Section 1.

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Stress is a state of affair involving demand on physical or mental energy and covers a huge range of phenomena from mild irritation to the kind of severe problems that might result in a real breakdown of health. In a challenging situation the brain prepares the body for defensive action e.g. the fight or flight response, by releasing stress hormones like epinephrine (EPI) and norepinephrine (NE). Therefore, plasma concentration of these hormones increases markedly in stress conditions. They are also termed as catecholamines as they contain a catechol group, and are derived from the amino acid tyrosine. Catecholamines are the major circulating biogenic amines that act as hormones in peripheral tissues (especially EPI) and neurotransmitters in sympathetic nervous system (mainly NE). Many other global and metabolic threats such as burns, bacterial or viral infections, elevated sound levels, intense light, severe exercise, hypoglycemia, emotional distress and shock can also result in enhanced secretion of catecholamines. Following release into blood, EPI and NE bind differential adrenergic receptors on target cells. These receptors are prototypical examples of seven-pass transmembrane proteins that are coupled to G-proteins and stimulate or inhibit intracellular signaling pathways. Complex physiologic responses result from stimulation of these receptors because there are multiple receptor subtypes (mainly: α1, α2, β1, β2, β3), which are differentially expressed by different tissues and cells. The major responses of EPI and NE include increase in force and rate of contraction of heart muscles, contractions of small blood vessels raising the blood pressure, increased blood flow towards skeletal muscles, dilation of bronchioles, increased metabolic rate and inhibition of certain non-essential processes like gastrointestinal secretion and motor activity.

Epinephrine is known to play an important role in augmenting hepatic glucose production during acute stresses (e.g., hypoglycemia) by increasing hepatic gluconeogenesis (glucose synthesis) and glycogenolysis (the conversion of glycogen to glucose) in several tissues including liver and skeletal muscle cells. Norepinephrine (NE) is a neurotransmitter that mediates chemical communication in the sympathetic nervous system. Like other neurotransmitters, it is released at synaptic nerve endings to transmit the signal from a nerve cell to other cells. Other actions of norepinephrine include increased glycogenolysis in the liver, increased lipolysis in adipose tissue, and relaxation of bronchial smooth muscle to open up the air passages to the lungs. All of
these actions represent a mobilization of the body's resources in order to meet the stressful challenge.

Iron plays a central role in energy metabolism due to its unique chemical properties. It is widely known that rate limiting enzymes of energy generating pathways, such as TCA cycle and glycolysis, are dependent on iron for their activities as they require iron as cofactor. Evidences show that a few enzymes of TCA cycle contain iron responsive elements in their transcripts. In iron deficient conditions TCA cycle activity goes down resulting in decreased NADH and ATP generation, which results in increased glycolysis and lactate production, in order to meet the cellular energy requirement. Although, the role of iron in energy metabolism is known for so long, regulation of iron homeostasis by catecholamines have never been explored. Evolution has developed very complex and coordinated mechanisms to regulate iron homeostasis in the body as iron is highly reactive and so can be toxic when present in excess. This is because free iron can generate toxic hydroxyl radicals in the presence of reactive oxygen species by Fenton reaction. Therefore, to understand the effects of stress on iron homeostasis, the regulations of two important iron homeostasis genes, transferrin receptor 1 (TfR1) and ceruloplasmin (Cp), by catecholamines were investigated and described in chapter 1 and chapter 2, respectively.

Transferrin receptor 1 (TfR1) is the major route of iron entry into the cells and it is expressed on cell membrane of all most every cell type in vertebrates. Diferric holo-transferrin (Fe-Tf) binds to TfR1 on the cell membrane, and then transferrin-iron enters into the cell by endocytosis of the receptor-holo-transferrin TfR1-(Fe-Tf) complex in clathrin-coated pits to form specialized endosomes. Endosomal acidic pH (pH~5.5) facilitates the release of Fe$^{3+}$ from the TfR1-(Fe-Tf) complex and TfR1-Tf complex returns to the cell surface where neutral pH facilitate the release of Tf from the receptor. Previous studies have demonstrated the regulation of TfR1 expression by intracellular iron levels, hypoxia and infections but data are lacking on its regulation by catecholamines. In chapter 1, the details of cellular and molecular mechanisms of catecholamines mediated regulation of TfR1 expression in liver and skeletal muscle cells have been shown.

