Section 6.

Summary And Discussion
6. Summary and Discussion:

The secretion of 'catecholamines' or 'stress hormones' represents the primary physiologic defense mechanism of body in response to metabolic, psychological or environmental insults. Norepinephrine (NE) is the major neurotransmitter in the peripheral sympathetic nervous system, whereas epinephrine (EPI) is the primary hormone secreted by the adrenal medulla in mammals. Release of catecholamines is stimulated by a variety of stressors including hypoglycemia, cold exposure, severe exercise, fear, asphyxia, burns and bacterial or viral infections (Radikova et al., 2003; Toso et al., 1993; Schwarz and Kindermann, 1990; Lyte and Bailey, 1997; Goldstein and Kopin, 2008). As part of the response to stress, release of both the catecholamines may be stimulated. Once released in the bloodstream, EPI and NE rapidly prepare the body for action in emergency situations by boosting the supply of oxygen and glucose to the brain and muscles, while suppress other non-emergency bodily processes (digestion in particular) (Selye and Fortier, 1950; Mersmann, 1998). They increase heart rate and stroke volume, dilate the pupils, and constrict arterioles in the skin and gastrointestinal tract while dilating arterioles in skeletal muscles. In addition to these physiological responses catecholamines also influence a number of important metabolic processes (Nonogaki, 2000) in order to release energy required to cope up with the stressful events. They stimulate glycogenolysis in several organs, gluconeogenesis in liver (Viguerie et al., 2004) and have a well-characterized effect on adipose tissue lipolysis that increase in plasma free fatty acid levels (FFA) (Langin et al., 2000). Catecholamines also increase energy expenditure in humans (Blaak et al., 1993), which can be explained by a moderate increase in myocardial energy expenditure, an increase in adipose tissue lipolysis and an increase in FFA oxidation, most notably in skeletal muscles (Nonogaki, 2000; Cha et al., 2005).

Evidences show that the key enzymes of energy generating pathways are dependent on iron for their activities (Gray et al., 1996; Oexle et al., 1999). For instance, iron deficiency modulates the expression of the Krebs cycle enzyme mitochondrial aconitase by a translational mechanism (Oexle et al., 1999). Another citric acid cycle enzyme, succinate dehydrogenase (SDH), may also be susceptible to iron-mediated regulation as it contains an IRE in its 5'UTR (Kohler et al., 1995). Oexle et al. has demonstrated that reduced activities of these enzymes can also lead to reduced activities of citrate synthase and isocitrate dehydrogenase resulting in decreased
formation of NADH and reduced ATP generation by oxidative phosphorylation (Oexle et al., 1999). Thus glycolysis and lactate formation are significantly increased in order to compensate the low ATP generation by mitochondria. All these evidences strongly suggest that the citric acid cycle or energy metabolism and iron homeostasis are tightly interconnected. Therefore, iron homeostasis genes should be potential targets to be regulated by high catecholamine-levels, particularly in various stress conditions.

Although catecholamines are known to play essential role in energy metabolism for a very long time, data are lacking concerning the effects of these hormones on cellular iron homeostasis. Cellular iron homeostasis requires molecules involved in iron import and export across the cell membrane, including transporters and ferroxidases, as well as iron storage proteins (Mukhopadhyay et al., 1998; Levy et al., 1999; Donovan et al., 2000; Sarkar et al., 2003; Mims and Prchal, 2005). The present study focuses on the changes in the expression of transferrin receptor 1 (TfR1: the major iron uptake protein) in human hepatoma cells HepG2 and mouse skeletal myoblasts C2C12 and expression of the ferroxidase ceruloplasmin (Cp: converts toxic ferrous iron to its nontoxic ferric form) in HepG2 cells. It was observed that the expression of both TfR1 and Cp was up-regulated by catecholamines, in concentration as well as in time dependent manner. TfR1 represents the major route through which iron enters into the cells and it is expressed by all most all the cell types. TfR1 synthesis is mainly regulated by interaction of the iron regulatory proteins (IRPs) with the iron-responsive elements (IREs) present on the 3'UTR of TfR1 mRNA, binding which, in iron deficient conditions, stabilizes TfR1 mRNA that result in its enhanced translation (Kuhn and Hentze, 1996; Rouault and Klausner, 1997). The IRE-IRP interactions can also be induced by other factors like hypoxia, reactive oxygen species and nitric oxide generation and phosphorylation (Pantopoulos and Hentze, 1995; Pantopoulos and Hentze, 1998; Hanson et al., 1999; Brown et al., 1998). Several lines of evidence presented in this study to establish the role of catecholamines on TfR1 regulation in at least two of their target cell types. First, the present study demonstrates that catecholamines regulate TfR1 by a post transcriptional mechanism by increasing the half life of its mRNA. EPI and NE increased TfR1 half life from about 2.1 h to more than 5 h, as analyzed by RTPCR in HepG2 cells. Second, catecholamines increased
the binding of IRPs to the IREs present at the 3'UTR of TfR1 mRNA resulting in transcript stability and hence increased translation of TfR1. Catecholamines mediated complex was identified to be consisting of IRE-IRP interactions by super shift analysis using anti IRP1/2 antibodies. Similar results were found in skeletal muscle cells. Third, evidences showed that catecholamines induced TfR1 expression was dependent on ROS generation in the target cells.

