment. After incubating the cells for 16 h, immunoblot analyses were performed with the conditioned media. Results showed that prazosin could not block EPI mediated Cp regulation (Fig. 6A), while β-AR antagonist propranolol was able to block the Cp induction completely (Fig. 6B); indicating the role of β-AR in EPI mediated Cp expression in HepG2 cells. To characterize further the β-AR subtypes involved in Cp regulation by EPI, HepG2 cells were treated with 10 μM metoprolol, which is a well defined β1-AR blocker, 30 min prior to the EPI treatment for 16 h and immunoblot analysis was performed with conditioned media. It was observed that metoprolol failed to inhibit the Cp expression induced by EPI (Fig. 6C) suggesting that EPI specifically activates β2-AR to induce Cp synthesis. Similar strategies were used to elucidate the specific NE sensitive adrenergic receptor. Thus, HepG2 cells were treated with 10 μM prazosin, propranolol and metoprolol for 30 min before the addition of NE (10 μM) and after 16 h immunoblot analyses were performed with conditioned media. It was observed that prazosin could not block the NE mediated Cp up-regulation while propranolol completely inhibited the Cp induction (Fig. 7A and 7B). Interestingly, metoprolol also blocked NE mediated increase in Cp expression that indicates that unlike EPI, NE increases Cp expression by activating β1-AR (Fig. 7C).
Figure 6. Identification of specific adrenergic receptor(s) involved in Cp regulation by EPI. HepG2 cells were pretreated with α-AR antagonist prazosin (10 μM) (A), β-AR antagonist propranolol (10 μM) (B) and β1-AR antagonist metoprolol (10 μM) (C) for 30 min, the medium was replaced with fresh medium before 10 μM EPI treatment and incubated for 16 h. Immunoblot analyses were performed using conditioned media using anti Cp antibody (upper panels). The blots were subsequently incubated with Coomassie Blue to verify uniform loading of all samples (lower panels). Densitometric analyses are represented as the mean ± SD of three independent observations (side panels).
Figure 7. Identification of specific adrenergic receptor(s) involved in Cp regulation by NE. HepG2 cells were untreated or treated with α-AR antagonist prazosin (10 μM) (A), β-AR antagonist propranolol (10 μM) (B) and β1-AR antagonist metoprolol (10 μM) (C) for 30 min. Then the medium was replaced with fresh medium and cells were treated with 10 μM NE for 16 h. Immunoblot analyses were performed with conditioned media using anti Cp antibody (upper panels). The blots were subsequently incubated with Coomassie Blue to verify uniform loading of all samples (lower panels). Densitometric analyses are represented as the mean ± SD of three independent observations (side panels).
5.4 Discussion:

Catecholamines, particularly epinephrine and norepinephrine are major modulators of energy metabolism (Viguerie et al., 2004; Sanghani and Scarpace, 1994; Nonogaki, 2000) and also may have role in iron homeostasis as described in previous chapter where EPI and NE both have shown to up-regulate TfR1 expression by increasing IRE-IRP interactions in liver and skeletal muscle cells. The role of multicopper oxidase Cp is well established in mammalian iron homeostasis due to its ferroxidase activity (Osaki, 1966; Klomp and Gitlin, 1996; Osaki and Johnson, 1969). In this study it was revealed that EPI and NE regulate Cp expression in the human hepatic cell line HepG2 by different mechanisms. In support of this conclusion, it was found that catecholamines, EPI and NE, both increased Cp protein and mRNA in HepG2 cells by increasing its transcription. EPI increased the activity of a larger heterologous reporter gene containing -4774bp long 5'flanking region of human Cp gene about 5-fold in comparison to approx. 3.5-fold increase by NE. Only EPI was found to increase the activity of shorter heterologous reporter constructs containing -3639 and -800bp long 5'flanking region of Cp gene. An APRE sequence is previously defined between -3701 and -3639bp upstream of translation start site in Cp promoter (Das et al., 2007). Both EPI and NE increased the binding at Cp-APRE as detected by EMSAs. In addition to APRE, EPI also increased binding at newly defined C/EBP binding sequence identified between -700 and -650bp upstream of translation start site in Cp 5'flanking region. A radiolabelled probe containing the C/EBP binding sequence specifically binds to C/EBPα and C/EBPβ as observed in EMSAs and supershift analyses. Interestingly, it was further identified by using specific inhibitors for α- and β-ARs that the increased Cp expression by NE is mediated specifically by β1-adrenergic receptors, while EPI possibly activates β2-adrenergic receptors in order to up-regulate Cp expression in liver cells.