The present study has provided evidence that catecholamines increased the steady state expression of transferrin receptor 1 (TfR1) in both human hepatocarcinoma cell line HepG2 and mouse skeletal myoblast cell line C2C12 by increasing the half life of
TfR1 mRNA. A marginal increase in TfR1 mRNA levels was observed within 4 h of catecholamines treatment that further increased and could be detected at least up to 16 h. TfR1 mRNA is unique to contain five iron responsive elements (IREs) in its 3’untranslated region (UTR) and binding of iron regulatory proteins (IRPs) to these elements increases the stability of the transcript. IRE-IRP complex formation protects the mRNA from endonuclease mediated degradation. Both EPI and NE increased IRE-IRP interaction at 3’UTR of TfR1 that stabilized the transcript and enhanced the translation. Two cytosolic IRPs: IRP1 and IRP2 are known so far to mediate IRE-IRP complex formation. In response to catecholamines, increased IRE-IRP complex in HepG2 cells was confirmed by super-shift assay using anti IRP1 and anti IRP2 antibodies. IRP1 is a [Fe-S] cluster containing bifunctional protein that acts as a cytosolic aconitase in iron sufficient conditions that lack RNA binding activity. Catecholamines significantly decreased aconitase activity suggesting the activation of IRP1 that lead to increased IRE-IRP interaction. Unlike IRP1, IRP2 lack any aconitase activity and readily undergoes proteasomal degradation in iron sufficiency. It was further identified that catecholamines induced IRE-IRP interaction was due to intracellular reactive oxygen species (ROS) generation as confirmed by pretreatment of antioxidant N-acetyl cysteine (NAC) that completely blocked catecholamine-mediated up-regulation of TfR1 in C2C12 cells. These findings are the first demonstration of regulation of TfR1 expression by stress hormones and established catecholamines as novel modulators of iron metabolism in target cells. This observation might be implicated to higher energy generation in cellular stress conditions.

Ceruloplasmin (Cp), a copper-containing protein, primarily expressed by the liver and secreted in plasma. Evidences showed that in brain, astroglial cells express a glycosylphosphatidylinositol (GPI) -anchored form of Cp on their membranes. Both forms of Cp have been detected in retinal cells and lymphocytes. Cp plays a significant role in body iron homeostasis as aceruloplasminemia patients and Cp knock-out mice exhibit iron overload in several tissues including liver and brain, which suggest that Cp is required for efficient iron release from cells and tissues. In contrast, Cp has been shown to increase iron uptake by iron-deficient cells of hepatic and erythroid origin and by glioblastoma cells. Several other functions as ferroxidase, amine oxidase, as pro-oxidant, as antioxidant, and in nitric oxide metabolism are also
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attributed to Cp. Cp has been shown to be regulated by iron deficiency, hypoxia, inflammation, redox active copper and reactive oxygen species.

Increased catecholamine levels during stress condition can produce cellular toxicity by several means. For instance catecholamines are known to generate oxidative stress in their target tissue either by generating intra cellular ROS or auto-oxidation of catecholamines that produce reactive metabolites, which leads to oxidative tissue damage and apoptosis. Although, amine oxidase activity of Cp is known for so long its significance with respect to catecholamines has not yet been explored. Thus to investigate the effect of catecholamines on Cp expression, a study was carried out in human hepatocarcinoma cell line HepG2 as liver is the major source of circulating Cp. The detailed mechanism of regulation of Cp by catecholamines has been described in chapter 2.

The study demonstrates that both EPI and NE can induce Cp expression in HepG2 cells on transcriptional level but by differential mechanisms. 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 has been described previously in response to cellular redox change. To test whether this APRE is involved in catecholamine-mediated Cp expression cells were transfected with reporter gene constructs containing Cp-APRE cloned upstream of promoter less luciferase gene and then treated with catecholamines. It was found that both EPI and NE increased the expression of reporter gene in HepG2 cells. The role of Cp-APRE in catecholamine-mediated Cp expression mediated was further confirmed by mobility shift assay as an increased binding at Cp-APRE was identified by both EPI and NE treatments. 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-ARs, while, EPI may induce Cp expression by activation of β2-ARs. As catecholamines increases TfR1 and preferentially iron uptake, the increase of Cp by catecholamines present an enigma to explain as increased Cp expression may lead to increase in simultaneous iron release. But these studies potentially signify the amine oxidase activity of Cp, as plasma Cp may plays a
protective role against toxicity generated by catecholamines by oxidizing these biogenic amines.

In summary, catecholamines EPI and NE up-regulated TfR1 expression in human hepatoma cells HepG2 and mouse skeletal muscle cells C2C12 by post transcriptional mechanism. Catecholamines induced TfR1 mRNA stability via IRE-IRP interactions at 3’UTR of its mRNA, which was found to be ROS dependent. On the other hand, NE and EPI induced Cp expression in HepG2 cells at transcriptional level, but by different mechanisms. NE seemed to activate β1-ARs to increase binding at APRE in Cp promoter region in order to increase Cp expression, however, EPI stimulated binding at APRE and C/EBP binding site in Cp promoter region possibly via β2-ARs. 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.