Line of evidences indicates that catecholamines induced IRE-IRP interaction could be due to activation of IRP1. IRP1 contains [4Fe-4S] cluster and exists in cytoplasm as aconitase that lack RNA binding property, in iron sufficient conditions. The RNA binding activity of IRP1 can be induced by iron deficiency, ROS or NO generation and hypoxia. Unlike IRP1, IRP2 lack any [4Fe-4S] cluster and undergoes a proteasome-mediated degradation in iron sufficient conditions. Catecholamines had no effect on IRP1 and IRP2 expression indicating the direct activation of IRPs from the cytoplasmic pool of IRP1/2. Results failed to reveal any iron deficiency in EPI or NE treated cells even after 16h of treatment, whereas a significant amount of ROS generation was detected by both EPI and NE in HepG2 cells within 30 min of treatment, as analyzed by the fluorescent microscopy. As IRE-IRP interactions can be induced by NO generation also, thus, catecholamines mediated expression of TfR1 was analyzed in presence of inhibitors of ROS and NO generation, NAC and L-NAME, respectively and it was observed that NAC and not L-NAME could block catecholamines mediated increased expression of TfR1, completely, confirming the role of ROS in catecholamines mediated TfR1 expression.

Several ROS-generating systems, including the mitochondrial electron transport chain, nitric oxide synthase, xanthine oxidase, and cyclooxygenase have been shown to be involved in intracellular signaling cascades leading to changes in cell structure, function, and proliferation (Wolin, 2000). However, the NAD(P)H oxidase is a much larger source of induced ROS in the vascular wall than these other enzymes. There are reports of role of NAD(P)H oxidase mediated ROS generation in response to adrenergic stimulations in splanchnic arteries from cirrhotic patients (Salcedo et al., 2007) and in catecholamine- stimulated proliferation of cultured vascular smooth muscle cells (VSMCs) (Bleeke et al., 2004). It can be presumed that NAD(P)H oxidase might be involved in catecholamines mediated ROS generation and TfR1 regulation also, which has not been established in the present study. Future studies are
needed to establish whether NAD(P)H oxidase is actually involved and if at all then which of its subunits are involved in this process. Studies on HepG2 and C2C12 cells demonstrated a similar mechanism of regulation of TfR1 expression by catecholamines, indicating an inclusive mode of regulation of TfR1 in stress conditions throughout the body, which is not tissue or species specific.