The role of AP-1 in the regulation of Cp has been described previously (Lee et al., 2004; Das et al., 2007). Das et al. has shown the role of intracellular copper induced activation of AP-1 in Cp expression and also have shown in involvement of c-Jun and c-Fos. AP-1 is known to play a crucial role in cellular growth, differentiation and tumorigenesis (Kim and Jeoung, 2009; Kajanne et al., 2009; Wagner, 2010). Also, AP-1 has been shown to mediate apoptosis in various cells types (Ameyar et al., 2003). Catecholamines, especially NE, are known to apoptosis in cardiomyocytes by
generating reactive oxygen species (Fu et al., 2004; Remondino et al., 2003; Gupta et al., 2006; Neri et al., 2007) leading to the pathogenesis of cardiotoxicity. Several reports are available to show the catecholamine-mediated activation of AP-1 and its role in apoptosis and hypertrophy in cardiomyocytes (Taimor et al., 2004; Gupta et al., 2006). Catecholamines also generate ROS in liver cells and have shown to be associated with liver toxicity and pathogenesis. It will be interesting to find out whether the activation of AP-1 in liver cells, by catecholamines is one of the potential mechanisms for catecholamine-mediated liver toxicity.

Major difference between EPI and NE mediated response was found to be the increase in binding at the newly defined potential C/EBP binding sequence, present in proximal 5'flanking region of Cp gene, by EPI. C/EBP binding sequences are shown to be present on promoter regions of many acute phase proteins (Poli et al., 1990; Akira et al., 1990) and its role in energy metabolism has been extensively studied (Landschulz et al., 1988; McKnight et al., 1989; Friedman and McKnight, 1990; Croniger et al., 2001). EPI is known to be the prime modulator of energy metabolism in liver and skeletal muscles (Viguerie et al., 2004) whereas NE is potent regulator of energy metabolism in adipocytes (Nonogaki, 2000). Thus, activation of C/EBP by EPI can also describe the specificity of EPI-mediated regulation of energy metabolism in liver cells. C/EBP family is consisting of six isoforms, expressed differentially in different tissues. C/EBPα and C/EBPβ are highly expressed in liver and can bind to C/EBP binding sequences with similar affinity. Results revealed that anti C/EBPα and C/EBPβ antibodies could inhibit the EPI-induced complex formation at C/EBP binding element that again confirmed the EPI-mediated complex formation to be consist of C/EBP. So far there is no report of any direct role of Cp in energy metabolism, therefore, it may be speculated that catecholamine-induced Cp expression is to modulate iron homeostasis during stress conditions. Earlier studies have shown the role of Cp in cellular iron release in normal cells (Harris et al., 1999; Hellman and Gitlin, 2002) and iron uptake in iron deficient cells (Mukhopadhyay et al., 1998; Qian et al., 2001). Catecholaimes increased TfR1 expression in liver and skeletal muscle cells (as described in previous chapter) possibly to match the high iron demand during stressful events, which suggest the role of catecholamine-induced Cp in iron uptake in liver cells.
Another possible explanation for the Cp regulation by catecholamines could involve the amine oxidase activity of Cp (Holmberg and Laurell, 1951; Fellman et al., 1962) that is known for quite long but never been explored in context to catecholamines that are the major biogenic amines in the circulation. Catecholamines can undergo auto-oxidation with production of unstable catecholamines-O-quinones. These quinones give rise to the respective adrenochromes and leads to the production of ROS, like superoxide radical. The superoxide radical was proposed to be responsible for the damage observed in the catecholamine-induced cardiomyopathy (Tappia et al., 2001; Bolli, 1991; Behonick, et al., 2001), toxicity to neurons as well as glia (Rosenberg, 1988; Smythies and Galzigna, 1998) and in liver fibrogenesis (Hsu, 1992). Thus Cp may be released into the circulation to oxidize EPI and NE due to its amine oxidase capacity, probably to protect cells from catecholamine-mediated cellular toxicity. This is to note that Cp has previously shown to oxidize EPI without generating harmful superoxide radical (Ryan et al., 1993).

In conclusion, the present study demonstrated the increased transcription of multicopper oxidase Cp by EPI and NE involving different mechanisms in liver cells. NE increased the Cp expression by inducing binding at Cp-APRE located in 5’flanking region of Cp gene. NE response was found to be mediated by activation of β1-ARs. On the other hand, EPI increased Cp expression by increasing binding to two distinct cis-acting elements in Cp promoter mediated through its binding to β2-ARs. Results demonstrated that in addition to Cp-APRE, EPI also induced binding at newly defined C/EBP binding element in Cp promoter. Significance of catecholamines induced expression of Cp can be explained by virtue of its amine oxidase activity that may be required to oxidize and eliminate these biogenic amines from circulation as their auto-oxidation can lead to generation of harmful reactive oxygen species. Understanding the complete mechanism of cellular protection by Cp in catecholamine-induced toxicity is the subject of future studies.