The present work is the first demonstration of the regulation of any iron homeostasis gene by catecholamines. These results indicate that during stress conditions the cellular iron requirement may increase. By increasing TfR1 expression, these stress hormones enhance the cellular iron uptake in order to facilitate the energy generating pathways, potentially to meet high energy demand of the body. Catecholamines increased IRE-IRP interactions, which may also have direct effect on ferritin synthesis. Ferritin mRNA contains a single IRE in its 5'UTR region and binding of IRP to it blocks the efficient translation of ferritin transcript resulting in low levels of ferritin. Allen et al. has shown that catecholamines can release iron from ferritin (Allen et al., 1994). Thus catecholamines, by blocking ferritin synthesis and inhibiting iron storage, facilitate iron release from iron stores to make it available for enzymes of energy generating pathways. On the other hand catecholamines may have little effect on iron release mediated by ferroportin expressed on plasma membrane. Since, ferroportin also contains a single IRE in its 5'UTR, so catecholamines by all probability would also increase IRP binding to ferroportin-IRE resulting into blocking of ferroportin translation in these cells. Thus, activation of IRP and its interaction with IRE actually can ensure TfR1 mediated iron uptake to be utilized for cellular need than for its storage or release from cells (by blocking ferritin and ferroportin synthesis). Ferroportin is in general known to be regulated by post-translationally through internalization and degradation following binding of the hepcidin (Ganz and Nemeth, 2006), a hormone produced by the liver in response to iron levels, erythropoiesis, hypoxia and inflammation. Role of catecholamines on this important hormone hepcidin regulation is not reported so far in the literature.

The other iron homeostasis protein was undertaken for the present study is ceruloplasmin (Cp). Cp is a six domain multi-copper oxidase that catalyzes the conversion of Fe$^{2+}$ to Fe$^{3+}$ (Zaitsev et al., 1999; Bento et al., 2007). The resulting Fe$^{3+}$ is thought to move to a holding site in ceruloplasmin from where it can be loaded onto iron binding proteins for transport (Zaitsev et al., 1999; Bento et al., 2007). By virtue
of its multi-copper oxidase activity, Cp is also capable of oxidizing biogenic amines like EPI and NE in vitro, but its in vivo implication has never been established. Hepatocyte generated soluble Cp is an acute phase protein, whose concentration in serum increases with infection and systemic inflammation. Its properties as an antioxidant are well described (Gutteridge, 1978; Vassiliev et al., 2005), which is attributed to it due to its ferroxidase activity. In the CNS, however, Cp is expressed as GPI-anchored variant by astrocytes (Patel et al., 2000) that partners with ferroportin to efflux iron from these cells (Jeong and David, 2003). Multi-copper oxidase capacity of GPI-Cp in astrogial cells has been found important for stability of iron release protein ferroportin (De Domenico et al., 2007). The role of Cp in iron homeostasis has been confirmed by findings of abnormal iron metabolism and iron accumulation in various tissues in aceruloplasminemia patients with mutation in Cp gene (Miyajima et al., 1987) and in Cp knockout mice (Harris et al., 1999), suggesting that Cp is required for efficient iron release from cells and tissues. In contrast, Cp also has been shown to mediate iron uptake in several cell culture systems including hepatic, erythroid (Mukhopadhyay et al., 1998; Attieh et al., 1999), and glioblastoma cells (Xie et al., 2002; Ke et al., 2006) particularly in iron deficient conditions.

Thus, regulation of Cp in response to various stimuli is important for iron distribution in different tissues by aiding iron-loading into transferrin. Other way, in central nervous system, relative expression of Cp may control iron release from astrocytes accordingly and thus may play a critical role in protecting neurons from iron-mediated damage. Earlier work has described the regulation of Cp mostly at the transcriptional level in response to various stimuli like hypoxia and iron deficiency (Mukhopadhyay et al., 1998; Mukhopadhyay et al., 2000), redox-active copper (Das et al., 2007), insulin (Seshadri et al., 2002), and hyperoxia (Fleming et al., 1991). However, Cp has also reported to be regulated at post transcriptional level by reactive oxygen species and during deficiency of cellular antioxidant glutathione (Tapryal et al., 2009; Tapryal et al., 2010). Cp expression in monocytes was shown to be regulated both at the transcriptional (Mazumder et al., 1997) and at the translational level by interferon-γ (Mazumder and Fox, 1999) in a time dependent response.

In the present study it was found that EPI and NE up-regulated Cp protein and mRNA expression by a transcriptional mechanism, in HepG2 cells. A chimeric construct containing 4774 bp long Cp 5′flanking region of Cp promoter cloned upstream of a
luciferase gene in pGL3 basic vector was transfected into cells and then treated with catecholamines. EPI and NE induced the luciferase activity of the construct by about 4.5 and 3 fold, respectively, suggesting that the regulation of Cp by catecholamines was at the transcriptional level. In quest to identify the responsive element and transcription factors involved in Cp regulation, further deletion constructs of Cp promoter were utilized. Results demonstrated that EPI and NE increase Cp transcription using different mechanisms. Both EPI and NE increased the binding at the APRE (AP-1 responsive element) in the distal 5'flanking region of Cp promoter that was sufficient for NE mediated Cp regulation, while, EPI also induced binding at a putative CCAAT/enhancer-binding proteins (C/EBPs) binding site located within -800 bp proximal Cp promoter region. Previous work done by Das et al. has shown the role of same Cp-APRE in regulation of Cp expression by red-ox active copper (Das et al., 2007) and binding of C/EBP to the rat Cp promoter for basal expression has also been shown earlier (Bingle et al., 1993) but significance of C/EBP binding for inducible Cp expression was never described before. The binding at Cp-APRE and C/EBP binding site were confirmed by mobility shift analysis and cold competition assays with wild type and mutant sequences. The specificity of C/EBP binding was confirmed by super shift analysis using anti C/EBPα and C/EBPβ antibodies that significantly block the DNA-protein complex formation. C/EBP family members are basic-leucine-zipper transcription factors which recognize specific C/EBP elements as either homodimers or heterodimers (Landschulz et al., 1988). The C/EBP family includes, at least, six isoforms namely C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε and CHOP-10 that has tissue-restricted expression patterns. Among all C/EBPα, C/EBPβ and C/EBPδ have been studied in detail that bind to C/EBP consensus sequence at 5'flanking regions of genes of several acute phase proteins with similar specificity and shown to be involved in the regulation of metabolic homoeostasis (Cao et al., 1991; Roesler, 2001) including energy metabolism (McKnight et al., 1989; Friedman and McKnight, 1990; Croniger et al., 2001). However, involvement of C/EBP for inducible Cp expression was not reported before.

Although, ceruloplasmin has been shown to facilitate iron release from the cells its role in iron release in response to catecholamines is very unlikely as evidences have shown that EPI and NE significantly increased TfR1 (an iron uptake protein) expression in liver and skeletal muscle cells through IRE-IRP interactions. The iron
export protein ferroportin contains an IRE in its 5′UTR and thus catecholamine-induced IRE-IRP complex formation might also block iron release by blocking ferroportin synthesis. However, role of Cp in iron uptake has also been reported in iron deficient conditions, thus it may be assumed that Cp may increase iron uptake by loading iron into apo-transferrin. Since, iron loading of transferrin is dependent on Cp ferroxidase activity (Osaki et al., 1966), catecholamine-mediated increase in Cp expression in hepatic cells ensures iron-loaded holo-transferrin formation. TfR1 may then bind holo-transferrin and take up iron for cellular utilization.

On the other hand, catecholamines can be the potential targets for amine oxidase activity of Cp (Holmberg and Laurell, 1951; Fellman et al., 1962) that may also explain the catecholamine-induced Cp expression. Catecholamines are the major circulating biogenic amines and their prolonged exposure can be toxic to the cells (Iwai-Kanai et al., 1999; Zaugg et al., 2000; Rosenberg, 1988; Smythies and Galzigna, 1998). These biogenic amines can easily undergo autoxidation to produce unstable catecholamines-O-quinones. These quinones give rise to the respective adrenochromes and lead to the production of ROS like superoxide radical (Behonick et al. 2001). The catecholamines are well known to cause toxicity to neurons as well as glia (Rosenberg, 1988; Smythies and Galzigna, 1998). Also, the superoxide radical was proposed to be responsible for the damage observed in the catecholamine-induced cardiomyopathy (Tappia et al., 2001) and liver fibrogenesis (Hsu, 1992). Cp has been shown to bind and oxidize serotonin and EPI into non toxic metabolites (Ryan et al., 1993). The amine oxidase activity of Cp was never considered seriously for any in vivo activity so far because of its higher Km in comparison to its ferroxidase activity. But higher Km towards EPI and NE could be important to allow the regular functioning of these biogenic amines in lower concentrations. In stress conditions, when both of these catecholamines are also increased then Cp may act to metabolize these amines by its amine oxidase activity to protect cells from their toxicity. This may practically ensures NE and EPI to function within their physiological range.

Activation of AP-1 can also increase the expression of heme oxygenase1 (HO1) by binding its promoter region in response to various stresses. Increased HO1 might lead to increased heme degradation and release of free iron into cytoplasm. Thus prolonged release and exposure to catecholamines may lead to iron accumulation in the cells due to increased TfR1 expression (bringing more iron into cell), increased
iron release from ferritin (as ferritin synthesis might be blocked) and increased cellular iron levels due to increased heme degradation. Free iron is highly toxic, in excess, as in the presence of reactive oxygen species it can form highly reactive hydroxyl radicals (Halliwell and Gutteridge, 1990) by Fenton reaction and is implicated in several pathological conditions including hepatic injury-related cancer, neurodegenerative diseases and cardiovascular diseases (de Valk and Marx, 1999). Therefore, amine oxidase activity of Cp is of very high significance that helps in removal of catecholamines from the system that may protect cells from iron mediated toxicity.

Cytokine interleukin-6 (IL-6) is known to regulate Cp expression on transcriptional level via IL-6 response element located within -800 bp in Cp promoter (Fey and Fuller, 1987; Seshadri et al., 2002; Conley et al., 2005) and several reports have described the role of C/EBPs in IL-6 mediated regulation of many acute phase genes (Poli et al., 1990; Akira et al., 1990; Baumann et al., 1992; Agrawal et al., 2001). Thus it is very likely that EPI and IL-6 might be using same or overlapping responsive elements. To resolve this, Cp expression was tested by both EPI and IL-6 independently and in combination. Result showed that EPI and IL-6 induced Cp expression most likely by different pathways as combined treatment of EPI and IL-6 induced Cp expression to greater extent in comparison to EPI and IL-6 alone.

Catecholamines elicit a wide variety of important physiological responses in target tissues and their actions are mediated by α- and β-ARs. Several reports indicated that adrenergic receptors in human liver plasma membranes are predominantly of the β2- and α1-subtypes (Kawai et al., 1986), while skeletal muscle is equipped uniquely with the β2-ARs and very little α1-ARs. It was hypothesized that differential mode of EPI and NE mediated Cp regulation might be due to differential activation of cell surface adrenergic receptors by these catecholamines. Use of α-AR and β-AR subtype selective inhibitors prazosine and propranolol, respectively, demonstrated that both EPI and NE mediated Cp expression in HepG2 cells was through β-ARs. Further investigation illustrated that NE induced Cp regulation was mediated by β1-ARs, while EPI may utilize β2-ARs to up-regulate Cp as β1-AR specific inhibitor metoprolol completely blocked NE mediated Cp upregulation while EPI mediated Cp regulation remained unaltered. Thus, in the light of these evidences it can be concluded that β1-AR activation lead to increased binding at Cp-APRE and resulted
Catecholamines stimulated β-Adrenergic receptors transduce signals to G_s-protein, which in turn activates adenylyl cyclase to produce the second messenger cAMP (Dohlman et al., 1991; Kobilka, 1992) and cAMP-dependent protein kinase A (PKA), its major cellular effector. However, several biological processes mediated by G_s-protein have been reported that could not be accounted by the PKA pathway. For instance inhibition of magnesium uptake in S49 cells by isoproterenol or prostaglandin E1 had previously been shown to be G_s-dependent, but cAMP and PKA-independent (Maguire and Erdos, 1980). There are reports of PKA independent regulation of L-type calcium channels by G_s that activates intracellular PI3 kinase pathway (Wickman and Clapham, 1995). PI3-K can activate several downstream targets including PKB, PKC, MAPK and p70^{S6K} (Chan et al., 1999). Although β-AR stimulated responses are generally mediated by the G_s-protein and cAMP/PKA signaling pathway, β2-ARs are also shown to interact with G_i-proteins leading activation of PI3-K/MAPK pathway (Crespo et al., 1994; Lopez-Illasaca et al., 1997; Maier et al., 1999). Role of β-AR and these down-stream signaling pathways have been described in several metabolic processes (Fraeyman and van Ermen, 1993; Tosh and Agius, 1994) including gluconeogenesis and glycogenolysis, but till now no report is there in literature describing role of any of these pathways in induction of IRE-IRP interactions. Thus, it will be interesting to find which of these kinase pathways is involved in TfR1 stability mechanism or other way to activate cellular iron sensors like IRP. In future studies are needed to reveal specific adrenergic receptors and downstream signaling pathways responsible for catecholamines mediated induction in IRE-IRP interactions.

Several reports have shown that β-AR stimulation causes apoptosis in cardiomyocytes (Communal et al., 1999; Iwai-Kanai et al., 1999; Zaugg et al., 2000; Zhu et al., 2003; Remondino et al., 2003). Catecholamines also have shown to cause toxicity to neurons and glial cells (Rosenberg, 1988; Smythies and Galzigna, 1998). And in both the cases there have been reports of generation of reactive oxygen species (ROS) (Remondino et al., 2003; Bolli, 1991; Smythies and Galzigna, 1998) and iron accumulation in the respective tissues. Catecholamines can easily undergo auto-
Summary and Discussion

oxidation with production of unstable catecholamines-O-quinones. These quinones give rise to the respective adrenochromes and leads to the production of reactive oxygen species (Behonick et al. 2001). It is well known that liver inflammation precedes and promotes the progression of liver fibrosis. Catecholamines are the part of repair and pro-inflammatory system (Oben and Diehl, 2004). Therefore, it has been suggested that the pro-inflammatory effect of catecholamines on liver cells may play a role in promoting hepatic inflammation in patients with chronic liver diseases, thus favoring fibrosis development (Hsu, 1992; Sancho-Bru et al., 2006). Similar to cardiovascular diseases and neurodegeneration, iron plays an integral role in the progression of hepatic fibrosis (Bassett et al., 1986; de Valk and Marx, 1999). In excess free iron catalyses the formation of highly reactive and damaging ROS by Fenton reaction. The damage done by ROS includes lipid peroxidation, protein and DNA modification leading ultimately to apoptosis and necrosis. It has been suggested that the accumulation of iron may lead to development of oxidative stress in ageing skeletal muscle further resulting in the development of sarcopenia (Janssen et al., 2004). One common phenomenon associated with age- and disuse-induced muscle atrophy is an increase in apoptosis and oxidative stress and iron has shown to play an important role in this process (Kondo et al., 1992; Powers et al., 2005). The present study provides the evidence of iron overload in target tissues of catecholamines as it has been shown that in response to catecholamines iron uptake protein TfR1 is up-regulated due to increased IRE-IRP interactions. As mentioned previously, increased IRE-IRP interactions can decrease ferritin levels and increase free iron levels in the cytoplasm. Catecholamines induced Cp may also facilitate iron uptake to some extent by loading iron to apo-transferrin. In case of prolonged catecholamine release and exposure that may happen in case of certain chronic diseases, infections or prolonged mental stress conditions, iron overload can happen due to high rate of iron uptake than its utilization in metabolic processes. Thus accumulated iron in cells can facilitate hydroxyl radical generation in presence of ROS that further leads to development of pathological conditions. Hence, in response to catecholamines Cp expression was increased may be to protect organs and tissue from ROS and iron mediated toxicity. Cp possesses amine oxidase activity that targets the circulating catecholamines and oxidizes them without generating harmful super oxide radical and quinines, those are the major cause of oxidative stress in target tissues.
In summary, catecholamines or stress hormones EPI and NE have shown to up-regulate the major iron uptake protein TfR1 by stabilizing its transcript via IRE-IRP interactions at its 3'UTR in human hepatoma cells HepG2 and mouse skeletal muscle cells C2C12. Catecholamines induced IRE-IRP complex formation was found to be dependent on ROS generation. On the other hand NE and EPI induced Cp expression in HepG2 cells on transcriptional level, but by different mechanisms. NE seemed to activate β1-AR to increase binding at APRE in Cp promoter region sufficient for Cp up-regulation, however, EPI stimulated binding at APRE and C/EBP binding site in Cp promoter region possibly via β2-AR. The exact significance of these novel regulations of iron homeostasis genes in response to catecholamines are to be understood and forms the basis for future investigation.